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ORIGINAL PAPER

Highly Sensitive Visualization of Inorganic Mercury in Mouse Neurons Using a Fluorescent Probe

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Abstract In the present study, we used the previously developed fluorescence probe, **EPNP**, to generate the first image of the distribution of mercuric ion in primary mouse neuron cultures. At postnatal day 1 (P1), the mice were intraperitoneally (IP) injected with mercuric chloride in doses ranging from 0.05 to 0.6 μg/g body weight. After 1, 2, 3, and 4 days exposure, primary nervous cell cultures and frozen brain and spinal tissue sections were prepared and dyed using **EPNP**. On the third day of repeated injections, Hg^{2+} was visualized in primary cerebral neuron cultures as an increase of Hg^{2+}-induced fluorescence at the doses≥0.1 μg/g. A similar accumulation of Hg^{2+} was observed in frozen hippocampus tissue sections. In contrast, no Hg^{2+} was observed in spinal cord neurons and spinal tissue sections. The detection of a low dose of IP injected mercury in mouse cerebral neurons facilitated the evaluation of the exposure risk to low-dose Hg^{2+} in immature organisms. Moreover, the highly sensitive **EPNP** revealed Hg^{2+} in the cerebral neurons of mice younger than P4, while the presence of Hg^{2+} was not detected until≥P11 in previous reports. Thus, this technology and the results obtained herein are of interest for neurotoxicology.

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Introduction

Inorganic mercury is a common environmental pollutant resulting from mining release, smelting, and industrial discharge. However, compared with methylmercury, the toxicity of inorganic mercury has not been well studied. As an electrophilic toxin, Hg^{2+} shows the strong potency of neurotoxicity, as neurons lack an adequate inducible defense mechanism against this chemical [1]. Consequently, low-dose exposures to Hg^{2+} could potentially alter nervous behavior in a development-dependent manner. Several studies have suggested that the toxicity of organic mercury in vivo reflects demethylation, which increases toxic Hg^{2+} [2, 3], and Hg^{2+} is the proximate toxic species upon exposure to Hg^0 [4]. Therefore, evaluating the risk of exposure to Hg^{2+} is of interest for neurotoxicity, particularly in immature organisms. Although mercury toxicity has been well studied, there is a knowledge gap concerning the risks of Hg^{2+} toxicity at low concentrations in vivo.

Compared with autometallography [5], fluorescence imaging is currently the most powerful technique available for the continuous observation of intracellular processes in living cells [6]. Herein, we used a previously developed molecular fluorescence probe, **EPNP** (2,6-bis-(4′-peperazino-N′-butyl-1′,8′-naphthalimide) dimethylpyridine), to develop a new technique for real time and on-line neurotoxicity studies. The sensitivity and selectivity of this probe for intracellular nanomolar concentrations of Hg^{2+} have been confirmed through the imaging of cultured human kidney tubular epithelial cells exposed to mercuric chloride [7] and Hg^{2+} in live plant cells [8].

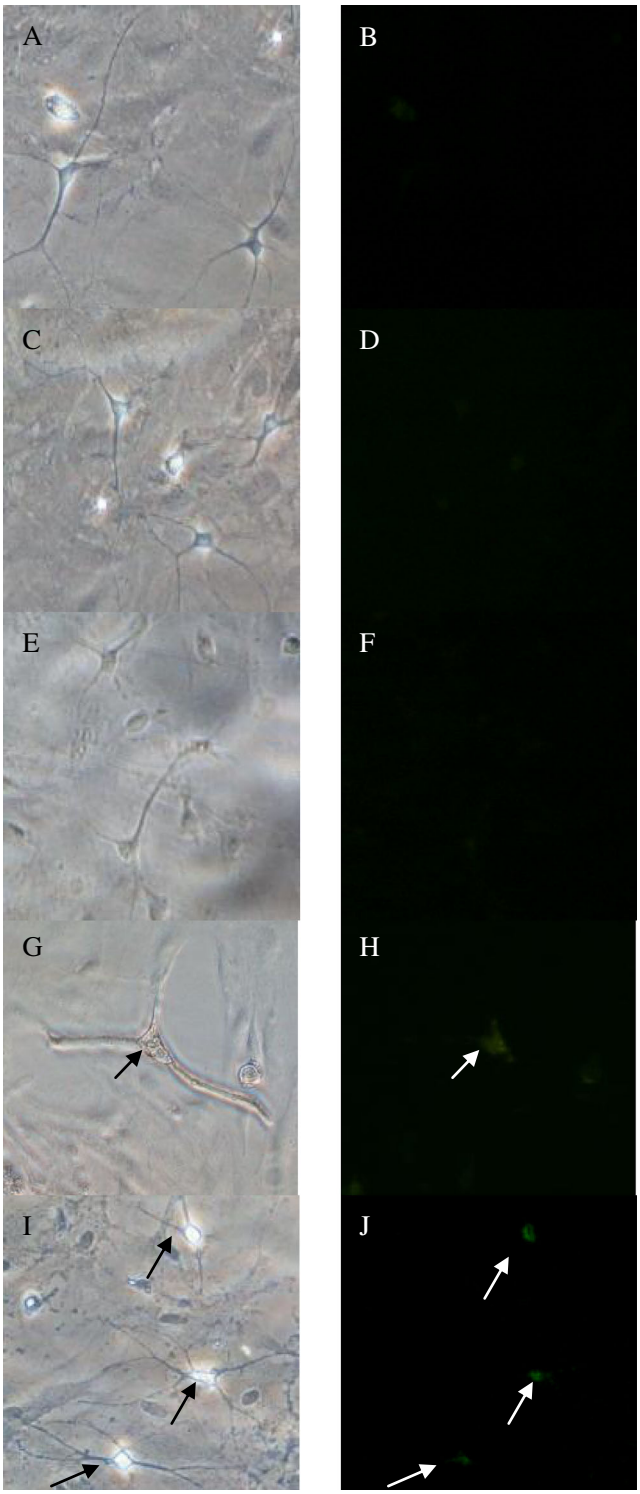

Fig. 1 Images of cerebral neurons. Phase contrast (**a**, **c**, **e**, **g**, **i**) and fluorescence (**b**, **d**, **f**, **h**, **j**) images are shown. The two images is the same field and the fluorescence photos were captured under the same conditions. **a** and **b** shows the cerebral neurons of control mice (no $HgCl_2$ injection). **c** and **d**: repeated injections with 0.05 μg $HgCl_2$/g body weight for 4 days. **e** and **f**: repeated injections with 0.1 μg $HgCl_2$/g body weight for 2 days. No fluorescence was observed in these fields. **g** and **h**: repeated injection with 0.1 μg $HgCl_2$/g for 3 days. The arrows indicate the obvious green fluorescence observed in neurons. **i** and **j**: repeated injections with 0.6 μg $HgCl_2$/g body weight for 4 days

In the present study, we applied **EPNP** to visualize the distribution of Hg^{2+} in primary cultured cerebral and spinal cord cells and frozen tissue sections obtained from neonatal mice after the systemic IP injection of a low dose of $HgCl_2$. Using this fluorescence probe, we aimed to (1) Observe the distribution of Hg^{2+} in neurons on-line; (2) Determine the lowest dose of Hg^{2+} absorbed in the neurons of neonatal mice; and (3) Rationalize the application of the fluorescence probe for neurotoxicity.

Materials and Methods

Animals

Healthy P1 pathogen-free NIH mice (weighing 1.8-2.0 g) were obtained from the Dalian Laboratory Animal Center (Dalian, China). Neonatal and maternal mice were housed in cages, and the maternal mice were fed a breeding diet during the entire experimental period. The mice were maintained at room temperature (20±2 °C) under a 12-h light/12-h dark cycle.

Intraperitoneal Injection of $HgCl_2$

Stock solutions of $HgCl_2$ (pro analysis grade; Merck, Germany) were dissolved in distilled water, followed by

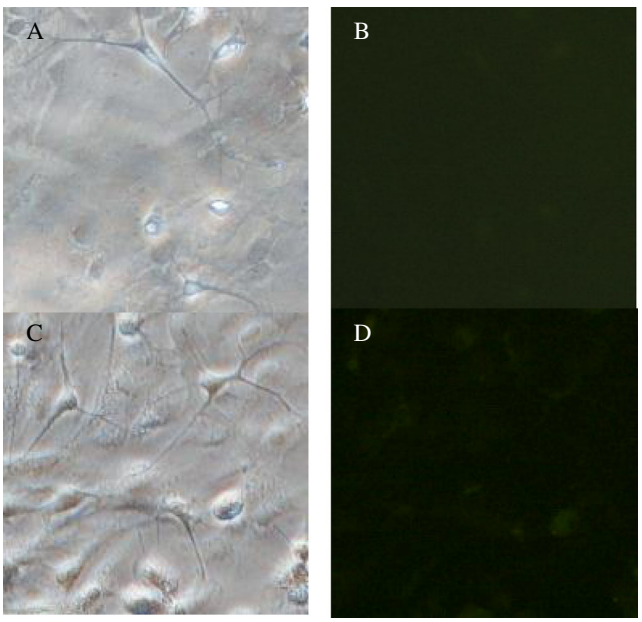

Fig. 2 Images of spinal cord neurons. Phase contrast (**a**, **b**) and fluorescence (**c**, **d**) images are shown. The two images is the same field and the fluorescence photos were obtained under the same conditions. **a** and **b**: spinal cord neurons of control mice. **c** and **d**: spinal cord neurons of neonatal mice IP injected with $HgCl_2$ at 0.6 μg $HgCl_2$/g body weight for 4 days. No fluorescence was observed in these fields

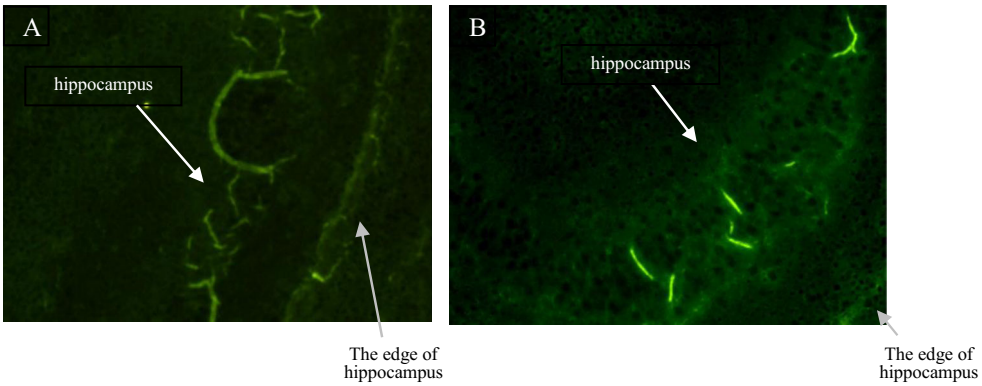

Fig. 3 Imaging of **EPNP** stained neonatal mice hippocampus. Photos were taken under the same conditions. (A) Image of the control mice. (B) Image of the mice injected IP with 0.1 μg $HgCl_2$/g body weigh $HgCl_2$ for 3 days. In the hippocampus of the control mice, only fluorescence noise is found inside hippocampus. As shown in B, the bright green fluorescence was increased, indicating Hg^{2+} is present

filter sterilization. Neonatal mice at P1 were IP injected with 0.02 ml of aqueous $HgCl_2$ using a 1-mL syringe and a 27 G needle. Doses of 0.05, 0.1, 0.2, 0.4, and 0.6 μg $HgCl_2$/g body weight were administered at 4 mice per treatment. Control neonatal mice were injected with 0.02 mL of distilled water. The mice were sacrificed after treatment for 1, 2, 3, and 4 days. Brain and spinal cord samples were obtained for use in subsequent experiments. No systemic ill-effects were observed after these treatments.

Neuronal Cultures

Primary cerebral and spinal cord cell cultures were prepared as previously described [9]. Briefly, the cells were dissociated through mild trypsinization at 37 °C, followed by trituration in a DNase I solution (0.004 % wt/vol) containing soybean trypsin inhibitor (0.05 % wt/vol). The cells were suspended in culture medium (DMEM/F12 with 20 % fetal calf serum) supplemented with *p*-aminobenzoate, insulin, and penicillin. The cell suspension (1.6×10^6 cell/mL) was seeded in 24-multiwell plates and incubated for 8 days in a humidified 5 % CO_2/95 % air atmosphere at 37 °C. The culture medium was replenished every other day.

Preparation of the Freezing Sample

Neonatal mice were sacrificed using 0.02 mL of intraperitoneal pentobarbitone and immediately dissected to remove the brain and spinal cord. The tissues were embedded using Tissue-Tek (OCT) and immersed in liquid nitrogen for rapid freezing. Cryostat-cut sections (4 μm thickness) were obtained using a Leitz-1512 slicer (German).

Visualization of Mercury

The mercuric ions were visualized in neuronal cells and tissues using the fluorescence probe **EPNP** according to a previous publication [7, 8]. Briefly, the cultured cells were incubated in the presence of 0.5 μM **EPNP** for 30 min, washed three times with PBS and observed using a Nikon TE2000 inverted fluorescence microscope (Nikon, Japan) fitted with a wide-band cube U-MWU filter. The excited light was WB 450–480 nm, and the magnification was 20×. A total of 20 μL of **EPNP** solution was applied onto the frozen sections using a micropipette. After dying for 30 min, the samples were rinsed with PBS, and observed using a Nikon fluorescence microscope. The fluorescence images were

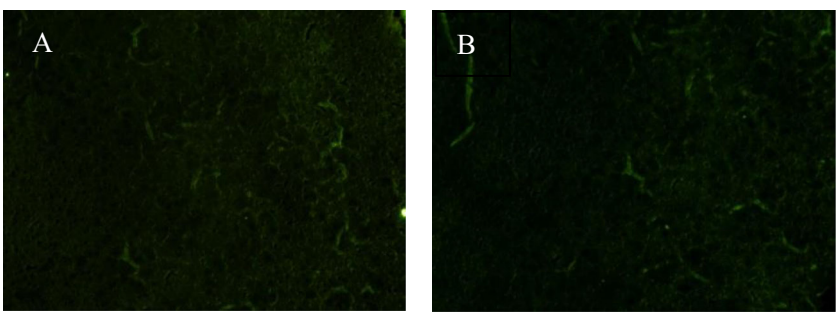

Fig. 4 Imaging of **EPNP** stained neonatal mice spinal cord. (A) Image of the control mice. (B) Image of the mice injected with $HgCl_2$ at a dose of 0.6 μg $HgCl_2$/g body weight for 4 days. No specific fluorescence was seen in the spinal cord

captured under the same conditions, using an exposure time of 1/3 s.

Results

Dose and Time-Dependent Hg^{2+} Distribution in Neurons

The distribution of Hg^{2+} in cerebral and spinal cord neurons at various dosages and series of exposure times is shown in Figs. 1 and 2, respectively. No obvious fluorescence was detected in the cerebral and spinal cord neurons obtained from control mice. Green fluorescence was initially observed in cerebral neuron bodies on the third day after repeated injections with 0.1 μg $HgCl_2$/g (Fig. 1h). At higher doses or subsequent injections, the fluorescence intensity was obviously increased. Figure 1j shows the fluorescence image at 4 days after repeated injections with 0.6 μg/g mercury.

No mercury-induced fluorescence was observed in spinal cord neurons, even after 4 days of repeated injections with 0.6 μg/g Hg^{2+} (Fig. 2d).

Hg^{2+} Distribution Within the Hippocampus

The frozen brain and spinal cord sections were dyed with **EPNP**. Hg^{2+}-induced fluorescence was visible in the hippocampus (Fig. 3) at 3 days after repeated injections with 0.1 μg $HgCl_2$/g. No obvious fluorescence was observed in other regions of the brain, and no mercury was detected in the spinal cord (Fig. 4) at all dosages and time courses used in these experiments.

Discussion

Fluorescent probes are emerging as key tools for the visualization of trace elements in live species. Using the well-established fluorescent probe, **EPNP**, we presented the first on-line analysis of the real-time distribution of inorganic mercury in the CNS of neonatal rats after IP injection with $HgCl_2$.

At 3 days after repeated IP injection with $HgCl_2$ at 0.1 μg/g body weight, Hg^{2+} was observed in cerebral neurons as an increase in fluorescence. The lowest previously recorded dose of systemically injected $HgCl_2$ required for the detection of mercury granules through autometallography in the neurons of adult mice was 0.2 μg/g body weight for 5 days [10]. The high sensitivity of the fluorescence probe updated this limitation 3 times. The difference between developed and developing organisms must also be considered. Previous studies have shown that mercury uptake in neurons is age-related. In adult mice, retrograde axonal transport (from the neuromuscular junction to spinal and brainstem motoneurons) is the only pathway for Hg^{2+} uptake in neurons [11], as the polar electricity of Hg^{2+} prevents the passage of this chemical through the intact blood–brain barrier (BBB). In contrast, **EPNP** showed that Hg^{2+} directly passed through the BBB in infant mice and was subsequently absorbed by cerebral neurons, as no Hg^{2+} was observed in spinal neurons, even at 4 days after repeated IP injection with 0.6 μg/g Hg^{2+}, a 8-fold higher dosage than that observed in cerebral neurons. Indeed, (1) during early development, the rapid growth of the CNS might necessitate a functionally "leaky" BBB to accommodate the high demands of blood-borne nutrients for brain growth [12], through which Hg^{2+} could also enter. (2) Moreover, $HgCl_2$ could act as a direct barrier toxicant to facilitate the immediate breakdown of the BBB [13, 14].

Notably, Pamphlett R et al. [15] reported that infant mice could not uptake Hg^{2+} until≥P11. However, using **EPNP**, Hg^{2+} was detected in the cerebral neurons of mice younger than P4, demonstrating the high sensitivity of this fluorescence probe.

Consequently, EPNP was used to examine the distribution of Hg^{2+} in frozen cerebral and spinal cord slices. Chemical-induced brain damage is highly selective to some brain areas, but not to others. For example, inorganic forms of Pb and Mn distinctly accumulate in the hippocampus and basal ganglia, respectively. The results obtained in the present study suggest that Hg^{2+} precisely accumulated in the hippocampus, consistent with the results of the previous study [16]. Similar results were observed in primary neuron cultures, but no Hg^{2+} was observed in the spinal cord.

The new fluorescent probe **EPNP** facilitates the sensitive and easy detection of intracellular Hg^{2+}. The lowest injected dose of mercury accumulated in mouse neurons was updated. Thus, subsequent neurotoxicity studies of Hg^{2+} could benefit from the use of this fluorescence technique. Because the toxicological effects of Hg^{2+} on brain structure and neurobehavioral function are remarkably similar across a range of mammalian species (i.e., humans, rodents, and river otters), examining the distribution of Hg^{2+} in neonatal mice after mercury exposure will advance the current understanding of the mechanisms underlying Hg^{2+} toxicity in children.

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