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Methylmercury elicits rapid inhibition of cell proliferation in the developing brain and decreases cell cycle regulator, cyclin E

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Abstract

The developing brain is highly sensitive to methylmercury (MeHg). Still, the initial changes in cell proliferation that may contribute to long-term MeHg effects are largely undefined. Our previous studies with growth factors indicate that acute alterations of the G1/S-phase transition can permanently affect cell numbers and organ size. Therefore, we determined whether an environmental toxicant could also impact brain development with rapid (6–7 h) effects on DNA synthesis and cell cycle machinery in neuronal precursors. *In vivo* studies in newborn rat hippocampus and cerebellum, two regions of postnatal neurogenesis, were followed by *in vitro* analysis of two precursor models, cortical and cerebellar cells, focusing on the proteins that regulate the G1/S transition. In postnatal day 7 (P7) pups, a single subcutaneous injection of MeHg (3 μg/g) acutely (7 h) decreased DNA synthesis in the hippocampus by 40% and produced long-term (2 weeks) reductions in total cell number, estimated by DNA quantification. Surprisingly, cerebellar granule cells were resistant to MeHg effects *in vivo* at comparable tissue concentrations, suggesting region-specific differences in precursor populations. *In vitro*, MeHg altered proliferation and cell viability, with DNA synthesis selectively inhibited at an early timepoint (6 h) corresponding to our *in vivo* observations. Considering that G1/S regulators are targets of exogenous signals, we used a well-defined cortical cell model to examine MeHg effects on relevant cyclin-dependent kinases (CDK) and CDK inhibitors. At 6 h, MeHg decreased by 75% levels of cyclin E, a cell cycle regulator with roles in proliferation and apoptosis, without altering p57, p27, or CDK2 nor levels of activated caspase 3. In aggregate, our observations identify the G1/S transition as an early target of MeHg toxicity and raise the possibility that cyclin E degradation contributes to both decreased proliferation and eventual cell death.

Keywords: Mercury; Neurogenesis; Neural stem cell; Cell cycle; Cyclin E; Cell survival; Cerebral cortex; Cerebellar granule precursors; Hippocampus; Proliferation

1. Introduction

Methylmercury (MeHg) exposure produces widespread abnormalities in the developing brain, including reduced organ size and altered neuronal migration. *In utero* exposure leads to clinical deficits at exposure levels not associated with maternal symptoms (Chang et al., 1977; Choi et al., 1978; Choi, 1989). Considering the distinct sensitivity of the fetus, recent epidemiological studies have investigated the possible developmental effects of early exposure to dietary levels of MeHg (Davidson et al., 1998; Grandjean et al., 1997; Myers et al., 2003). While these studies have produced ambiguous, often contradictory, results (Spurgeon, 2006), animal studies reveal behavioral deficits at MeHg exposures that do not produce histologic changes (Sakamoto et al., 2004), implying that subtle MeHg effects may occur at subtoxic doses.

Mercury damages cells in multiple ways, by generating oxidative stress, binding critical sulfhydryl groups, depolymer-

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izing microtubules, and altering neurotransmission (Castoldi et al., 2001). Apoptosis or necrosis ensues depending on the level of exposure (Kuo and Lin-Shiau, 2004). In proliferating neuronal precursors of the cerebellum, MeHg impedes the production of new neurons by disassembling spindle microtubules and producing metaphase arrest (Rodier et al., 1984). MeHg effects at earlier phases of the cell cycle have been identified but are less well defined (Faustman et al., 2002; Roy et al., 1991). In addition, many MeHg studies on cellular toxicity (Castoldi et al., 2001), neurogenesis (Faustman et al., 2002), and long-term effects (Goulet et al., 2003; Rossi et al., 1997; Sakamoto et al., 2002) have used chronic dosing or timepoints $\geq$24 h. Few studies have investigated early, single-dose effects on neurogenesis.

Neurogenesis refers to the production of new cells, both neurons and glia, in the developing brain. Extracellular signals can alter this process by stimulating or inhibiting cell cycle progression from G1 into DNA synthetic S-phase. Under favorable growth conditions, mitogen-dependent cyclins interact with cyclin-dependent kinases (CDKs) to promote entry into S-phase and facilitate cell proliferation. Conversely, endogenous anti-mitogenic signals act via CDK inhibitors, such as p27 and p57, to reduce cyclin-CDK complex activity and prevent G1/S transition (Cunningham and Roussel, 2001; Sherr and Roberts, 1999). Previous studies have identified positive and negative regulators of G1/S transition that acutely (8 h) enhance or restrict cell proliferation, respectively (Carey et al., 2002; Li and DiCicco-Bloom, 2004; Wagner et al., 1999). Whether neurotoxicants can act in similar fashion is unknown.

Cell cycle regulation influences the number and position of neurons in the cerebral cortex (Levitt et al., 1997; Takahashi et al., 1995, 1999; Vaccarino et al., 1999), hippocampus (Altman and Bayer, 1990), and cerebellum (Hatten and Heintz, 1995). Within these structures, there are spatially distinct regions of proliferating cells that generate neurons at specific developmental periods: the forebrain ventricular zone produces neurons in the embryonic cerebral cortex, while granule neurons are produced postnatally in the hippocampal dentate gyrus and the external germinal layer (EGL) of the cerebellum (reviewed in DiCicco-Bloom and Sondell (2005)). Acute mitogenic regulation of precursor populations can have long-term effects. In postnatal hippocampus and cerebellum, enhanced neurogenesis follows basic fibroblast growth factor (bFGF) administration to newborn rats and produces sustained growth in both regions (Cheng et al., 2001, 2002). Interestingly, these neurogenetic effects do not extend to adult hippocampus, suggesting that developing neurons are uniquely susceptible to neurogenetic signals (Wagner et al., 1999), which may include environmental toxicants.

Based on these observations, MeHg could interact with cell cycle machinery to acutely disrupt proliferation and consequent cell numbers, leading to subtle effects during development. To investigate this possibility, we followed the bFGF model used in previous studies *in vivo*, and found that MeHg decreases DNA synthesis acutely in hippocampus but not cerebellum (Burke et al., 2004) and leads to long-term changes in cell number. We used a single subcutaneous injection of MeHg because it allowed highly reproducible control of mercury levels as well as precise temporal analyses of cell cycle and cell death pathways. We then focused on cell cycle regulators, using well-defined culture models to assess changes in DNA synthesis, cell survival, G1/S transition, and CDK levels. While changes in cell viability paralleled reductions in DNA synthesis at 24 h, earlier reductions at 6 h occurred independently of cell death. Furthermore, effects at 6 h were associated with a selective decrease in cyclin E, a molecule crucial for S-phase entry (Sherr and Roberts, 1999) but also involved in apoptosis (Mazumder et al., 2002). Our data support an additional cell cycle locus, namely the G1/S transition, for MeHg toxicity and raise the possibility that these rapid, region-specific effects depend on altered cell cycle machinery and lead to long-term consequences on regional brain development.

2. Materials and methods

2.1. Materials

Methylmercury chloride (CH_3HgCl) was purchased from Spectrum (Gardena, CA) or MP Biomedicals (Irvine, CA). A 1 mg/ml stock solution in 0.1 M phosphate buffered saline (PBS) was prepared immediately before use and dissolved by sonication. Dilutions were made in PBS for injections or in defined media for addition to cell culture.

2.2. Animal treatment

Time-mated Sprague–Dawley rats were obtained from Hilltop Lab Animals, Inc. (Philadelphia, PA) with plug date designated as embryonic day 0 (E0) or litter birth date as postnatal day 0 (P0). For *in vivo* studies, P7 rats were injected subcutaneously (sc) with vehicle or MeHg (0.1–30 μg/g) in a 50–100 μl bolus. Animals were sacrificed by decapitation at 7 h, 24 h, and in some cases, at 2 weeks. The hippocampus and cerebellum were removed from whole brains, cleaned of meninges, and frozen at −80 °C until processing. Cell cultures were obtained from P7 pups (for granule cells) or E14.5 embryos (for cortical precursors). Pregnant females were sacrificed by CO_2 inhalation and embryos removed immediately. All animal procedures were approved by the Robert Wood Johnson Medical School institutional animal care and utilization committee and conformed to NIH Guidelines for animal use.

2.3. Cortical precursor culture

To obtain a homogeneous population, dorsolateral cerebral cortex was separated from basal ganglia, hippocampus, and overlying meninges. Cells were mechanically dissociated, plated on 0.1 mg/ml poly-D-lysine coated culture dishes, and incubated at 37 °C with 5% CO_2 in defined media (Lu and DiCicco-Bloom, 1997) composed of DMEM and F12 (50:50 (v/v); Invitrogen, Grand Island, NY) and containing penicillin (50 U/ml), streptomycin (50 μg/ml), transferrin (100 μg/ml) (Calbiochem, La Jolla, CA), putrescine (100 μM), progesterone (20 nM), selenium (30 nM), glutamine (2 mM), glucose (6 mg/ml), and

bovine serum albumin (10 mg/ml). Unless otherwise noted, components were obtained from Sigma (St. Louis, MO). Specifically, 10^5 cells were added to 24-well plates for [^{3}H]-thymidine ([^{3}H]-Thy) incorporation studies or 35 mm dishes for cell counting and BrdU labeling. Protein analysis required 6–8 million cells plated in 60 mm dishes. In all cortical cell experiments, MeHg was added to the media 1 h after plating the cells so that initial adhesion was not disturbed by the toxicant.

2.4. Granule cell isolation

Cerebellar granule cells were isolated from P7 rat pups according to Hatten (1985), as previously described (Nicot et al., 2002; Tao et al., 1996). Briefly, a single cell suspension was obtained by incubating the tissue in trypsin-DNase solution for 3 min followed by dissociation in DNase solution by trituration. Cells were pelleted, filtered through nylon (20 μm), and centrifuged on a 35/60% Percoll step gradient at 3200 rpm for 13 min. Cells present at the gradient interface were removed, washed, and pre-plated to remove glia and finally plated in 35 mm or 24-well plates. Granule cells were obtained in two major experimental paradigms. In the first, pups were treated with MeHg at zero time and [^{3}H]-Thy at 5 h. Then at 7 h, granule cell precursors were isolated from three pups in each control and MeHg treated groups. Six aliquots of 10^5 cells for each group were added to 24-well plates and immediately collected onto filter paper, as below, to assess incorporation that had occurred *in vivo* (see Fig. 1C). In the second paradigm, cells were isolated from untreated, naive pups and were plated into culture plates to assess effects of MeHg *in vitro*.

2.5. [^{3}H]-thymidine (Thy) incorporation: in vitro

Plated cells were incubated with [^{3}H]-Thy (5 μCi/ml; Amersham Pharmacia) during the last 2 h or 4 h of total incubation, lifted with a trypsin-EDTA solution, and collected onto filter paper with a semi-automatic cell harvester (Skatron). After addition of the luminating solution Eco-Lite (MP Biomedicals), radioactivity was measured by scintillation spectrophotometry.

2.6. [^{3}H]-Thy incorporation: In vivo

[^{3}H]-Thy (5 μCi/g body weight) was injected sc into animals 2 h prior to analysis. DNA synthetic rate was evaluated using a 'percent incorporation' assay, as outlined in Wagner et al. (1999). Frozen tissues were manually homogenized in distilled water using a 22 gauge needle and syringe. An aliquot was removed for determination of total isotope uptake into the tissue. In an equal aliquot, DNA was precipitated with 10% trichloroacetic acid, sedimented by centrifugation, and washed by resuspension and resedimentation. The final pellet was dissolved and counted along with the original aliquot in a scintillation spectrophotometer. Since radiolabel incorporation into DNA depends on the amount of label taken up by the tissue, incorporation was calculated as the fraction of total tissue uptake. This method assures that experimental effects do not reflect possible differences in tissue region dissection or individual animal injection, absorption or blood flow, but rather changes in specific regional DNA synthesis.

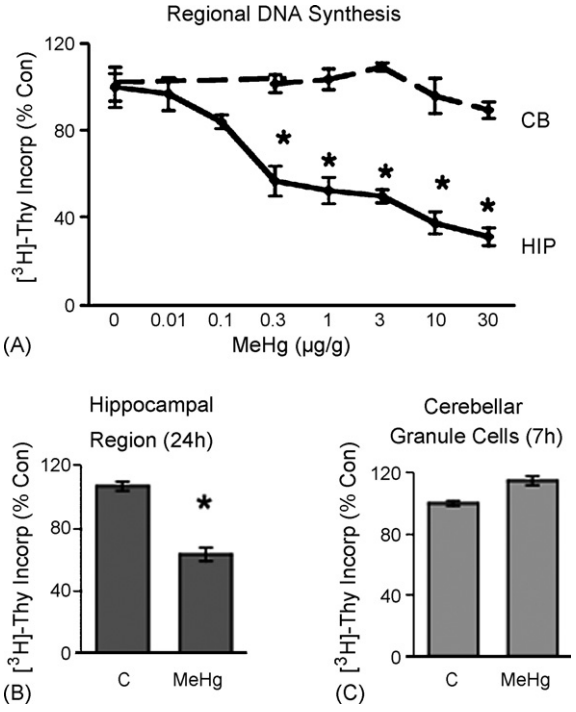

Fig. 1. Effect of MeHg on brain region-specific DNA synthesis in P7 rat pups. (A) Seven hours after subcutaneous (sc) injection of MeHg over a range of doses, percent [^{3}H]-Thy incorporation was assessed in the hippocampal (HIP) and cerebellar (CB) regions. DNA synthesis was reduced in hippocampus but was unaltered in cerebellum. $N = 4$–9 animals per group. Data are derived from two experiments. Data are expressed as % of mean control value. (*) Differs from control at $p < 0.05$ (B) At 24 h, hippocampal DNA synthesis was also reduced by MeHg (3 μg/g) injection. Figure shows one of the three experiments with similar results, each with $N = 3$ or more animals per group in each experiment. (*) Differs from control at $p < 0.01$ (C) The proliferative granule cell precursors in cerebellum were unaffected after *in vivo* MeHg (3 μg/g) exposure, consistent with whole organ analysis shown in (A). Results are the sum of data from two experiments that were combined and expressed as percent of control. $N = 6$–7 animals per group.

2.7. DNA quantitation

P21 hippocampus and cerebellum were manually homogenized in distilled water. DNA was pelleted with 10% trichloroacetic acid (TCA) and centrifugation. After the pellet was hydrolyzed with 1N KOH and neutralized, DNA was resedimented with 5% TCA and denatured at 90 °C. Samples were quantified with a diphenylamine reagent that reacts proportionally with DNA to produce a colored reaction product that can be assessed by spectroscopy for each experiment. Sample values were converted to total DNA based on a standard curve run in parallel for each assay, as previously described (Cheng et al., 2001, 2002).

2.8. BrdU labeling

Bromodeoxyuridine (BrdU), a thymidine analog, was used to identify cells in S-phase. Cells were incubated with BrdU

(10 μM) during the last 2 h of total incubation time and fixed in 4% paraformaldehyde. To detect nuclear incorporation, cells were exposed to 2N HCl for 30 min, incubated with monoclonal anti-BrdU (1:100; Becton-Dickinson, San Jose, CA) followed by biotinylated anti-mouse (1:100) secondary and then visualized with the Vectastain ABC kit using the DAB substrate (Vector, Burlingame, CA). Using a Leica inverted microscope at 200× magnification, total cells in three randomly selected 1 cm strips [(3% of area)/dish] were counted using phase microscopy and simultaneously BrdU (+) cells were counted under brightfield optics, assessing approximately 100 BrdU (+) cells per dish. Data are expressed as a percent of total cells exhibiting nuclear BrdU labeling, the labeling index (LI), as reported previously (Lu and DiCicco-Bloom, 1997).

2.9. Cell counting

Cell counts were performed on a Leica inverted phase microscope. Living cells in three randomly selected 1 cm strips at 200× magnification [(3% of area)/dish, 3–4 dishes/group] were counted, using previously reported methods (Lu and DiCicco-Bloom, 1997; Zhou et al., 2001). Using microscope stage micrometers, the counted regions were randomly positioned by setting the *Y*-axis at predetermined arbitrary locations (135, 140, 145) and all cells with intact cell membranes and morphology observed on the culture dish surface were counted as the stage was advanced from right to left over a 1 cm distance. These positions correlated roughly to one strip in the upper, middle and lower thirds of the 35 mm dish. Cell numbers within these fields were routinely within 10% of each other, indicating that initial cell plating was equally distributed. Cell viability was based on appearance, including cell size measured using a micrometer eyepiece, presence of processes, and membrane integrity as assessed with trypan blue exclusion. MeHg disruption of cell cytoskeleton led to some variability in size and appearance, without altering trypan blue reactivity in 7 h cultures.

2.10. Protein extract preparation

Cells were detached from 60 mm dishes in PBS (pH 7.4) with a rubber policeman, pelleted, and resuspended in buffer (20 mM HEPES-KOH, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$) with 0.5 mM dithiothretiol (DTT), 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM leupeptin, and 3.5 μg/ml aprotinin. Samples were stored at −80 °C. For western blotting, defrosted samples were lysed by sonication and centrifuged to separate the pellet. Supernatant was removed and normalized to 0.1 M NaCl.

2.11. Western blotting

Protein extracts (30–100 μg per lane) were analyzed by 12% SDS-PAGE. Protein was transferred from gel to polyvinylidene diflouride (PVDF) membrane by transfer apparatus at 30 V for 4 h or 100 V for 45 min. The membrane was blocked with 5% milk and incubated with primary antibody against cyclin E (1:1000, SC-481), p27 (1:1000, SC-528), p57 (1:1000, SC-8298), CDK2 (1:1000, SC-163, Santa Cruz, CA) or cleaved caspase-3 (1:1000, Cell Signaling Biotech., MA). After incubation with anti-rabbit (polyclonal) or anti-mouse (monoclonal) horseradish peroxidase-conjugated secondary antibody, protein was visualized with an enhanced chemiluminescence system (Pierce, Rockford, IL), as previously reported (Carey et al., 2002; Li and DiCicco-Bloom, 2004).

2.12. Measurement of brain tissue total Hg

Total mercury in brain tissue was measured using a Thermal Decomposition (Gold) Amalgamation Atomic Absorption Spectrophotometer (TDA/AAS) designed for direct mercury analysis (DMA-80, Milestone, Inc., Pittsburgh, PA). Tissue was obtained from P7 cerebellum and hippocampus or from cortical precursor culture. Briefly, for *in vitro* measurements, 10–12 million cells were added to 60 mm plates and treated with 3 μM MeHg for 6 h. Cells were harvested in 0.05% Trypsin-EDTA (Sigma), washed and then centrifuged to form a pellet. Measurements using this technique (see Section 3) corresponded to previous concentrations obtained in culture at similar doses (Meacham et al., 2005). Prior to analysis, tissue and cell samples were weighed and frozen at −80 °C. For [Hg] determination, cortical precursor cells were diluted to 1 mg/50 μl and 25 μl were analyzed in triplicate. Hippocampus and cerebellum were thawed on ice and (approximately 100 mg) were then analyzed individually for total Hg content within each brain region. Samples were dried in the DMA-80 at 300 °C for 90 s, decomposed on a catalytic column at 850 °C for 300 s. Mercury vapor was collected on a gold amalgamation trap and subsequently desorbed for quantitation. Mercury content was determined using atomic absorption spectrometry at 254 nm, and compared to external Hg reference standards. The lower limit of detection was 0.01 ng total Hg.

2.13. Statistical analysis

Data are reported as means ± S.E.M. For *in vivo* experiments, statistical comparisons were performed with Statview using ANOVA and Scheffe *F*-test posthoc. For *in vitro* studies, raw data was compared by unpaired *t*-test using Excel or Statview. p values <0.05 were considered statistically significant. Following analysis, grouped data is presented in its raw form or as percentage of control depending on variation between experiments. Sample sizes are indicated in the figure legends.

3. Results

3.1. MeHg selectively decreases DNA synthesis in hippocampus but not cerebellum in vivo

A single injection of bFGF can elicit long-term changes in neurogenesis of hippocampus and cerebellum during postnatal brain development (Cheng et al., 2001, 2002). MeHg, a neurotoxicant that permanently damages developing brain

(Choi et al., 1978; Choi, 1989; Reuhl and Chang, 1979), may similarly affect developmental neurogenesis. To address this possibility, P7 rat pups were given a single subcutaneous injection of MeHg and evaluated for acute changes in DNA synthesis in hippocampus and cerebellum at 7 h. Regional proliferation was assessed by percent [^{3}H]-thymidine (Thy) incorporation, a marker of DNA synthesis (Wagner et al., 1999). In the hippocampus, [^{3}H]-Thy incorporation decreased progressively with increasing concentrations of MeHg above 0.1 μg/g body weight (Fig. 1A). Specifically, DNA synthesis decreased 43% at 0.3 μg/g, 50% at 3 μg/g, and 69% at 30 μg/g. To determine whether anti-mitogenic effects of MeHg were transient, or alternatively, sustained, we examined DNA synthesis at 24 h. One day after exposure to 3 μg/g MeHg, hippocampal [^{3}H]-Thy incorporation remained depressed by 41% (Fig. 1B), suggesting that initial reductions in DNA synthesis are sustained or alternatively, replaced by other effects, such as mitotic arrest or cell death.

In marked contrast to observations in the hippocampus, DNA synthesis in the cerebellum was unchanged across the entire dosage range (Fig. 1A), suggesting region-specific responses. Significantly, these differential effects of MeHg *in vivo* did not depend on different concentrations of the metal in the two brain regions, as total Hg levels were very similar in hippocampus and cerebellum 7 h after subcutaneous injection of 10 μg/g MeHg (Table 1). To examine whether data obtained from homogenates of the whole cerebellum accurately represented the response of proliferating granule cells, the precursors alone were isolated from brains under conditions identical to those used in Fig. 1A: P7 pups were injected with MeHg (3 μg/g) at zero time and with [^{3}H]-Thy at 5 h, and then sacrificed at 7 h. Granule cells were then isolated and incorporation was assessed. In this isolated precursor population, DNA synthesis again was unchanged 7 h after MeHg injection compared to control (Fig. 1C), indicating that the neurotoxicant *in vivo* affects brain precursors in a highly region-specific fashion at the doses assessed.

3.2. Acute effects on DNA synthesis predict long-term outcome in brain region cell number

Considering brain formation relies on the production of new neurons, the acute decrease in hippocampal DNA synthesis may have permanent effects. Potentially, fewer cells will be produced, and long-term cell number will be decreased accordingly. Alternatively, it is possible that proliferating cells can compensate for an acute insult, leading to recovery of normal cell numbers. Previous studies show that changes, both increases and decreases, in total DNA content *in vivo* parallel those defined by stereological cell counting, and therefore can be used to accurately represent cell number (Cheng et al., 2002). We measured total DNA content 2 weeks after acute MeHg-induced insults to the precursor proliferation at two doses, 0.3 μg/g and 3.0 μg/g. In the hippocampus, MeHg exposure on P7 decreased cell number by 17% at P21 (Fig. 2), indicating that acute changes in cell proliferation lead to long-term consequences for regional cell composition. In marked contrast, parallel studies of the cerebellum demonstrated no changes in cell number 2 weeks after MeHg injection (data not shown), suggesting that acute changes in regional DNA synthesis are useful indices of long term consequences for brain region neurogenesis.

Table 1
[Hg] in cerebellum and hippocampus 7 h after 10 μg/g MeHg injection

Tx	Brain region	*n*	Weight (g)	Hg (ng)	[Hg] ± S.E. (ng/g = ppb)
Con (saline)	HIP	4	0.038	0.11	3.05 ± 0.88
Con (saline)	CB	5	0.047	0.07	1.53 ± 0.33
Hg (10 μg/g)	HIP	4	0.041	32.4	714 ± 68
Hg (10 μg/g)	CB	5	0.049	43.1	920 ± 122

Note: Tx, treatment group; HIP, hippocampus; CB, cerebellum; ppb, parts per billion.

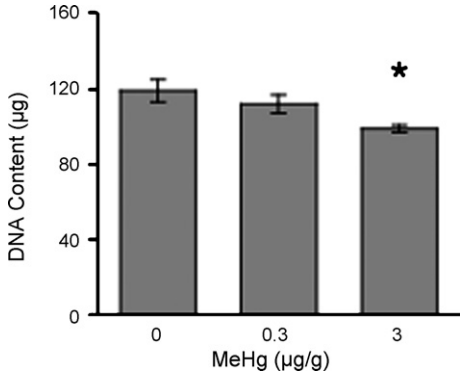

Fig. 2. Effect of acute MeHg exposure on long-term hippocampal DNA content. Following injection of vehicle or MeHg (0.3 μg/g or 3.0 μg/g) at P7, hippocampal DNA content (μg/region) was assessed at P21. Data are expressed as μg DNA per region. In previous studies, observed decreases in DNA content accurately represented quantitative changes in cell number defined by stereological assessment (Cheng et al., 2002). Data represent two experiments, each with $N = 3$ pups per group. Thus the total $N = 6$ per group. (*) Differs from control at $p < 0.05$.

3.3. Cerebellar granule cell precursors are susceptible to MeHg effects in vitro

In vivo, the cerebellum was unaffected by the concentrations of MeHg that decrease hippocampal cell proliferation. To determine whether cerebellar resistance reflects cellular characteristics versus regional tissue differences, we isolated granule cells and assessed directly effects of MeHg *in vitro*. In isolated precursor cells that had been resistant to MeHg effects on cerebellum *in vivo*, [^{3}H]-Thy incorporation was decreased by metal exposure at both 6 h and 24 h (Fig. 3A). MeHg was more potent in eliciting effects as duration of exposure was increased: 0.3 μM MeHg elicited no change at 6 h but decreased incorporation 17% at 24 h; 3 μM led to a 43% decrease at 6 h and 92% decrease at 24 h. The decrease in DNA synthesis *in vitro* indicates that granules cells are sensitive to direct MeHg exposure, in contrast to their resistance *in vivo*. Furthermore, these *in vitro* studies emphasize the differential responsiveness of cerebellum and hippocampus to MeHg

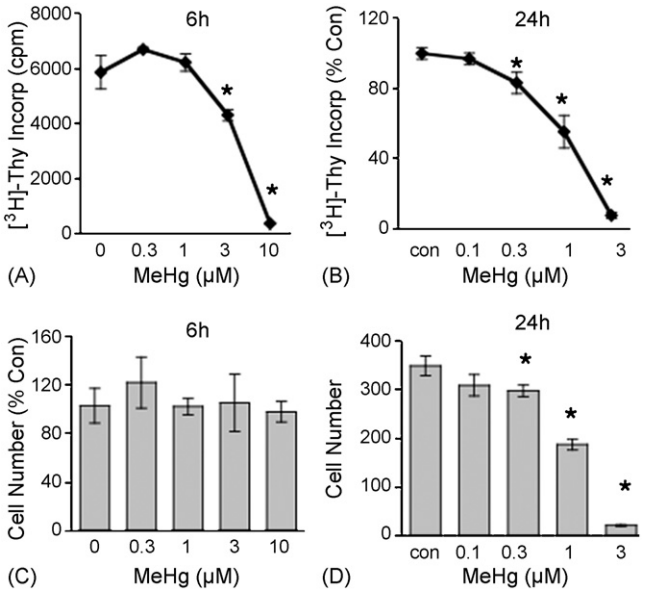

Fig. 3. Effects of MeHg on DNA synthesis of cerebellar granule cell precursors *in vitro*. Granule cell precursors were isolated from P7 rats and exposed to different concentrations of MeHg in defined medium. Incorporation of [^{3}H]-Thy (A and B) as well as cell number (C and D) were assayed at 6 h and 24 h. MeHg decreased DNA synthesis with increasing potency with increasing incubation time. Decreased DNA synthesis occurred without cell loss at 6 h (A and C) but paralleled cell death at 24 h (B and D). Data are expressed as mean incorporation ±S.E.M. (A), mean cell number ±S.E.M. (D), or percent of control ±S.E.M. (B and C). For each graph, data were derived from three to four experiments, showing similar effects. Thymidine incorporation was measured in quadruplicate and cell numbers in triplicate for each experiment, with the total $N = 12–16$ for incorporation and $N = 9–12$ for cell counts. (*) Differs from control at $p < 0.05$.

exposure in the intact, living animal, further supporting region-specific sensitivities.

3.4. Cortical precursor cultures also exhibit decreased DNA synthesis in response to MeHg exposure

To assess *in vitro* MeHg effects on DNA synthesis in another proliferating population and better define relevant cell cycle machinery, cortical precursors were treated with a range of MeHg concentrations for 6 h or 24 h. While cerebellar studies allowed for the isolation of analogous proliferative precursors, hippocampal results *in vivo* are not as easily paralleled in culture. Specifically, it is difficult to achieve survival of cells obtained from the postnatal mature hippocampus due to damage by mechanical disruption and absence of yet undefined trophic factors. While isolation of prenatal E18 hippocampal cells has been the standard approach, these cultures contain a mixture of neurons, glia, and diverse precursors (Robinson et al., 1993) and do not faithfully represent precursors that generate dentate gyrus granule neurons (Cheng et al., 2001, 2002). Thus, we turned attention to precursors from embryonic cerebral cortex, a well-defined model composed almost exclusively of neuronal precursors, with glial markers expressed by <1% of cells at 3 days incubation (Carey et al., 2002). This cortical model has been used to identify alterations in G1/S transition with anti-mitogenic PACAP (Carey et al., 2002; Lu and DiCicco-Bloom, 1997). Thus we used this well-defined forebrain population as a surrogate model for hippocampus and found effects similar to cerebellar cultures: MeHg exposure elicited dose-dependent reductions in [^{3}H]-Thy incorporation at 6 h and 24 h. The lowest dose required for significant effect, 3 μM, which decreased incorporation by 48% (Fig. 4), was selected for further cell cycle studies.

3.5. Changes in cell viability and apoptosis parallel decreased DNA synthesis at 24 h but not 6 h following MeHg

Since MeHg exposure is known to affect neuronal survival, cell numbers were analyzed at 6 h and 24 h to determine the contribution of cell death to decreased DNA synthesis. Changes in cell number have been shown to parallel results from apoptosis assays in this model using another environmental toxicant (Davidovics and DiCicco-Bloom, 2005). After 24 h MeHg exposure, both cerebellar and cortical cell numbers paralleled the decrease in [^{3}H]-Thy incorporation (Figs. 3D and 4D), suggesting that reduced DNA synthesis at this time reflects diminished numbers of mitotic precursors. In contrast, minimal changes in cell number were observed at 6 h (Figs. 3C and 4C), an earlier time point associated with reduced DNA synthesis *in vivo*. Thus, acute 6 h reductions in DNA synthesis at 3 μM

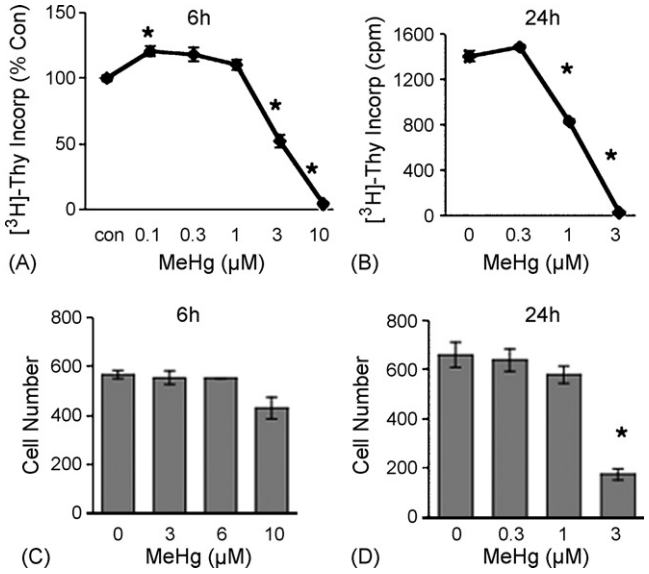

Fig. 4. Effects of MeHg on DNA synthesis of cortical precursor cells *in vitro*. Precursors were isolated from the cortex of E14.5 rat pups and exposed to different concentrations of MeHg in defined medium. Incorporation of [^{3}H]-Thy (A and B) and cell number (C and D) were assayed at 6 h (A and C) and 24 h (B and D). MeHg decreased DNA synthesis with increasing potency with increasing incubation time. Decreased DNA synthesis occurred without cell loss at 6 h but paralleled cell death at 24 h. Data are expressed as mean incorporation ±S.E.M. (B), mean cell number ±S.E.M. (C and D), or percent of control ±S.E.M. (A). For each graph, data were derived from three to four experiments, showing similar effects. Thymidine incorporation was measured in quadruplicate and cell numbers in triplicate for each experiment, with the total $N = 12–16$ for incorporation and $N = 9–12$ for cell counts. (*) Differs from control at $p < 0.05$.

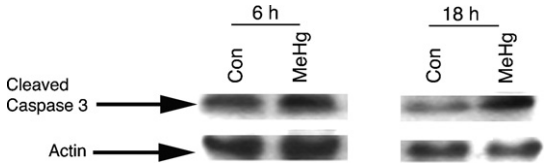

Fig. 5. Effect of MeHg on protein levels of activated (cleaved) caspase 3. Precursors were isolated from the cortex of E14.5 rat pups and exposed to 3 μM MeHg in culture for 6 h or 18 h and protein extracts were collected for western analysis. At 6 h, there were no differences in the level of cleaved caspase 3, whereas there were major increases at 18 h. The marked increase in this apoptotic pathway only after 6 h parallels changes in cell counts described in Fig. 4. The blot represents one of the two westerns revealing similar results.

MeHg may reflect toxicant effects on cell cycle progression of neurogenetic precursors.

While morphological assessment of cells at 6 h suggested that cell death does not contribute to early reductions in DNA synthesis, it is possible that the apoptotic cascade may already be engaged. To further examine this issue, we assessed levels of activated (cleaved) caspase 3, a major mediator of cell death, by western analysis in cultures exposed to MeHg for both 6 h and 18 h. We chose 18 h for the assay, rather than 24 h, because elevation of this molecular signal likely precedes the major cell loss observed at 24 h. While there were major increases in activated caspase 3 at 18 h, we did not observe increases in this marker of apoptosis at 6 h. These observations suggest that MeHg exposure activates the cell death pathway at times later than 6 h (Fig. 5).

3.6. MeHg inhibits S-phase entry in cortical and cerebellar precursors

MeHg elicited decreased DNA synthesis independent of cell death at 6 h in both cortical and cerebellar precursors, since greater than 50% reductions in [^{3}H]-Thy incorporation occurred in the absence of cell loss or activation of caspase 3. Mechanistically, MeHg may either reduce the entry of cells into S-phase, as previously suggested (Hayes et al., 1996), or alternatively, decrease the rate of ongoing DNA synthesis in the cohort of cells already replicating their DNA. These different mechanisms can be distinguished by using BrdU labeling to assess the number of cells in S-phase during the last 2 h of MeHg incubation. In 6 h cerebellar granule cultures, MeHg exposure elicited a 73% reduction in [^{3}H]-Thy incorporation (Fig. 3), and a 48% decrease in BrdU labeling (Fig. 6), consistent with the hypothesis that the toxicant prevented the G1- to S-phase transition. Studies in cortical precursors followed a similar pattern with MeHg decreasing BrdU labeling by 37% at 6 h (Fig. 6). These observations suggest that MeHg allows fewer cells to enter S-phase, possibly by regulating cell cycle machinery required for the G1/S transition.

3.7. MeHg selectively reduces pro-mitogenic regulator, cyclin E

Considering the decrease in BrdU labeling, we assessed MeHg effects on cell cycle proteins involved in the G1/S

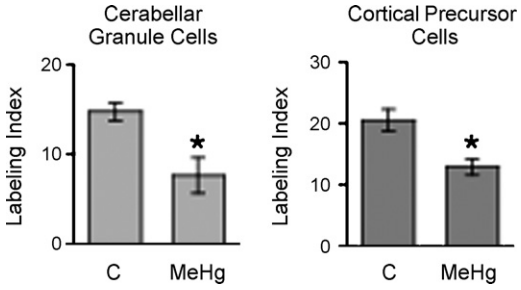

Fig. 6. Effect of MeHg on S-phase entry *in vitro*. Cerebellar granule cells (A) and cortical precursor cells (B) were incubated with 3 μM and 6 μM MeHg, respectively, and assessed for nuclear BrdU incorporation at 6 h. Data represent the percent of total cells exhibiting nuclear labeling and were derived in triplicate dishes from two experiments each, $N = 6$ per group. (*) Differs from control at $p < 0.01$.

transition, using the cortical precursor model. Since endogenous anti-mitogenic PACAP selectively increased CDK inhibitor p57 to inhibit mitosis in cortical cells (Carey et al., 2002), we wondered whether MeHg may employ the same mechanism to block the G1/S-phase transition. Cortical precursors were incubated in control or MeHg containing medium under conditions associated with decreased S-phase entry independent of cell death (3 μM MeHg for 6 h). Protein extracts were subjected to Western analysis to measure levels of p27, p57, cyclin E and CDK2. MeHg exposure elicited a 59% reduction in the levels of cyclin E, but did not alter the levels of p27, p57, and CDK2 (Fig. 7). While one might hypothesize that a general MeHg toxicity in cortical precursors could lead to overall cell protein degradation, these data suggest that cyclin E is targeted specifically. Considering the crucial role of cyclin E in G1/S transition (Sherr and Roberts, 1999), our observations suggest that at 6–7 h, MeHg effects on DNA synthesis are promoted by reductions in cyclin E.

3.8. Tissue accumulation of mercury in vivo and in vitro

As described above, while MeHg exhibited region-specific effects in hippocampus and cerebellum, the regional mercury

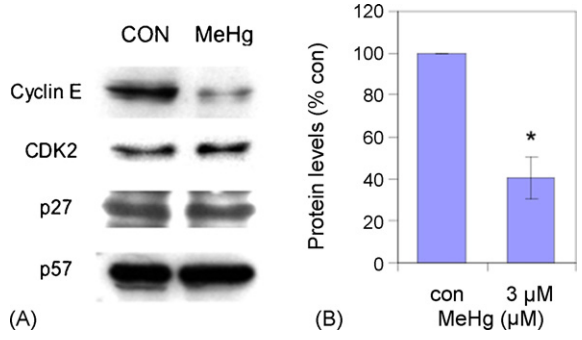

Fig. 7. Effect of MeHg on protein levels of cell cycle regulators p27, p57, CDK2 and cyclin E. Cortical precursor cells were exposed to 3 μM MeHg for 6 h. Exposure to MeHg induced a marked reduction in levels of cyclin E only. (A) Proteins were isolated by Western blot analysis. One representative blot is shown of three for cyclin E and CDK2 and two for the CDK inhibitors. (B) Densitometric analysis of cyclin E proteins was obtained from three separate experiments. (*) Differs from control at $p < 0.01$.

concentrations did not differ significantly, with cerebellum accumulating 714 ng/g and hippocampus accumulating 920 ng/g for 7 h after MeHg injection (Table 1). These data demonstrate rapid peripheral uptake and central transport of MeHg to the developing brain. The higher concentration of MeHg used for *in vivo* assessment (10 μg/g) was intended to ensure adequate detection of possible regional differences. *In vitro* mercury concentrations were also measured to allow comparison of culture models to observed effects in the developing rat brain. Previous studies indicate that addition of MeHg to culture media leads to significant uptake (Meacham et al., 2005), an observation confirmed by our experiments. At 6 h, a time when DNA synthesis and levels of cyclin E are decreased, 3 μM MeHg exposure produced 9.31–10.84 μg/g (mean Hg = 10.04 ± 0.44 μg/g cells; mean ± S.E.M.; $N = 3$) Hg in cells. Our assessment of mercury accumulation corresponds to the prediction by Lewandowski et al. (2002) that 1 μM of MeHg is equivalent to ∼3 μg/g in tissue.

4. Discussion

MeHg has profound effects on brain development, suggesting that component processes in nervous system formation are particularly vulnerable. We assessed one such component, precursor proliferation, and found that MeHg rapidly alters DNA synthesis and cell production selectively in the hippocampus. While MeHg effects on mitosis, in particular, inducing metaphase arrest, are well-defined (Rodier et al., 1984; Sager et al., 1984), the knowledge that extracellular signals act during G1 to control cell division raised the possibility that toxicants also alter proliferation at the G1/S transition. Indeed, in cerebellar and cortical precursor cultures, MeHg decreased G1/S progression prior to alterations in cell number and elicited selective reductions in pro-mitogenic cyclin E, suggesting that MeHg may decrease proliferation by altering cell cycle regulators. Consequently, these studies demonstrate early neurogenetic effects of MeHg and identify novel cell cycle mechanisms for environmental factors during brain development.

4.1. MeHg has rapid, differential effects on proliferation in developing brain regions

Our observations indicate that DNA synthesis was decreased in the postnatal hippocampus 7 h after a single peripheral injection of MeHg. In contrast to hippocampus, the developing cerebellum was unaffected acutely and long term by MeHg exposure *in vivo* using the measures we employed. Since levels of mercury were distributed equally in both regions at 7 h (Table 1), differential effects in hippocampus and cerebellum reflect selective vulnerabilities. To our knowledge, few studies have investigated the accumulation and effect of mercury in the nervous system at <24 h (Harry et al., 2004). In recent work by Lewandowski et al. (2003), comparable brain mercury concentrations (∼1–3 μg/g) did not alter cell cycle kinetics in embryonic rat midbrain. Our results indicate that region-specific responsiveness is an important aspect of mercury toxicity, potentially accounting for these differences.

While both hippocampus and cerebellum have been considered MeHg targets, differences we detect may reflect their distinct courses of neurogenesis during development (DiCicco-Bloom and Sondell, 2005; Wakabayashi et al., 1995). In neonatal rat brain, mercury accumulation (Sakamoto et al., 2002) and pathology (Wakabayashi et al., 1995) are regionally dependent on postnatal age. At P1, mercury distributes equally (Sakamoto et al., 2002) and produces histological disruption in hippocampus but not cerebellum (Wakabayashi et al., 1995). Our study defined similar differential effects on proliferation in hippocampus and cerebellum at P7. Previous studies in postnatal mice (Rodier et al., 1984; Sager et al., 1984) and humans (Choi, 1989) have observed cerebellar susceptibility, contrasting with our results and potentially reflecting species differences (Burbacher et al., 1990).

In vitro, both cerebellar and cortical precursors were equally affected by MeHg, results not parallel to those *in vivo*. Similarly, while midbrain cells are resistant to MeHg exposure *in vivo* (Lewandowski et al., 2003), they show cell cycle alterations in culture (Ponce et al., 1994). The inconsistency between *in vitro* and *in vivo* responses may represent a deficiency in cell culture models, differences on Hg levels to which cells are exposed, or the presence of protective factors *in vivo*. Based on tissue Hg measurements under both conditions, cells were exposed to mercury levels at least 10-fold higher *in vitro* than *in vivo*. However, the level of 700–900 ng/g in brain tissue does not reveal what precursor cells themselves may experience in their microenvironment, whether higher or lower, as Hg may partition differentially in vascular cells, neurons, glia and extracellular spaces. Meanwhile the lack of effect on cerebellum is not likely due to Hg levels per se, since even very high doses, 30 μg/g MeHg, were without effect on DNA synthesis. Based on the equivalent MeHg exposures *in vivo*, another mechanism may lie with region-specific glial cells, absent from culture, which can differ in number, maturity or specific metabolic capacities. While culture models indicate that MeHg alters neuronal precursor proliferation, *in vivo* changes in cell proliferation may reflect not only neuronal but also glial susceptibility (Garg and Chang, 2006) to the toxicant. In addition, glial cells alter markedly the sensitivity of neurons in culture to metal toxicants, including both MeHg (Aschner, 1996; Morken et al., 2005; Shanker et al., 2003) and lead (Tiffany-Castiglioni and Qian, 2001; Zurich et al., 2002). Also, while glutathione may play a protective role following oxidative stress, both regions exhibit comparable levels of antioxidant defenses that protect cells from neurotoxicant insults (Li et al., 1996). Considering regional characteristics and previous studies *in vivo,* differences in hippocampus and cerebellum most likely reflect individual courses of neurogenesis (lifelong in hippocampus but only 3 weeks in cerebellum) and regional glial factors.

4.2. Long-term effects of MeHg suggests alteration in brain region formation

Our previous studies indicate that changing the availability of endogenous growth factors such as bFGF *in vivo* can acutely

regulate precursor proliferation and produce long term changes in brain cell composition (Cheng et al., 2001, 2002). While bFGF is an endogenous factor with a physiological role in development (Tao et al., 1996), MeHg is an environmental toxicant usually present in trace amounts in human brain. Still, like bFGF, a single injection of the toxicant can elicit both acute and long-term consequences: MeHg decreased DNA synthesis acutely and reduced total cell number 2 weeks later. It should be noted that the single MeHg injection may still allow a continuous slow release for a period of time. The acute and long-term changes elicited by MeHg suggest that the developing brain is impaired in its ability to recover following toxic insult and therefore, initial effects on precursor proliferation may lead to permanent alterations in brain cell composition. However, the long half-life of mercury in the brain (Burbacher et al., 2005; Magos and Butler, 1976) supports an alternative possibility that tissue levels remain high weeks after dosing and continually affect proliferation. While Sager et al. (1984) found that P2 mice given 4 mg/kg MeHg had 1.8 μg/g Hg after 24 h but <0.1 μg/g at P21, future studies need to address Hg levels, compartmentation and neurogenesis in the P21 rat following early postnatal exposure. Furthermore, while bFGF studies indicate a relatively permanent change in brain cell populations after newborn treatments, studies on adult rats after early MeHg exposures will be needed to explore this issue.

Our observations of cell cycle effects of MeHg *in vivo* occur at Hg levels (probably ~200–300 ng/g, given use of 3 mg/kg MeHg for proliferation studies and a measured level of 700–900 ng/g after 10 mg/kg) achieved with chronic dosing and also associated with long-term behavioral effects (Newland and Reile, 1999; Rossi et al., 1997; Sakamoto et al., 2002). Whether MeHg is delivered as a single injection (our study) or a chronic low dose oral exposure, similar mercury levels are associated with altered cell production and future behavioral effects (Castoldi et al., 2001; Goulet et al., 2003; Newland and Reile, 1999; Rossi et al., 1997). Significantly, Hg levels in human infants from fish-eating populations also range from 50 ng/g to 250 ng/g (Lapham et al., 1995), suggesting that our injection model better approximates environmental exposures than the consequences of overt metal toxicity. Moreover, our approach reveals a remarkably rapid and robust effect of a single MeHg exposure on neurogenesis, raising concern regarding transient spikes in the levels of metals, usually not considered in setting environmental exposure limits (Tchounwou et al., 2003). This robust response may now enhance our ability to identify proximate mechanisms at both the tissue and cellular levels *in vivo* with a focus on the cell cycle mechanisms we have defined in culture.

4.3. Decreased DNA synthesis in vitro reflects reduced entry into S-phase and rapid changes in regulatory cyclin E

Measurements of DNA synthesis reflect both the number of cells entering S-phase and the rate of [^{3}H]-Thy incorporation. The parallel decrease in BrdU labeling suggests that fewer cells entered S-phase following MeHg exposure, similar to earlier preliminary results (Hayes et al., 1996). Since extracellular signals such as PACAP decrease G1/S transition in this culture model by altering cycle regulators (Carey et al., 2002), we assessed levels of cyclin E, p27, p57, and CDK2 to define MeHg effects. MeHg exposure selectively reduced levels of cyclin E, a critical promoter of G1/S progression (Sherr and Roberts, 1999). In contrast to PACAP, which increased CDK inhibitor p57, MeHg decreased a positive cyclin regulator, suggesting that environmental toxicants can target cell cycle machinery, a novel molecular mechanism. Studies in other systems have reported MeHg effects on G1/S transition and regulatory molecules. In primary embryonic midbrain cultures, MeHg affected G1/S only at 4 μM, when all cycling had ceased (Ponce et al., 1994). However, in fibroblasts and splenocytes, MeHg apparently decreased the rate of the G1/S transition, with the underlying mechanism undefined (Roy et al., 1991; Vogel et al., 1986).

Activation of key G1/S regulatory genes by MeHg, which induces *GADD45* and *GADD153*, may contribute to cell cycle arrest after DNA damage in embryonic precursors (Faustman et al., 2002). Furthermore, CDK inhibitor p21 is upregulated by MeHg at 30 h of exposure (Ou et al., 1999), a change that inhibits cycle progression but not the G2/M transition (Mendoza et al., 2002), suggesting MeHg halts G1 progress by modifying cell cycle machinery. The decrease we observe in cyclin E following MeHg exposure for 7 h identifies a molecular change at a timepoint earlier than previous studies. While additional studies are needed to characterize the role of cyclin E in MeHg-induced G1/S arrest, our results raise the possibility that neurotoxicants may alter positive regulators, a mechanism that contrasts with the increases in negative regulators observed in previous MeHg studies and the model of PACAP. Finally, oxidative stress, a known effect of mercury, can interfere with thymidine kinase (Junod et al., 1985) whose activity prepares DNA bases for re-use in DNA synthesis, the so-called salvage pathway. Considering the brain's dependence on purine and pyrimidine salvage (Barsotti et al., 2002), this mechanism could potentially limit production of endogenous deoxynucleotide pools as well as modified exogenous tracers, [^{3}H]-Thy and BrdU, for incorporation, possibly leading to decreased DNA synthesis, an model worthy of additional study in primary brain cells.

4.4. Decreased precursor proliferation may reflect MeHg effects on cell death and cell cycle machinery

The decreases in DNA synthesis and cell number elicited by MeHg exposure *in vivo* may reflect multiple cellular mechanisms. To evaluate the potential role of cell death in decreased [^{3}H]-Thy incorporation, we examined cell viability and activated caspase 3 *in vitro* at timepoints corresponding to mitotic inhibition *in vivo*. While reductions in cell number (i.e., cell death) and increases in caspase 3 *in vitro* paralleled changes in incorporation after 24 h exposure, cell numbers and an apoptotic pathway were unchanged at 6 h when we observed marked mitotic inhibition. In turn, initial decreases in DNA synthesis *in vivo* may similarly occur independently

of cell death. In contrast, changes observed at 24 h may represent more general cell damage to proliferating precursors. MeHg is known to reduce cell viability at 24 h in PC12 cells (Parran et al., 2001), neuroepithelial cells (Faustman et al., 2002; Ponce et al., 1994) and dorsal root ganglion neurons (Wilke et al., 2003) through mechanisms including glutathione depletion, reactive oxygen species production, microtubule damage, and apoptosis. MeHg-induced cell death is highly dependent on p53, a protein commonly associated with apoptosis but also involved in the G2/M and G1/S transitions (Gribble et al., 2005), raising the possible connection between G1/S regulation and cell death following MeHg exposure. Actively cycling cells, such as those in the developing brain, are more susceptible to cell death (Bogner et al., 2003) and much work has focused on MeHg effects on precursor proliferation and cell number in terms of mitotic arrest. However, recent studies of adult brain neurogenesis reveal complex and non-uniform relationships between S-phase entry and subsequent apoptosis depending on the nature of the insult, such as vascular compromise or ischemia (Kuan et al., 2004). More relevant to our study, cyclin E has been linked to both G1/S regulation and apoptosis. Caspase-dependent cleavage of cyclin E is associated with increased levels of apoptosis (Mazumder et al., 2002). As apoptosis ensues, fewer intact molecules are detectable, implying that our observed reduction in cyclin E may reflect fragmentation and increased elimination versus decreased production. The dual role of cyclin E poses the question of whether early alterations in the G1/S transition are related to subsequent cell death, a well-recognized event in MeHg toxicity. Furthermore, how these molecular effects may contribute to altered proliferation and cell number *in vivo* is unknown.

In summary, the inhibitory effects of MeHg exposure on cell cycle progression and precursor proliferation are relevant to concerns about prenatal and childhood exposure to environmental levels of MeHg on the developing human brain (Myers and Davidson, 2000; Myers et al., 2003). Our studies build on epidemiological data by identifying a component of neurodevelopment that is susceptible to mercury at levels approaching ambient exposure. We demonstrate rapid, region-specific alterations in cell proliferation after a single exposure to MeHg with associated decreases in cell number 2 weeks later. Culture models reveal disruption of G1/S transition at comparable time points and MeHg doses, suggesting that environmental toxicants could alter neurogenesis through cell cycle regulation. Finally, we identify a new locus of MeHg toxicity, cyclin E, and raise the possibility that early cell cycle arrest and subsequent apoptosis are linked by a common molecular mechanism.

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