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Low level prenatal exposure to methylmercury disrupts neuronal migration in the developing rat cerebral cortex

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ABSTRACT

We determined the effects of low-level prenatal MeHg exposure on neuronal migration in the developing rat cerebral cortex using *in utero* electroporation. We used offspring rats born to dams that had been exposed to saline or various doses of MeHg (0.01 mg/kg/day, 0.1 mg/kg/day, and 1 mg/kg/day) from gestational day (GD) 11–21. Immunohistochemical examination of the brains of the offspring was conducted on postnatal day (PND) 0, PND3, and PND7. Our results showed that prenatal exposure to low levels of MeHg (0.1 mg/kg/day or 1 mg/kg/day) during the critical stage in neuronal migration resulted in migration defects of the cerebrocortical neurons in offspring rats. Importantly, our data revealed that the abnormal neuronal distribution induced by MeHg was not caused by altered proliferation of neural progenitor cells (NPCs), induction of apoptosis of NPCs and/or newborn neurons, abnormal differentiation of NPCs, and the morphological changes of radial glial scaffold, indicating that the defective neuronal positioning triggered by exposure to low-dose of MeHg is due to the impacts of MeHg on the process of neuronal migration itself. Moreover, we demonstrated that *in utero* exposure to low-level MeHg suppresses the expression of Rac1, Cdc42, and RhoA, which play key roles in the migration of cerebrocortical neurons during the early stage of brain development, suggesting that the MeHg-induced migratory disturbance of cerebrocortical neurons is likely associated with the Rho GTPases signal pathway. In conclusion, our results provide a novel perspective on clarifying the mechanisms underlying the impairment of neuronal migration induced by MeHg.

1. Introduction

Methylmercury (MeHg) is a toxic heavy metal which poses a serious threat to human health and ecological environments (Grandjean and Landrigan, 2006). The primary source of human expose to MeHg is *via* the consumption of seafood and fish products (Clarkson and Magos, 2006). Currently, exposure to low levels of MeHg is common amongst the majority of human populations (Mahaffey et al., 2011). Therefore, toxicological knowledge acquired under conditions of low-level MeHg exposure would be of value because this exposure protocol imitates the most common type of human exposure.

MeHg can readily pass the placental and the blood–brain barriers, and the developing nervous system is highly susceptible to the toxic effects of MeHg (Grandjean et al., 2010). In the poisoning incidents which occurred in Minamata, Japan and rural Iraq, many infants who had been maternal exposed to MeHg were severely affected, exhibiting ataxia, mental retardation, and epilepsy (Amin-Zaki et al., 1974; Bakir et al., 1973; Harada, 1995). Notably, histological examinations of two infantile brains from the Iraq outbreak revealed that the extensive disruption of the cytoarchitecture of the cerebrum was one of the major pathological changes, which was characterized by numerous ectopic neurons located in the cerebral white matter and irregular groupings of cortical neurons in many regions, indicating an apparent disturbance in migration of cerebrocortical neurons (Choi et al., 1978; Choi, 1989). Similar observations were made in two animal studies (Kakita et al., 2001, 2002) mimicking the circumstances underlying the Iraqi accident. There have also been several studies showing that MeHg can disrupt neuronal migration in the developing cerebral cortex in experimental animals (Peckham and Choi, 1988; Geelen et al., 1990) and in organotypic slices of human fetal cerebrum (Choi et al., 1981). These findings suggest that the migratory defect of cerebrocortical neurons is one of the principal reasons

Abbreviations: Rho, Ras homolog gene; Rac1, Ras-related C3 botulinum toxin substrate 1; Cdc42, cell division cycle 42; RhoA, Ras homolog gene family member A.

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contributing to MeHg-induced developmental neurotoxicity. Consequently, further investigation of the influence of MeHg exposure on the migration of cerebrocortical neurons is needed. Owing to the lack of available data, however, the specific effects of MeHg on neuronal migration in the developing cerebral cortex and the underlying mechanisms are unknown, especially at low exposure levels.

During the early stage of corticogenesis, the post-mitotic neurons produced in the ventricular zone (VZ) and subventribular zone (SVZ) migrate radially to the brain surface to form the cortical plate (CP) (Kriegstein and Alvarez-Buylla, 2009). A change in the number of neuronal cells resulting from altered proliferation of neural progenitor cells (NPCs), induced apoptosis of NPCs and/or newborn neurons, as well as abnormal differentiation of NPCs, may bring about a variation in the population of neurons which settle in their appropriate target layers, generating a phenotype similar to the aberrant neuronal migration. Thus, the impaired neuronal migration could be a secondary effect of the above three cases (Pinheiro et al., 2011; Zheng et al., 2011). In addition, the proper neuronal migration is dependent of the radial glial scaffold (Ayala et al., 2007), the morphological changes of which can also lead to the mis-oriented migration and displacement of newborn neurons. In this context, it is likely that MeHg-induced defective migration of cerebrocortical neurons is also associated with these aspects as mentioned above. Therefore, a series of studies should be conducted to verify whether or not these possibilities really exist.

Neuronal migration is strictly controlled by multiple molecular cues (Ayala et al., 2007). The Rho family of small GTPases, known to be crucial for cytoskeletal dynamics and cell motility (Heasman and Ridley, 2008), is one of the famous families of instructive signals. Among the Rho GTPases, Rac1, Cdc42, and RhoA are the best characterized members and play critical roles in regulating the migration of cerebrocortical neurons, namely facilitating radial migration by Rac1 and Cdc42 and restraining radial migration by RhoA (Govek et al., 2011). Strikingly, two recent studies (Fujimura et al., 2009; Fujimura and Usuki, 2012) have indicated that MeHg exposure *in vitro* influences the level of expression of Rac1, Cdc42, and RhoA in cultured cerebrocortical neurons. Based on the findings obtained from *in vitro* experiments, coupled with the roles of all three Rho GTPases on neuronal migration, it is reasonable to speculate that MeHg-induced migratory defects of cerebrocortical neurons seem to be connected to the varying levels of expression of Rac1, Cdc42, and RhoA.

Thus, in the current study we explored the effects of prenatal exposure to low-level MeHg on the migration of neonatal cerebrocortical neurons and determined whether or not the MeHg-induced anomalous positioning of cerebrocortical neurons is due to the impacts of MeHg on the process of neuronal migration itself or on changed proliferation of NPCs, increased apoptosis of NPCs and/or newborn neurons, abnormal differentiation of NPCs, or the morphological changes of radial glial scaffold. We also determined whether or not the Rho GTPases signaling pathway might be involved in MeHg-induced defects of cerebrocortical neuronal migration. Together, the current study provides new insight into understanding the mechanisms mediating the impairment of neuronal migration caused by MeHg.

2. Materials and methods

2.1. Chemicals and reagents

Methylmercury chloride (MeHgCl) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The MeHgCl was dissolved in normal saline at a concentration of 5 mg/ml as a stock solution. Fresh MeHg (as MeHgCl) solutions were prepared by diluting the stock solution with 0.9% saline according to the principle of identical volume injection of 1 ml/kg body weight. Plasmids expressing enhanced yellow fluorescent protein (EYFP) were kindly provided by Professor Xiaobing Yuan (Institutes of Neuroscience, Shanghai Institutes for Biological Science, Chinese Academy of Science, China). Fast Green, bromodeoxyuridine (BrdU), Triton X-100, bovine serum albumin (BSA), 4′,6-diamidino-2-phenylindole (DAPI), and all reagents for electrophoresis were purchased from Sigma Chemical Co. ECL kits were purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA).

2.2. Animals

Timed pregnant Sprague-Dawley rats were provided by SLAC Laboratory Animal Co. Ltd (Shanghai, China). The morning after mating (as evidenced by the presence of a vaginal plug) was designated gestational day (GD) 1. Parturition generally occurs on GD22 and the day of birth was designated as postnatal day (PND) 0. All animals were housed under a 12-h light/dark cycle with free access to food and water at all times. Food and water consumption, body weight, physical health status, and the behavioral performance of pregnant dams were monitored daily during the present study. The litter size at birth and the postnatal body weight of pups were also recorded. All animal handling procedures were performed under the guidelines and with the permission of the Animal Care and Administration Committee of the Institute of Neuroscience (Shanghai Institute for Biological Sciences of the Chinese Academy of Sciences).

2.3. Experimental protocol

To determine the effects of exposure to low levels of MeHg on neuronal migration in the developing cerebral cortex, pregnant rats were randomly divided into 4 groups (18 dams per group), and treated with 0.9% saline and 0.01 mg/kg/day, 0.1 mg/kg/day, or 1 mg/kg/day of MeHg, respectively. The doses of MeHg were chosen according to previous studies (Coccini et al., 2006; Dasari and Yuan, 2010; Kakita et al., 2000; Sakamoto et al., 2004), which suggested that the rat dams and the offspring exposed to the doses used in the present study remained physically and behaviorally normal, and did not show any significant histological changes in the cerebral cortex. From GD11–21, saline or MeHg were given to the rat dams once daily in a volume of 1 ml/kg body weight by intraperitoneal (ip) injection to ensure that the drug was accurately administered and absorbed effectively. The period of dosing was selected because the neuronal migration in the developing rat cerebral cortex occurs principally between GD13 and GD21 (Miller, 1986; Paxinos, 2004) and toxicokinetic data (Lewandowski et al., 2002) showed that the peak mercury (Hg) concentration was achieved in rat embryonic tissues within 48 h of dosing. Therefore, this exposure regimen allowed for maximal MeHg exposure during the critical window of cerebrocortical neuronal migration. All dams underwent *in utero* electroporation (IUE) at GD16 to label the cerebrocortical neurons of embryonic rats, which were fated to upper cortical layers. Postnatal rats from each group were perfused at PND0, PND3, and PND7 (6 litters per time point) and the brains successfully transfected with EYFP were collected (3 brains per litter). Thereafter, 30-μm sections were prepared and processed by immunohistochemistry (IHC; 3 equally spaced sections per brain) using antibodies against green fluorescence protein (GFP). For quantitative analysis of neuronal migration, a specific region of the neocortex was examined. The neocortex on the electroporated side was subdivided into three regions (layers II/III, layers IV–VI, and the VZ/SVZ/white matter [WM]). The number of EYFP-positive cells in different regions was counted and the ratio of EYFP-positive cells in each region-to-the total number of EYFP-positive cells was calculated as a percentage. Disruption of neuronal migration was evaluated by a change in the percent distribution of EYFP-positive cells. In addition, some sections of PND3 pup brains were used to analyze the impacts of MeHg exposure on differentiation of NPCs and the morphology of radial glial scaffold, and some offspring brains (3 brains per litter) were harvested for evaluation of the total brain Hg concentration, which is the best biomarker for Hg exposure (Burbacher et al., 1990; Newland and Reile, 1999).

To determine whether or not the aberrant neuronal migration caused by low levels of MeHg intoxication originated from the change in proliferation of NPCs, an additional 48 pregnant rats were randomized into 4 groups (12 dams per group) treated with the same dose and route of administration of MeHg (*vide supra*). Saline or MeHg were administered once daily from GD11–18. On GD18, all dams received a single ip injection of BrdU at a dose of 150 mg/kg body weight dissolved in 0.9% saline. The dose of 150 mg/kg was chosen because 150 mg/kg is roughly equivalent to the saturating dose for a single injection of BrdU in the rat and produces virtually no toxic effects (Taupin, 2007). Embryos were perfused 4 h and 24 h after BrdU injection (3 embryos per litter and 6 litters per time point). Previous studies (Miller, 1986, 1988; Paxinos, 2004) showed that neurogenesis of the developing rat cerebral cortex is most active during the period from GD13 to GD19. Therefore, this experimental scheme guaranteed sufficient MeHg exposure throughout the key stages of cortical neurogenesis. Furthermore, it has been reported that a single BrdU injection marks cells in the S-phase for approximately 5–6 h (Hayes and Nowakowski, 2000), and the duration of the S-phase and the total cell cycle length in cortical precursor cells of GD18 rat embryos were approximately 8 h and 17 h, respectively (Miller and Kuhn, 1995). Thus, the 4 h survival time is adequate for the incorporation of BrdU into rapidly proliferating cells in the S-phase, but not sufficient for the completion of cell division. Nevertheless, several studies (Lewandowski et al., 2003; Miller and Kuhn, 1995; Miller and Nowakowski, 1991) have indicated that alternations in the proportion of BrdU-positive cells may be more easily probed with a longer duration of labeling. Thereby, the 24 h survival time was selected in this study to mark more dividing cells, and even those that had sufficient time to go through a single