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Prenatal exposure to bisphenol A interferes with the development of cerebellar granule neurons in mice and chicken

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ABSTRACT

In mice, prenatal exposure to low doses of bisphenol A has been shown to affect neurogenesis and neuronal migration in cortex, resulting in disturbance of both neuronal positioning and the network formation between thalamus and cortex in the offspring brain. In the present study we investigated whether prenatal exposure to bisphenol A disturbs the neurodevelopment of the cerebellum. Two different model systems were used; offspring from two strains of mice from mothers receiving bisphenol A in the drinking water before mating, during gestation and lactation, and chicken embryos exposed to bisphenol A (in the egg) on embryonic day 16 for 24 h before preparation of cerebellar granule cell cultures. In the cerebellum, tight regulation of the level of transcription factor Pax6 is critical for correct development of granule neurons. During the development, the Pax6 level in granule neurons is high when these cells are located in the external granule layer and during their migration to the internal granule layer, and it is then reduced. We report that bisphenol A induced an increase in the thickness of the external granule layer and also an increase in the total cerebellar Pax6 level in 11 days old mice offspring. In cultured chicken cerebellar granule neurons from bisphenol A injected eggs the Pax6 level was increased day 6 in vitro. Together, these findings indicate that bisphenol A may affect the granule neurons in the developing cerebellum and thereby may disturb the correct development of the cerebellum.

1. Introduction

The developing brain depends critically on the generation of distinct classes of neurons at defined sites within the nervous system. Early developmental, tightly regulated processes including cell division, cell death, cell differentiation, and cell migration determine this. Since disturbance of any of the developmental processes may result in altered neurodevelopment (Rice and Barone, 2000), the developing brain is more vulnerable to neurotoxicants than the adult brain (Eriksson, 1997; Grandjean and Landrigan, 2006). There is concern about the increasing incidence of neurodevelopmental diseases in children (Schettler, 2001) and the possible contribution of pollutants in the daily environment to this increase (Herbert, 2010).

Bisphenol A (BPA) is used as a monomer in the production of polycarbonate plastic. Exposure measurement data from several countries indicate that humans are widely exposed to low levels of BPA on a continuous basis (vom Saal and Hughes, 2005). BPA passes through the blood–brain barrier and the BPA brain tissue content in rat was 18–41% of that in plasma (Kim et al., 2004). BPA also passes through the placenta (Schonfelder et al., 2002) and has been detected in the human amniotic fluid (Ikezuki et al., 2002), indicating significant fetal exposure. BPA is weakly estrogenic (Steinmetz

Abbreviations: BPA, bisphenol A; DIV, days in vitro; ED, embryonic day; EGL, external granule layer; H&E, hematoxylin and eosin; IGL, internal granule layer.

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et al., 1997), and estrogen receptors are expressed and functional in the developing cerebellum (Jakab et al., 2001). BPA has been reported to act as a disruptor of rapid estradiol-induced actions in the developing rat cerebellum (Zsarnovszky et al., 2005). Using a non-human primate model, Leranth and coworkers showed that BPA abolished the synaptogenic effect of estradiol in hippocampus and the prefrontal cortex (Leranth et al., 2008). BPA also mediates non-estrogenic effects in the central nervous system. For instance, prenatal exposure to BPA altered the pattern of neuronal migration during cortical histogenesis and induced morphological changes during development of the mouse forebrain by changes in the intracellular concentration of thyroid hormone (Nakamura et al., 2006). Prenatal BPA exposure has also been shown to cause persistent aberrations in learning, memory and spontaneous behavior in rodents (Tian et al., 2010; Viberg et al., 2011; Nakamura et al., 2012).

The cerebellar cortex is an important model system for the development of the central nervous system (Solecki et al., 2006). Recent studies provide evidence that the cerebellum, in addition to function in balance and motor control, is central to motor learning, sensory discrimination, and complex cognitive tasks. The granule cells of the cerebellum are the most numerous neurons of the entire brain, and this allows for purification of large numbers of granule cell progenitors for cellular and molecular studies (Solecki et al., 2006). Cerebellar granule neurons are generated in the external granule layer (EGL) of the cerebellum, which covers the outer surface of the cerebellar cortex. Rapid proliferation in the EGL during development generates millions of granule neurons in the cerebellar cortex. Proliferating granule neurons are located in the outer layer of the EGL whereas newly postmitotic granule neurons are located in the internal EGL (Engelkamp et al., 1999). The postmitotic neurons in the EGL migrate to the internal granule layer (IGL) and differentiate to mature neurons (Rakic, 1971; Hatten and Heintz, 1995). Cultured cerebellar granule neurons are widely used as a model system for evaluation of potential developmental neurotoxicity of chemicals since key processes in the development such as cell proliferation, migration and morphological/functional differentiation can be evaluated (Hogberg et al., 2009; Radio et al., 2010). Granule neurons at different stages of maturation express stage-specific genes (Hatten and Heintz, 1995; Hatten et al., 1997). Thus, it is possible to determine if the neurons are EGL-like or IGL-like. It has been reported that freshly plated purified granule neurons represent an EGL-like population, and that the neurons can be considered IGL like after 4 days in culture (Manzini et al., 2006).

The transcription factor Pax6 (a paired homeobox DNA binding protein) is expressed in specific spatiotemporal patterns during mammalian brain development and is involved in brain patterning, neuronal migration, and neural circuit formation (Osumi, 2001; Simpson and Price, 2002). Pax6 has been shown to be a key regulatory gene for the proper differentiation of granule cells (Swanson et al., 2005), and is necessary for polarization and migration of the granule neurons from the EGL to the IGL (Yamasaki et al., 2001). High levels of Pax6 are expressed in granule neurons in the EGL and under migration (Engelkamp et al., 1999). Lower levels of Pax6 are detected in mature granule neurons in the IGL (Yamasaki et al., 2001). BPA has been reported to affect Pax6 in different model systems (Yamamoto et al., 2007; Baba et al., 2009), however, effects on the cerebellar level of Pax6 has not previously been studied although several reports describe effects of BPA on the cerebellum (Nishizawa et al., 2005; Zsarnovszky et al., 2005; Yamaguchi et al., 2006; Kimura-Kuroda et al., 2007).

In this study we investigate how prenatal exposure to BPA affects the cerebellar morphology and the Pax6 level in cerebellar granule neurons using two different model systems; mice (BALB/cA and non diabetic NOD/ShiLtJ) and chicken embryos. Since BPA exposure has previously been reported to influence both the cerebellum and the Pax6 level, the hypothesis tested presently was that BPA disturbs the Pax6 level in the developing cerebellar granule neurons and that this is accompanied by changes in the morphology of the cerebellum.

2. Materials and methods

2.1. Reagents

Bisphenol A (2,2-bis-(4-hydroxyphenyl)-propane, BPA) was purchased from Wako Pure Chemical Industry (Osaka, Japan). Basal Eagle's medium (BME), penicillin/streptomycin, and horse serum were from Gibco (Paisley, UK). Pax6 primary antibody was obtained from Millipore (Billerica, USA), PCNA primary antibody from Dako (Glostrup, Denmark), whereas β-actin primary antibody was from Sigma (St. Louis, USA). Cy-3 conjugated mouse anti-rabbit was purchased from Jackson ImmunoResearch Laboratories (West Grove, USA). BCA protein assay kit and HRP-conjugated Goat Anti-Rabbit were from Pierce (Rockford, USA). Hoechst 33342 was obtained from Invitrogen (Oregon, USA). Pure nitrocellulose membrane was from Bio-Rad (Hercules, USA). All other reagents were from Sigma (St. Louis, USA).

2.2. Animals

BALB/cA mice obtained from Harlan Ltd. (Horst, The Netherlands) and non diabetic NOD/ShiLtJ (NOD) mice from the Jackson Laboratory (Maine, USA) were housed in the animal research facility at the Norwegian Institute of Public Health (Oslo, Norway). In accordance with the 3R-principle for the use of animals in research we utilized cerebella originating from mice used in two different diabetes studies, the first of which is now published (Bodin et al., 2013). The mice were kept in BPA-free disposable cages (Innovive, San Diego, USA) and isolated with minimal handling to avoid stress and minimize contact to pathogens. The female mice were given BPA in their drinking water for 1 week before mating and throughout gestation and lactation. They were fed a diet containing minimal phytoestrogens (2919X, Harlan Laboratories, Indianapolis, USA). BPA was dissolved in autoclaved distilled water heated to 60 °C, and kept in BPA-free water bottles. The mice had free access to food and water, were exposed to a 12 h light and 12 h dark cycle with 35–75% humidity, and the room temperature was 21 ± 2 °C.

The BPA concentrations in the drinking water were 0.1, 1 or 10 mg/l for the NOD mice, and 10 or 100 mg/l for the BALB/cA mice. The amount of water the mice drank was measured and the average BPA dose ingested by each mouse was calculated. The average ingested BPA dose for mice given 10 mg/l BPA in the drinking water was 1.4 mg/kg bodyweight/day both before and during the gestation and 3.9 mg/kg bodyweight/day during lactation. For mice given 100 mg/l BPA in the drinking water the average dose was 13.7 mg/kg bodyweight/day both before and during gestation and 38.9 mg/kg bodyweight/day during lactation. Control mice were given autoclaved distilled water. The BALB/cA offspring were decapitated day 7 and 11, the cerebellum was removed and either homogenized for western analysis or the whole brain was fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for immunohistochemistry. NOD offspring were decapitated day 8, 15, or 22, and the cerebellum was removed and homogenized for western analysis.

Eggs (Gallus gallus, weight 50–60 g) from different hatches were obtained from Samvirkekylling (Våler, Norway). The eggs were incubated at 38 °C in a Covatutto 20 + 20 incubator (Novital). A single dose of the test substance was injected via openings of about 1 mm in the eggshell into the amniotic cavity of the egg on embryonic day 16 (ED16). BPA was dissolved in ethanol and diluted with 0.9% saline before injection. The final concentration of BPA in the egg was ~1 μM (~0.23 mg/kg), a dose within the range of environmentally exposed humans (Midoro-Horiuti et al., 2010). The ethanol concentration was approximately 0.01 μl/g egg. Control eggs were either not injected or injected with ethanol to a final concentration of 0.01 μl/g egg. To make sure that only eggs with live embryos were used, spontaneous movement by the embryo was confirmed by transillumination before injection. Light transillumination was also done to avoid the blood vessels when injection was performed. Before preparation of cultures, the eggs were placed on crushed ice for 7 min to anesthetize the embryos. Cultures of cerebellar granule neurons were prepared on ED17. The granule neurons were isolated as previously described (Jacobs et al., 2006) and then grown in serum free conditions as follows: After isolation 1.7×10^6 cells/ml were seeded onto poly-L-lysine-coated dishes and incubated in BME supplemented with heat inactivated horse serum (10%), KCl (25 mM), glutamine (2 mM), insulin (100 nM), and penicillin/streptomycin (100 Units/ml Penicillin; 100 μg/ml Streptomycin) at 37 °C and 5% CO_2 for 24 h. Then the culture medium was replaced with serum-free BME supplemented with KCl (25 mM), glutamine (2 mM), penicillin/streptomycin (100 Units/ml Penicillin; 100 μg/ml Streptomycin), insulin (25 μg/ml), transferrin (100 μg/ml), T3 (1 nM), and selenite (30 nM) (Barthel et al., 1996). The concentrations given in the brackets were the final concentrations in the media. The neurons were maintained for up to 6 days. Cell death was measured by trypan blue exclusion day 6 in vitro (DIV6). This method is suitable for primary neurons since it offers visual control of cell morphology and cell type.

All animals were handled in accordance with the Norwegian Animal Welfare Act and the EU directive 2010763/EU for animal experiments, and approved by the local representative of the Norwegian Research Authority.