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Developmental exposure of aflatoxin B_1 reversibly affects hippocampal neurogenesis targeting late-stage neural progenitor cells through suppression of cholinergic signaling in rats

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ABSTRACT

To elucidate the maternal exposure effects of aflatoxin B_1 (AFB_1) and its metabolite aflatoxin M_1, which is transferred into milk, on postnatal hippocampal neurogenesis, pregnant Sprague-Dawley rats were provided a diet containing AFB_1 at 0, 0.1, 0.3, or 1.0 ppm from gestational day 6 to day 21 after delivery on weaning. Offspring were maintained through postnatal day (PND) 77 without AFB_1 exposure. Following exposure to 1.0 ppm AFB_1, offspring showed no apparent systemic toxicity at weaning, whereas dams showed increased liver weight and DNA repair gene upregulation in the liver. In the hippocampal dentate gyrus of male PND 21 offspring, the number of doublecortin$^+$ progenitor cells were decreased, which was associated with decreased proliferative cell population in the subgranular zone at $\geq$0.3 ppm, although T-box brain 2$^+$ cells, tubulin beta III$^+$ cells, gamma-H2A histone family, member X$^+$ cells, and cyclin-dependent kinase inhibitor 1A$^+$ cells did not fluctuate in number. AFB_1 exposure examined at 1.0 ppm also resulted in transcript downregulation of the cholinergic receptor subunit *Chrna7* and dopaminergic receptor *Drd2* in the dentate gyrus, although there was no change in transcript levels of DNA repair genes. In the hippocampal dentate hilus, interneurons expressing CHRNA7 or phosphorylated tropomyosin receptor kinase B (TRKB) decreased at $\geq$0.3 ppm. On PND 77, there were no changes in neurogenesis-related parameters. These results suggested that maternal AFB_1 exposure reversibly affects hippocampal neurogenesis targeting type-3 progenitor cells. This mechanism likely involves suppression of cholinergic signals on hilar GABAergic interneurons and brain-derived neurotrophic factor-TRKB signaling from granule cells. The no-observed-adverse-effect level for offspring neurogenesis was determined to be 0.1 ppm (7.1–13.6 mg/kg body weight/day).

Abbreviations: AFB_1, aflatoxin B_1; AFM_1, aflatoxin M_1; BDNF, brain-derived neurotrophic factor; BW, body weight; CA3, Cornu ammonis 3; CALB1, calbindin-D-28K; CALB2, calbindin-D-29K; *Cdkn1a*, cyclin-dependent kinase inhibitor 1A; *Chat*, choline *O*-acetyltransferase; CHRNA7, acetylcholine receptor, nicotinic, alpha 7 (neuronal); CNTF, ciliary neurotrophic factor; C_T, threshold cycle; DAB, 3,3′-diaminobenzidine; DCX, doublecortin; *Drd2*, dopamine receptor D2; GABA, γ-aminobutyric acid; GFAP, glial fibrillary acidic protein; γ-H2AX, gamma-H2A histone family, member X; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; GCL, granule cell layer; GD, gestational day; GFAP, glial fibrillary acidic protein; HPLC, high-performance liquid chromatography; *Hprt1*, hypoxanthine phosphoribosyltransferase 1; NTRK2, neurotrophic tyrosine kinase receptor, type 2; NeuN, neuron-specific nuclear protein; NOAEL, no-observed-adverse-effect level; PAX6, paired box 6; PCNA, proliferating cell nuclear antigen; PFA, paraformaldehyde; PND, postnatal day; PVALB, parvalbumin; $p21^{Cip1}$, cyclin-dependent kinase inhibitor 1A; RT-PCR, reverse-transcription polymerase chain reaction; SGZ, subgranular zone; SST, somatostatin; TBR2, T box brain 2; TRKB, tropomyosin receptor kinase B; TUBB3, tubulin, beta 3 class III; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

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1. Introduction

Aflatoxin is one of most toxic mycotoxins produced by the *Aspergillus* species, which is known to contaminate a variety of human and animal food stuffs, such as corn, nuts, and cereals (WHO, 2008). Among *Aspergillus* fungus, *Aspergillus flavus* and *Aspergillus parasiticus* produce aflatoxin B_1 (AFB_1), which is known to have the strongest toxic, carcinogenic, and mutagenic potential (Hussein and Brasel, 2001). Ingested AFB_1 is metabolized into isoforms of aflatoxin Q_1, aflatoxin P_1, and aflatoxin M_1 (AFM_1) in the liver, and AFM_1 has been shown to be transferred to breast milk (Lutz et al., 1980). AFM_1 also has genotoxicity and carcinogenicity *via* similar mechanisms to AFB_1, but with lower potency (Cullen et al., 1987; Hsieh et al., 1984). AFM_1 is considered to be the main exposure source during lactation, because AFB_1 is excreted into milk primarily in the form of AFM_1 (Van Egmond, 1989). Because AFM_1 contamination has been widely reported mainly in dairy milk (WHO, 2008), there is a health hazard with the infantile population.

Many toxicity studies using AFB_1 and AFM_1 have focused on carcinogenicity, genotoxicity, and hepatotoxicity, revealing chronic toxicity profiles (Hussein and Brasel, 2001; WHO, 2008). However, our knowledge about toxicity effects on central nervous systems during development remains limited. AFB_1 is considered to pass the placental barrier and damage the fetal nervous system; intraperitoneal AFB_1 exposure at the late gestation period in rats results in a variety of tumors in central and peripheral nervous systems in the offspring (Goerttler et al., 1980). Chentanez et al. (1986) reported that intraperitoneal exposure of AFB_1 at 2 mg/kg during gestational days 8–10 resulted in a transient decrease of locomotive activity in offspring, which was associated with neuronal degeneration in the brain. Reports have shown that subcutaneous exposure to AFB_1 during gestational days 11–14 in rats showed decreased neurobehavioral performance in the offspring (Kihara et al., 2000). Because of the genotoxicity health hazard potential of AFB_1 and AFM_1, as well as the possibility of maternal exposure, it is important to assess developmental neurotoxicity.

Neurogenesis is an important mechanism for brain development, as well as spatial and memory information in the adult brain (Pan et al., 2013). In the hippocampus, the subgranular zone (SGZ) of the dentate gyrus continuously produces new neurons throughout adult life (Kempermann et al., 2004). In the SGZ, type-1 neural stem cells differentiate into type-2a, type-2b, and type-3 proliferative progenitor cells. Type-3 progenitor cells differentiate into postmitotic immature granule cells and then, finally, into mature granule cells that populate the granule cell layer (GCL; Hodge et al., 2008). It is reported that γ-aminobutyric acid (GABA) ergic interneurons in the hilus of the dentate gyrus control granule cell differentiation and support the maintenance of appropriate granule cell population (Lussier et al., 2009; Masiulis et al., 2011). In addition to GABAergic neuronal inputs, the dentate gyrus receives various types of projections from other brain regions, such as cholinergic, dopaminergic, noradrenergic, serotonergic, and glutamatergic inputs (Freund and Buzsáki, 1996; Masiulis et al., 2011). Both cholinergic and glutamatergic inputs to the SGZ are important for maintaining adequate proliferation and differentiation of granule cell lineages (Cameron et al., 1995; Freund and Buzsáki, 1996). Previous studies have reported that AFB_1 chronic exposure decreases cholinergic and dopaminergic transmissions in the adult rat brain (Coulombe and Sharma, 1985; Egbunike and Ikegwuonu, 1984).

Recently, we showed that a number of chemicals known to have neurotoxic profiles had deleterious effects on proliferation and differentiation of progenitor cells in the SGZ (Shibutani, 2015). We previously reported aberrations in the number of interneuron subpopulations producing reelin or calcium-binding proteins, such as calbindin-D-28K (CALB1), calbindin-D-29K (CALB2), and parvalbumin (PVALB) (Shibutani, 2015). Adult neurogenesis is a multistep process comprising self-renewal of stem cells and the generation of progenitor cells, proliferation, differentiation, and formation of dendrites and axons (Hodge et al., 2008; Kempermann et al., 2004). Therefore, evaluating the effects on neurogenesis by analyzing populations of granule cells, in combination with interneurons, could reveal the mechanical targets of neurotoxins.

The present study was designed to examine the maternal exposure effect of AFB_1 on developmental neurotoxicity, focusing on hippocampal neurogenesis in rat offspring. The effect on offspring is regarded to be a reflection of transplacental AFB_1 exposure and lactational AFM_1 exposure. For risk assessment purposes, the dose–response information and reversibility of neuropathological effects on neurogenesis, with regard to distribution, proliferation, and apoptosis of granule cell lineages in the SGZ and distribution of GABAergic interneuron subpopulations in the dentate hilus, were examined at the end of developmental exposure and during the adult stage.

2. Materials and methods

2.1. Chemicals and animals

Aflatoxin B_1 (AFB_1; CAS No. 1162-65-8) was extracted from medial and mycelial fractions of cultivated *A. flavus* in M_1 medium and purified by high-performance liquid chromatography (HPLC) as previously described (De Jesus et al., 1988). According to the AOAC official method 970.44, the purity of aflatoxin B_1 was calculated to be approximately 90% according to absorption peak ratios of ultraviolet measurements on methanol (AOAC, 2005). Forty-eight pregnant Crl:CD (SD) rats were purchased from Charles River Japan (Yokohama, Japan) at gestational day (GD) 1 (appearance of vaginal plug was designated as GD 0). Animals were individually housed in polycarbonate cages with paper bedding until day 22 after delivery [postnatal day (PND) 22 (day of delivery was PND 0)]. Animals were maintained in an experimental animal room under the following conditions: $23 \pm 3\,^{\circ}C$ relative humidity, $55 \pm 15\%$ 12-h light/dark cycle. Animals were allowed access to a powdered basal diet (CRF-1; Oriental Yeast Co. Ltd., Tokyo, Japan) (AFB_1 concentration: under the detection limit of 5 ppb) until the start of AFB_1 exposure and tap water *ad libitum* during the experimental period. From PND 21 onwards, offspring were housed with three or four animals per cage and provided with a pelleted CRF-1 basal diet (AFB_1 concentration: under the detection limit of 5 ppb) and tap water *ad libitum*.

2.2. Experimental design

Pregnant rats were randomly assigned to four groups of 12 animals per group and treated with a diet containing 0, 0.1, 0.3, or 1.0 ppm AFB_1 from GD 6 to PND 21. The high AFB_1 dose was a dose determined to induce slight maternal toxicity according to the OECD guideline for the testing chemicals (Test No. 426: Developmental Neurotoxicity Study; OECD, 2007). For this purpose, a preliminary maternal exposure study, which consisted of groups of dams subjected to AFB_1 exposure by feeding at 0, 0.1, and 0.5 ppm, was conducted ($N = 3$ dams, 0 and 0.1 ppm groups; 4 dams, 0.5 ppm group). Because male offspring exposed to 0.5 ppm showed transiently suppressed body weight (BW) on PND 14, 1.0 ppm was selected as the highest dose level expected to show slight offspring toxicity. Lower doses were subsequently set to 0.3 and 0.1 ppm. In the preliminary study, AFM_1 concentrations in milk removed from offspring stomach on PND 14 ($N = 3$ each for 0 ppm controls and 1.0 ppm group) were measured using HPLC-mass