博士論文

Molecular and morphological analysis of developmental toxicity in the cerebral cortex upon *in utero* exposure to environmental chemicals

(胎仔期環境化学物質曝露による大脳皮質発生毒性の

分子・組織学的解析)

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Abstract

Developing brain is highly sensitive to environmental chemicals. The prevalence of developmental disorders has been suggested to be partly associated with exposure to the environmental chemicals. The cerebral cortex plays a key role in higher brain functions such as learning, memory and emotion. It is composed of highly organized cellular layers established by neuronal migration, an essential step during cerebral cortex development. Orchestrated formation of the cerebral cortex architecture is crucial for normal brain functions, and disruption in cortical organization is thought to cause cognitive impairment and neurological disorders. Thus, the objectives of this study were aimed to investigate whether prenatal exposure to environmental chemicals, bisphenol A (BPA) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), affected cerebral cortex development using neuronal migration as a representative endpoint of developmental neurotoxicity, and explore the molecular mechanisms underlying the developmental abnormities. It was found that prenatal exposure to low doses of BPA or TCDD interrupted cerebral cortex development which possibly results in abnormal higher brain function later in adulthood, suggesting the association between chemical exposure and abnormal behavior may have its origins at the early stage of brain development.

Abbreviations

5-HIAA	5-hydroxyindole-3-acetic acid
5-HT	Serotonin
ADHD	Attention Deficit Hyperactivity Disorder
ADI	Acceptable daily intake
ARNT	Aryl hydrocarbon receptor nuclear translocator
AhR	Aryl hydrocarbon receptor
AhRR	Aryl hydrocarbon receptor repressor
Aldh1a1	Aldehyde dehydrogenase family 1, subfamily A1
Apc	Adenomatosis polyposis coli
Atm	Ataxia telangiectasia mutated homolog
Atrx	Alpha thalassemia/mental retardation syndrome X-linked homolog
BDNF	Brain-derived neurotrophic factor
BPA	Bisphenol A
c-fos	FBJ osteosarcoma oncogene
Cdk4	Cyclin-dependent kinase 4
Cdk5	Cyclin-dependent kinase 5
Cdkl2	Cyclin-dependent kinase-like 2
Cdkl3	Cyclin-dependent kinase-like 3
Cdkn1b	Cyclin-dependent kinase inhibitor 1B
Comt	Catechol-O-methyltransferase
Cux2	Cut-like homeobox 2
Cyp1a1	Cytochrome P450, family 1, subfamily a, polypeptide 1
Cyp1b1	Cytochrome P450, family 1, subfamily b, polypeptide 1

DA	Dopamine
DAPI	4', 6-diamidino-2-phenylindole
DISC1	Disrupted in schizophrenia 1
DOPAC	3,4-dihydroxyphenylacetic acid
Drd1a	Dopamine receptor D1A
Drd4	Dopamine receptor D4
E	Embryonic day
Erdr1	Erythroid differentiation regulator 1
G530011O06Rik	RIKEN cDNA G530011O06 gene
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
Gad1	Glutamate decarboxylase 1
Gh	Growth hormone
Grin2a	Glutamate receptor, ionotropic, NMDA2A
HVA	Homovanillic acid
Hoxa5	Homeobox A5
IUE	*In utero* electroporation
Icam1	Intercellular adhesion molecule 1
Itga3	Integrin alpha 3
IZ	Intermediate zone
Kif2a	Kinesin family member 2A
Kitl	Kit ligand
LOAEL	Lowest observed adverse effect level
Lhx2	LIM homeobox protein 2
Lhx5	LIM homeobox protein 5

MZ	Marginal zone
Malat1	Metastasis associated lung adenocarcinoma transcript 1
Map2	Microtubule-associated protein 2
Mapt	Microtubule-associated protein tau
Mbnl1	Muscleblind-like 1 (Drosophila)
Mbnl2	Muscleblind-like 2 (Drosophila)
Mdga2	MAM domain containing glycosylphosphatidylinositol anchor 2
NMDR	Non-monotonic dose responses
Ncam1	Neural cell adhesion molecule 1
Ntrk2 (TrkB)	Neurotrophic tyrosine kinase, receptor, type 2
Otx2	Orthodenticle homolog 2
PBS	Phosphate-buffered saline
PBST	PBS containing 0.05% Triton X-100
PCB	Polychlorinated biphenyls
PCDD	Polychlorinated dibenzo-p-dioxins
PCDF	Polychlorinated dibenzofurans
PFA	Paraformaldehyde
Pf4 (Cxcl4)	Platelet factor 4
Pisd-ps3	Phosphatidylserine decarboxylase, pseudogene 3
Pitx3	Paired-like homeodomain transcription factor 3
RFP	Red fluorescent protein
Ralgapa1	Ral GTPase activating protein, alpha subunit 1
Reln	Reelin
Rhox4	Reproductive homeobox 4A
Robo1	Roundabout homolog 1

SP	Subplate
SVZ	Subventricular zone
Sema3g	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3G
Slc15a2	Solute carrier family 15 (H+/peptide transporter), member 2
Slc6a3	Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3
Slc6a4	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4
Slitrk1	SLIT and NTRK-like family, member 1
Stxbp1	Syntaxin binding protein 1
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TDI	Tolerable daily intake
TEF	Toxic equivalency factor
TEQ	Toxic equivalency
Th	Tyrosine hydroxylase
Tle3	Transducin-like enhancer of split 3, homolog of Drosophila E (spl)
Top2b	Topoisomerase (DNA) II beta
Trip11	Thyroid hormone receptor interactor 11
Ube3a	Ubiquitin protein ligase E3A
VZ	Ventricular zone
Vav3	Vav 3 guanine nucleotide exchange factor
Wnt1	Wingless-related MMTV integration site 1
XRE	Xenobiotic-responsive elements
Xlr3b	X-linked lymphocyte-regulated 3C

Chapter 1 General Introduction

Developing brain is highly susceptible to various external stresses. Occurrence of serious diseases caused by environmental pollution has been considerably reduced in modern societies due to the implementation of legislative and administrative actions. However, the prevalence of developmental disorders that cannot be fully explained by genetic factors or expansion of diagnostic criteria, have been suggested to be partly associated with environmental chemical exposure (Grandjean and Landrigan, 2014). Recent statistics showed that approximately 6.5% children in elementary and junior high school in Japan had substantial learning difficulty or behavior problems (MEXT, 2013), and approximately 15% of children in the United States had one of the developmental disabilities including attention deficit hyperactivity disorder (ADHD), learning disability and autism (Boyle et al., 2011). Actually, pregnant women are exposed to a variety of chemicals, such as polychlorinated biphenyls, pesticides, phenols, phthalates, polycyclic aromatic hydrocarbon, perchlorate, lead, mercury and toluene (Woodruff et al., 2011), and many of these chemicals can pass through the placental barrier and enter into the fetal bloodstream (Barr et al., 2007). The low-dose environmental chemical exposure that do not induce manifest toxicity in dams could have adverse outcomes in offspring resulting in irreversible changes of brain function (Eriksson, 1997). A great number of epidemiologic studies have suggested a possible

link of the environmental chemical exposure during embryonic period or early childhood with neurodevelopmental disorders such as learning disability, autism, and ADHD (Colborn, 2004). Among various kinds of environmental chemicals, bisphenol A (BPA) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are suggested to influence the higher brain functions (described in the Section 2.1.2 and Section 3.1.3, respectively). Therefore, in my thesis, the neurodevelopmental toxicity of BPA and TCDD were studied.

Cerebral cortex plays a crucial role in higher brain functions. It consists highly organized 6 cellular layers having distinct populations of neurons and unique morphologies. The precisely laminated structure was formed by neuronal migration, an essential process extremely important for brain functions. Excitatory projection neurons are born in the ventricular zone (VZ) and migrate radially to the surface of the brain in a birth-date-dependent “inside-out” manner to form the cortical plate (CP) (Molyneaux et al., 2007, Kwan et al., 2012). Unlike the excitatory projection neurons, GABAergic interneurons are generated mainly within the medial and caudal ganglionic eminences and adopt tangential migration to enter the cortex (Molyneaux et al., 2007, Kwan et al., 2012). Any disruption during the complex dynamic processes of neuronal migration is considered to be responsible for susceptibility to neuronal migration disorders (NMDs), including lissencephaly, schizencephaly, heterotopia, polymicrogyria, pachygyria, and

polymicrogyria, that are associated with cognitive impairments (Verrotti et al., 2010, Liu, 2011). Genetic abnormities and environmental factors are suggested to account for NMDs (Metin et al., 2008). Neuronal migration is guided by various molecular cues, such as Reelin, a crucial factor for cortical lamination. Reelin deficiency exhibits disturbed cortical structure as in reeler mouse, and has been associated with several neurodevelopmental diseases, such as schizophrenia and autism (Fatemi, 2001). Environmental factors interacted with intrinsic genes also can interrupt neuronal migration. Disc1, a crucial gene for neuronal migration (Bradshaw and Porteous, 2012), can be affected by the lead (Abazyan et al., 2013). Several studies using animal models showed association of the environmental chemicals exposure with neuronal migration. For example, prenatal methylmercury exposure altered neuronal migration in the developing cerebral cortex in rats (Guo et al., 2013) and inhibited the migration of cerebellar granule cells during early postnatal development in mice (Fahrion et al., 2012). In a mouse model prenatally exposed to cocaine, both radial and tangential migration of neurons were interrupted in the embryonic mouse forebrain (McCarthy et al., 2011). Toluene-exposed rat model revealed abnormal neuronal proliferation and migration in somatosensory cortex (Gospe and Zhou, 2000).

Environmental chemicals like BPA and TCDD have been suggested to impair brain development as well as cognitive function (described in the Section 2.1.2 and Section

3.1.3, respectively), but how these chemicals disturb the process of brain development in embryonic period is largely unknown. Neuronal migration, an important process for cerebral cortex construction, is suggested to be a target for chemical exposure but has not been widely studied in identification of developmental toxicity. Thus, the objective of this study was proposed to investigate whether prenatal exposure to low doses of BPA or TCDD affects cerebral cortex development using neuronal migration as representative endpoint of developmental neurotoxicity with the supposition that there may be the underlying morphologically abnormal alterations which can be correlated with the abnormal phenotype of higher brain function, and explore the molecular mechanisms underlying the developmental abnormities in the brain.

Chapter 2 Bisphenol A

2.1 Introduction

2.1.1 BPA and human exposure

Bisphenol A (BPA, 4,4'-dihydroxy-2,2-diphenylpropane), a high volume chemical produced worldwide, is used as a monomer to manufacture polycarbonate plastics (approximately 65% of consumption), epoxy resins (approximately 30% of consumption), and as an additive to other plastics (Burridge, 2003). Global BPA production in 2002 was 2.8 million metric tons, and an estimated 5.5 million metric tons in 2011 (Bailin et al., 2008). A variety of consumer products, i.e., baby bottles, water bottles, food cans, tableware, dental sealants, thermal paper, compact discs, cell phones and computers, are composed of BPA. Thus, during the production, use and waste disposal processes, BPA can leach into food commodities, as well as the environment. The main route of BPA exposure is through diet which was estimated to contribute for more than 90% of total BPA exposure (Geens et al., 2012).

BPA was approved by the US Food and Drug Administration (FDA) in 1960s, but worries about the safety of BPA on human health have been raised in recent years. BPA has been assessed for its possible health risk by World Health Organization and domestic governmental health authorities. FDA's current perspective is that the low levels of BPA in foods is safe, but may have potential health effects on fetus and

children, because quite a few experimental studies reported that environmentally relevant levels of BPA given to pregnant animals may induce alterations in the developing organs, including brain in the offspring as described in the following Section 2.1.2, although some studies claimed that such observations could not be reproducible.

Humans have been extensively exposed to BPA, which was detected in 92.6% of the U.S. population (Calafat et al., 2008). Potential dietary exposure was 0.01-4.5 μg/kg b.w./day for infants from 0 to 6 months of age; 0.1-3.0 μg/kg b.w./day for infants from 6 to 36 months of age; 0.2-1.9 μg/kg b.w./day for children over 3 years of age (WHO, 2010b). Human exposure to BPA in the general population has been estimated to 0.16 μg/kg b.w./day in the U.S., and 0.048-0.08 μg/kg b.w./day in Japan (WHO, 2009). The human exposure levels are much lower than the current acceptable daily intake (ADI) of BPA, which is 50 μg/kg b.w./day in U.S., Europe, and Japan, based on the lowest observed adverse effect level (LOAEL) of 50 mg/kg b.w./d in animal studies (Richter et al., 2007). Thus, it is important to determine the health effects upon environmental low doses of BPA exposure.

2.1.2 Toxicity of BPA

A large number of epidemiological studies show that BPA exposure has been linked to adverse health outcomes (Rochester, 2013), including decreasing probability of

fertilization, reduction sperm quality, alteration sex hormone concentration, premature deliveries, metabolic diseases, cardiovascular diseases, childhood wheeze/asthma, and childhood behavioral abnormalities.

Considering BPA has been widely detected in urine and serum of pregnant women (Lee et al., 2008, Padmanabhan et al., 2008, Wolff et al., 2008, Woodruff et al., 2011) as well as umbilical cord blood of newborn infants (Lee et al., 2008), increasing studies have been focused on the potential effects of BPA exposure during critical periods of development. Developing brain is considered to be a target of BPA. BPA concentrations were found higher in fetal brains than in liver or serum in rats after a single dose exposure (Doerge et al., 2011). Exposure to BPA in the developmental period of life was considered to be associated with learning and behavioral problems in children (Harley et al., 2013, Hong et al., 2013). In animal studies, maternal BPA exposure disrupted spatial learning and decreased exploratory behavior (Galea and Barha, 2011, Jasarevic et al., 2011), impaired memory and learning (Miyagawa et al., 2007), increased in anxiety-like behavior (Matsuda et al., 2012), caused hyperactivity (Ishido et al., 2004), and enhanced contextual fear memory (Matsuda et al., 2013). In our laboratory, adult male mice born to BPA-exposed dam were found to exhibit impulsive behavior (Miyazaki W, et al. unpublished). However, the mechanisms of BPA induced developmental neurotoxicity are still largely unknown. In contrast, there are studies from other laboratories, which

claimed a large-scale animals studies did not produce any abnormalities in offspring born to dams exposed to BPA by a wide range of BPA (Tyl et al., 2002, Gray et al., 2004).

2.1.3 Objectives

The present study was attempted to elucidate the possible effects of maternal low-dose BPA exposure on the process of brain development and the underlying mechanisms.

2.2 Methods

2.2.1 Chemicals

BPA was purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2.2 Animals and exposure

Pregnant ICR mice were purchased from CLEA Japan (Tokyo, Japan). The day of vaginal plug observation was designated as E0.5. Mice were housed in an animal room maintained with temperature at 22-24˚C, humidity at 40-60%, and under 12h light : 12h dark cycles (lights on at 8:00 am). Food (Labo MR Stock, Nosan, Yokohama, Japan) and water were provided *ad libitum*. The pregnant mice were exposed to BPA at a dose

equivalent to 0, 40 or 400 μg/kg b.w./day from E12.5 to E18.5. BPA exposure was performed by implanting an osmotic pump (Alzet, Micro-Osmotic Pump, Model 1007D, Cupertino, CA, USA) according to the manufacturer's instructions. Briefly, the pump was filled with BPA solution or vehicle (DMSO) by a syringe and placed in PBS at 37˚C for 4 h before implantation. After mouse was anesthetized, an incision was made in the abdomen, and the filled osmotic pump was inserted into the peritoneal cavity. Finally, the musculoperitoneal layer and the skin were closed with needled sutures and wound clips, respectively. According to a peer review of National Toxicology Program's report, 'low dose' refers to doses resulting in biologic responses close to that occurred in human or doses lower than those used in standard toxicological testing for evaluating reproductive and developmental toxicity (Melnick et al., 2002). Based on the current LOAEL of BPA, 5 mg/kg/day is used as the cutoff dose for low-dose effects, regardless of the routes and durations of exposure (Melnick et al., 2002). Thus, in this study, I selected the doses which can be considered as low doses of 40 μg/kg b.w./day and 400 μg/kg b.w./day. According to the doses given to the dams, the control and BPA exposed groups were named as Control, BPA40, and BPA400, respectively. All animal experiments were performed according to the guidelines of the Animal Care and Use Committee of the Graduate School of Medicine, the University of Tokyo.

2.2.3 Plasmids

Plasmids (pCAG-mCherry, a kind gift from Dr. Masanori Matsuzaki at Division of Brain Circuits, National Institute for Basic Biology, Okazaki, Japan) were purified using the EndoFree Plasmid Kit (Qiagen, Hilden, Germany) according to the manufacture's protocol. Purified plasmids were diluted with PBS to a final concentration of 3μg/μl before use.

2.2.4 *In utero* electroporation

In utero electroporation (IUE) was performed as described previously (Tabata and Nakajima, 2001, Saito, 2006, Tabata and Nakajima, 2008). Briefly, timed-pregnant mice were deeply anesthetized by intraperitoneal injection of pentobarbital sodium (50mg/kg b.w., Dainippon Sumitomo Pharma, Osaka, Japan). After the uterus was carefully pulled out from the abdominal cavity, about 1 μl plasmid solutions colored by 0.01% Fast Green were injected into pup's lateral ventricle, and electroporation (30-35 V, 50 ms, 4 pulses) was delivered by a Square Wave Electroporator (CUY21SC, Nepa Gene, Chiba, Japan) with a forceps-type electrode (CUY650P5). Then, the uterus was replaced into abdominal cavity. Abdominal wall and skin were closed with sutures.

2.2.5 Brain sections preparation and staining

The embryos were anaesthetized in crushed ice. Brains were dissected, fixed with 4% paraformaldehyde (PFA) overnight at 4˚C, and sank in 20%, 30% sucrose in PBS at 4˚C. Then, brains were embedded in OCT compound (Sakura, Tokyo, Japan), frozen on a metal block cooled with liquid nitrogen, and stored at -80˚C until analysis. Frozen brains were cut into 20μm-thick coronal sections using a cryostat (CM3050S, Leica Microsystems, Wetzlar, Germany).

For Map2 immunostaining, the brain sections were washed in PBS containing 0.05% Triton X-100 (PBST) and fixed in 4% PFA for 10 min. After blocked with 3% bovine serum albumins (BSA) in PBST at room temperature for 1 hr, brain sections were incubated with Anti-MAP2 antibodies conjugated to Alexa Fluor 488 (Merck Millipore, Billerica, MA, USA) for 3 hr. Following additional PBST washes, the sections were mounted using mounting medium with 4', 6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). Brain sections were protected from light throughout the procedure.

Images were acquired using Leica microscope (DM6000 B, Leica Microsystems, Wetzlar, Germany), processed with Neurolucida (MBF bioscience, Williston, VT, USA) and Image-J (NIH, USA) software. Brains successfully transfected with fluorescent protein vectors and exhibited bright fluorescence in the lateral neocortex were selected

for further analysis. In each litter, 1-3 mouse embryonic brains were used for analysis.

2.2.6 Analysis of cell distribution

Cell distribution was evaluated using a bin analysis as described previously (Tomita et al., 2011). The CP was equally divided into 10 bins which were numbered as Bin 1 (the bin closest to the ventricle) to Bin 10 (the bin closest to the pia mater). The relative distance (Relative distance = the distance of the nucleus from the top of the SP / the thickness of CP) was utilized to distribute the mCherry-positive cells to each bin. For example, bin 1 included the cells whose relative distance ranged from 0 to 0.1. Bin 10 included cells whose relative distance ranged from 0.9 to 1. In each bin, the number of mCherry-positive cells was estimated as a percentage of the total number in all 10 bins. All measurement was determined by Image-J software (NIH, USA).

2.2.7 Tissue preparation

For monoamine and RNA analyses, one male and one female pup were randomly selected from each litter at P0, and a total 6 pups per sex in each dosed group were used. Brains were collected and dissected into the forebrain and mid-hindbrain (including midbrain and hindbrain), snap frozen in liquid nitrogen, and stored at -80˚C until analysis.

2.2.8 Monoamine detection

Dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), serotonin (5-HT) and its metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) were analyzed using high performance liquid chromatography (HPLC, Waters 2690, Milford, MA, USA) with electrochemical detector (Coulochem Ⅱ,ESA, Sunnyvale, CA, USA), according to the method described previously (Takeda et al., 1990). Briefly, brain tissues were homogenized in the solution containing 2.0 mM sodium hydrogensulfite, 0.1 M perchloric acid, and 0.02 mM EDTA. Homogenized samples were centrifuged at 15,000 x *g* for 5 min at 4˚C. The supernatants were removed to a centrifugal filter (low-binding hydrophilic PTFE membrane, 0.45μm, Millipore, Billerica, USA) and centrifuged at 12,000 x *g* for 2 min at 4˚C. The purified filtrates were injected to HPLC and monoamines were separated on a reversed-phase column (Acclaim 120, C18, 3 μm, 3.0 x 75 mm, Dionex) using the mobile phase, consisting of 0.1 M disodium hydrogenphosphate 12-water, 0.05 M citric acid monohydrate, 3.6 mM 1-heptanesulfonic acid sodium salt, 0.1 mM ethylenediamine-N,N,N',N'-tetraacetic acid disodium salt, 0.026% (v/v) acetonitrile and 0.063% (v/v) methanol. The column temperature was maintained at 20˚C, and the flow rate was 0.4 ml/min. The electrochemical detector conditions were as follows: guard cell (model 5020) potential, E = 450 mV; analytical cell (model 5011) potentials, E1 = -50 mV, E2 = 400 mV;

sensitivity, 5 μA. The protein contents of brain tissues were determined using BCA protein assay kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Concentration of monoamines and their metabolites were presented as μg/mg protein. Ratios of metabolite / monoamine were calculated as an index of turnover for DA and 5-HT (Scholl et al., 2010, Matsuda et al., 2013).

2.2.9 RNA quantification

Total RNAs were extracted from mice brain using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA synthesis was performed using PrimeScript RT reagent Kit (Takara, Otsu, Japan). Quantitative real-time PCR was performed using the Thunderbird qPCR mix (Toyobo, Osaka, Japan) and LightCycler (Roche, Basel, Switzerland). Primer design and specificity check were performed by the Primer-BLAST (NCBI, USA). Primer sequences are listed in Table 1. To confirm no cross-contamination, negative control was analyzed per RCR batch. The mRNA expression of the target gene in each sample was normalized with GAPDH.

2.2.10 Statistical analysis

For comparison two groups, Student's *t*-test was used. For comparison more than two groups, one-way or two-way ANOVA followed by Tukey-Kramer's or Bonferroni's *post*

hoc test was performed. All data are expressed as mean ± SEM. The p-values less than 0.05 were considered statistically significant.

2.3 Results

2.3.1 Litter size and body weight

Table 2 summarized the data on litter size and body weight of pups at P0. There was no significant difference in little size of pups born to control or BPA exposed dams (total, P=0.63; male, P=0.12; female, P=0.46). Although an increasing tendency of body weight was observed in BPA40 and BPA400 groups, there was no significantly statistical difference in body weight of both male and female born to BPA treated dams compared with the control group (total, P=0.11; male, P=0.09; female, P=0.16).

2.3.2 Effects of *in utero* exposure to BPA on neuronal migration

To investigate whether *in utero* BPA exposure affects neuronal migration, IUE was used to introduce fluorescent protein expression vectors (pCAG-mCherry) into neural progenitor cells to visualize the process of migration. IUE was performed at E14.5 and the distribution of mCherry positive neurons were analyzed at E18.5 in the three groups (Control, BPA40, and BPA400) (Fig. 1A). At E18.5, most of mCherry positive neurons were observed in layer II/III of the control group. mCherry positive neurons that entered

the CP were analyzed using a bin analysis (Fig. 1B). The percentage of mCherry positive neurons in Bin 9 (cortical layer II/III) of BPA40 group was significantly decreased compared with the control group and BPA400 group. No significant difference was found in BPA400 group compared with the control group (Fig. 1D). These data suggest that prenatal BPA exposure interrupted neuronal migration in dose-specific manner.

2.3.3 Effects of *in utero* exposure to BPA on monoamine concentration

The next question I addressed was whether prenatal BPA exposure altered monoamine level in the embryonic brain. To test this hypothesis, monoamine levels in pups' brain were examined at P0. Dopamine, serotonin and their metabolites were detected in forebrain and mid-hindbrain (including midbrain and hindbrain), respectively. In male mice, levels of dopamine and its metabolites (DOPAC, HVA) in the forebrain were significantly increased in BPA400 group compared with BPA40 group (Fig. 2A, B, C), but there was no significant difference in mid-hindbrain (Fig. 2F, G, H). Dopamine turnover, i.e., ratio of DOPAC to DA, and that of HVA to DA, did not have significant difference among three groups both in forebrain (Fig. 3A, B) and mid-hindbrain (Fig. 3D, E). Levels of serotonin and its metabolite (5-HT) did not have significant difference among control, BPA40 and BPA400 groups in both forebrain and

mid-hindbrain (Fig. 2D, E, I, J), but serotonin turnover, i.e., ratio of 5-HIAA to 5-HT, in the hindbrain was significantly decreased in BPA400 group compared with the control group (Fig. 3F). In female mice, no significant difference in the concentration of dopamine, serotonin and their metabolites was found in forebrain and mid-hindbrain among the three groups (Fig. 4A-J). Dopamine and serotonin turnover also did not show any significant difference in forebrain, and mid-hindbrain among three groups (Fig. 5A-F). These data suggest that prenatal BPA exposure altered monoamine levels in a sexually dimorphic manner.

2.3.4 Effects of *in utero* exposure to BPA on mRNA expression

Next, the mRNA expression of genes required for migration and layer formation (BDNF, TrkB, Reelin, Cdk5, DISC1, Cdkl3, Itga3, Robo1), laminar specific genes in the mouse neocortex of layer II/III (Tle3, Kitl, Lhx2, Cux2, Slitrk1) or layer I (Lhx5), gene as an indirect marker of neuronal activity (c-fos) and genes involved in dopaminergic neuron development (Wnt1, Otx2, Drd1a, Drd4) were analyzed in male mice forebrain (Fig. 6). TrkB, a receptor for neurotrophins, was significantly increased in the BPA400 group compared with the control group. Robo1, a neuronal guidance receptor gene, was significantly increased in the BPA40 group compared with the BPA400 group. There was no significant difference in mRNA expression of other genes

(BDNF, Reln, Cdk5, DISC1, c-fos, Cdkl3, Lhx5, Tle3, Itga3, Cux2, Kitl, Lhx2, Slitrk1, Drd1a, Drd4, Wnt1, Otx2) among the three groups.

2.4 Discussion

In the present study, IUE method was used to visualize and quantitatively evaluate migration of newborn neurons to the appropriate layer of the cerebral cortex. As a result, prenatal BPA exposure produced significant effects on developmental processes of cerebral cortex. The delayed neuronal migration may lead to malposition of projection neurons, suggesting potential impairment for neuronal projection in BPA-treated mice brains. This study is probably one of the few studies to show the potential use of the IUE method in the toxicology research fields. In the mammalian forebrain, Robo signaling regulates the correct position of pyramidal neurons (Gonda et al., 2013) and plays a key role in the axonal path finding (Andrews et al., 2006, Lopez-Bendito et al., 2007). Incorrect position of projection neurons may associate with the abnormal Robo1 signaling in the BPA40 group. However, whether the decrease in neuronal migration was a transient delay or a permanent deficit is unclear, that needs more future studies.

Our laboratory found that adult male mice born to BPA-exposed dams (40 μg/kg b.w./day) significantly exhibited impulsive behavior compared with the control group (Miyazaki W, et al. unpublished). The abnormal neuronal migration in embryonic brain

and impulsive behavior in adulthood are notable for their consistency, in both of which BPA40 group but not BPA400 group, showed significant difference compared with the control group. These results indicate that BPA may affect the cerebral cortex development in non-monotonic dose responses (NMDR). Quite a few studies on low-dose effects showed that the dose-response curve has an ‘inverted U-shape’ and cannot be simply applied to derive monotonic dose-depending effects (Myers et al., 2009, Vandenberg et al., 2012, Vandenberg et al., 2013). The non-monotonic dose responses curve with the mid-range BPA exposures associated with increased weight of newborns has also seen in human case (Philippat et al., 2012). However, the mechanism of BPA’s effects on neuronal migration and animal behavior in low dose specific manner (40 μg/kg b.w./day) is not fully understood. Compensatory mechanisms might have been triggered to offset the effects of the high dose BPA exposure (400 μg/kg b.w./day), such as increased expression of TrkB in forebrain found in the present study, a receptor for neurotrophins, which mediates neuronal migration, differentiation and survival with beneficial, trophic effects (Medina et al., 2004). Further studies are needed to elucidate the molecular mechanisms underlying the impairment of neuronal migration caused by low-dose BPA exposure, and the relationship between defective neuronal migration and impulsive behavior.

Prenatal BPA exposure was reported to accelerate neuronal differentiation and

migration (Nakamura et al., 2006). The present study shows that prenatal BPA exposure decreased neuronal migration in the cerebral cortex. The different outcomes may result from different dose and duration of BPA exposure, and different experimental method. In the previous study (Nakamura et al., 2006), BPA was given to dams at a daily dose of 20 μg/kg b.w. from E0, and the increased neuronal migration was observed in cortical deep-layer neurons labeled at E12.5. In the present study, BPA was administered at a daily dose of 40 μg/kg b.w. from E14.5 to E18.5 which was an important time period for the layer II/III neurons birth and migration, and the decreased neuronal migration was found in cortical upper-layer neurons labeled at E14.5. The BrdU method used in the previous study (Nakamura et al., 2006) can label all the proliferating cells in developing brain including excitatory projection neurons and GABAergic interneurons, making it difficult to discriminate the source of BrdU positive cells, while the IUE method used in the present study has an advantage in terms of labeling specific types of cells during a specific period of brain development. The present study is probably the first to show that *in utero* exposure to BPA altered radial migration of projection neurons of layer II/III. However, whether migration of other types of neurons such as early-born projection neurons (born at E12.5-E14.5), GABAergic interneurons, dopaminergic neurons is affected by prenatal BPA exposure needs further studies.

Because dopamine and serotonin system can also influence neuronal migration

(Riccio et al., 2009, Vasudevan et al., 2012) and mediate impulsive behavior (Dalley and Roiser, 2012), the neurotransmitter concentrations were determined in this study. To our knowledge, the present study is few in number to examine the effects of *in utero* BPA exposure on monoamine concentration in newborn mice, most of which were in adult mice. DA and its metabolites in forebrain were significantly increased in BPA400 group compared with BPA40 group, suggesting dose specific manner underlying BPA induces toxicity. Serotonin turnover (ratio of 5-HIAA to 5-HT) was significantly decreased in BPA400 group compared with control group. Reduced serotonin turnover in central nervous system was reported in children with dopa-nonresponsive dystonia (DND), the childhood dystonia that does not respond to treatment with levodopa (Assmann et al., 2002), but the mechanisms of the reduction of serotonin turnover in DND are unknown. BPA's effects on monoamine levels were observed in male mice, but no significant difference was found in female mice, indicating sex specific effects of BPA. The sex specific effects have also been reported in children study in which prenatal urinary BPA concentrations were associated with increased internalizing problems such as anxiety, depression in boys at age 7, but no association were seen in girls (Harley et al., 2013). The present studies suggest that BPA affects brain neurotransmitter system as early as in newborn mice and in a sex specific manner. Further studies are needed to clarify which brain regions such as the neocortex,

hippocampus, striatum and hypothalamus are vulnerable to BPA induced alteration in monoamine levels.

To date, little is known about how prenatal BPA exposure affects the developing brain, but the data obtained in my thesis work may support the possibility that abnormal behaviors observed in adulthood have fetal origins of perturbation in neuronal migration, and related metabolic alteration in monoamine levels, both of which were induced by *in utero* exposure to BPA.

Chapter 3 Dioxins

3.1 Introduction

3.1.1 Dioxins and related compounds

Dioxins and related compounds are a group of persistent organic pollutants (POPs) including 75 isomers of polychlorinated dibenzo-p-dioxins (PCDDs), 135 isomers of polychlorinated dibenzofurans (PCDFs), and 209 isomers of polychlorinated biphenyls (PCBs). These compounds are composed by two benzene rings joined by two oxygen atoms in PCDDs, one oxygen atom in PCDFs, or a single carbon bond in PCBs. Nonortho-substituted PCBs are called coplanar or dioxin-like PCBs. Among the 419 congeners, a total of 29 congeners, consisting of 7 PCDDs, 10 PCDFs, and 12 PCBs, are named as dioxins because they have toxicity more or less similar to the one that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has (Van den Berg et al., 1998). For risk assessment and management purposes, the World Health Organization (WHO) have adopted the concept of toxic equivalency factors (TEFs) for each dioxin congener relative to TCDD, the most toxic dioxin congener as prototype (TEF=1). The criteria for inclusion of a compound in the TEF concept are four-fold. First, a given chemical shows a structural relationship to TCDD. Second, it binds to the AhR (AhR was described in Section 3.1.4) to a certain extent comparable to TCDD. Third, it elicits AhR-mediated biochemical and toxic responses. Fourth, it is persistent and accumulates

in the food chain. (Van den Berg et al., 1998, Van den Berg et al., 2006). Since a variety of dioxin congeners are simultaneously present in the environment and food commodities, it is a sort of science-based practical judge to estimate a total of dioxin exposure level by this concept.

3.1.2 Human exposure

Dioxins release into the environment through burning waste, smelting, chlorine bleaching of paper pulp, manufacturing of some herbicides and pesticides, forest fires, and volcanic eruptions. Total releases of dioxins have dramatically decreased in recent years. In Japan, approximately 98% of dioxins emission has been reduced between 1997 and 2010 (Ministry of the Environment Japan, 2012).

Dioxins are highly fat-soluble, easily concentrated through food chains and accumulated in adipose tissues. Humans are mainly exposed to dioxin through food, such as fish, shellfish, meat and dairy products. The average daily intake for adult was 0.85 pg-TEQ/kg b.w. in Japan (Ministry of the Environment Japan, 2012), 2.2-2.4 pg-TEQ/kg b.w. in USA (Schecter et al., 2001), 1.09 pg-TEQ/kg b.w. in UK (Harrad et al., 2003), and 2.28 pg-TEQ/kg b.w. in Italy (Fattore et al., 2006). Although dioxins have significantly decreased in food products over the past several years (De Mul et al., 2008, Llobet et al., 2008), breast-feeding infants were found to intake the highest

estimated daily TEQ, which was reported 42 pg-TEQ / kg b.w. in USA (Schecter et al., 2001), 60.3-80.4 pg-TEQ / kg b.w. in Greece (Costopoulou et al., 2013), and 12.8 pg-TEQ/g of fat in mother's milk in Japan (Ministry of the Environment Japan, 2012), that were much higher than the tolerable daily intake (TDI) of 1-4 pg-TEQ / kg b.w. recommended by WHO (Rolaf van Leeuwen et al., 2000). These data suggest that dioxin is a threat particular to infants, and it is important to investigate the toxicity of low-dose TCDD exposure during the perinatal period.

3.1.3 Toxicity of dioxins

The health effects of dioxins in human mainly known from accidental or occupationally exposed populations, included chloracne (Jansing and Korff, 1994), change in sex ratio of children (Mocarelli et al., 2000), increasing cancer mortality rate (Wang et al., 2013a), immune function alteration (Miyashita et al., 2011), and diabetes (Uemura, 2012). Animal experiments reported that dioxin affected a wide range of tissues and physiological function, such as wasting syndrome (Linden et al., 2010), promoting hepatocarcinogenesis (Bock and Kohle, 2005), and thymic trophy (Besteman et al., 2005). Although dioxins are ubiquitous in environment, it is considered that the environmental level of dioxin exposure do not cause health effects on human (WHO, 2010a). However, dioxins can be transferred from mother to baby via transplacental and

lactational routes (Kakeyama et al., 2003), and have adverse effects in offspring born to dam exposed to very low dose of dioxin during gestation that do not induce manifest toxicity in dam themselves (Tohyama, 2006). A large number of epidemiological and animal studies reported that prenatal exposure to low dose of dioxin disturbed the developing brain. A Hokkaido study suggested that prenatal dioxin exposure was significantly negatively associated with mental or motor development in six-month-old infants (Nakajima et al., 2006). Several epidemiological studies suggested that children prenatally exposed to dioxins have poorer cognitive development (Chen et al., 1992, Guo et al., 1995, Patandin et al., 1999, Stewart et al., 2008). In rhesus monkeys, learning performance was impaired in offspring born to low-dose TCDD exposed dams (Schantz and Bowman, 1989). In rodents, perinatal exposure to TCDD disrupted executive function and sociality (Endo et al., 2012), brain sexual differentiation and sexual behavior (Mably et al., 1992, Kakeyama et al., 2003, Takeda et al., 2009), and learning behaviors (Seo et al., 2000, Mitsui et al., 2006, Hojo et al., 2008, Haijima et al., 2010, Kakeyama et al., 2013). Cerebral cortex which is largely responsible for higher brain functions is considered to be a TCDD target during fetal development. *In utero* TCDD exposure reduced cortical thickness (Mitsuhashi et al., 2010), altered cortical glutamate transmission (Tomasini et al., 2012) and molecular expression (Kakeyama et al., 2001, Nayyar et al., 2002, Kakeyama et al., 2003, Hood et al., 2006).

3.1.4 Aryl hydrocarbon receptor

The toxic effects of dioxin are notably mediated by aryl hydrocarbon receptor (AhR), a member of the basic helix-loop-helix Per-ARNT-Sim family (bHLH-PAS) that regulates gene expression involving in many physiological and developmental processes (Denison et al., 2011). AhR is a ligand-activated nuclear transcription factor, localized in the cytoplasm. Upon ligand binding, the chaperone proteins (e.g. the heat shock protein 90 (HSP90), the AhR-interacting protein (AIP), p23) are released and AhR translocates into the nucleus, where it complexes with the AhR nuclear translocator (ARNT). The AhR/ARNT heterodimer recognizes xenobiotic-responsive elements (XREs) in target genes, such as cytochrome P450 family members (e.g. Cyp1a1), and AhR repressor (AhRR) which competes with the ARNT for binding to AhR (Sorg, 2013).

AhR expression in mouse embryo is as early as E10, followed by widespread expression in most of organs including brain, heart, liver, kidney, lung, muscle, bone and epidermis (Abbott et al., 1995). In brain, AhR was widely distributed in cerebral cortex, hippocampus, hypothalamus, brainstem and cerebellum (Petersen et al., 2000).

There are four allelic variants of murine AhR: Ahr^{b-1}, Ahr^{b-2}, Ahr^{b-3} and Ahr^{d}. The Ahr^{b} alleles (Ahr^{b-1}, Ahr^{b-2} and Ahr^{b-3}), display high ligand binding affinity. The Ahr^{d} allele has a lower ligand binding affinity. AhR^{b-1} occurs naturally in C57BL/6 strain.

AhR^d occurs naturally in DBA/2 strains. There are 10 nucleotides, 5 amino acids different in d allele in DBA/2 from the b-1 allele in C57BL/6. The lower ligand binding affinity of AhR^d allele is attributable to the change of the amino acid alanine as found in Ahr^b alleles to valine at residue 375 (Poland et al., 1994). Ahr^{b-1} and Ahr^d strains show an approximately 10-fold difference in susceptibility to dioxins (Poland et al., 1994). The 50% lethal doses (LD50) for C57BL/6 and DBA/2 mice were 182 and 2,570 μg TCDD/kg b.w., respectively (Chapman and Schiller, 1985).

AhR has been studied for more than 30 years, and is well known as dioxin receptor, a mediator of toxic and carcinogenic effects. Over the years, AhR has been found to play an evolutionarily conserved role in physiological processes such as leg development in drosophila (Kozu et al., 2006, Cespedes et al., 2010), liver development (Schmidt et al., 1996), vascular development (Walisser et al., 2004), hematopoietic progenitor cells expansion and differentiation (Smith et al., 2013), plasmacytoid dendritic cell differentiation (Liu et al., 2013), and sperm development (Smith et al., 2013). During the last few years, increasing investigations support the new hypothesis that AhR is involved in cell migration (Barouki et al., 2007, Barouki and Coumoul, 2010). For example, AhR has been found to participate in migration of immortalized mouse mammary fibroblasts (Mulero-Navarro et al., 2005, Carvajal-Gonzalez et al., 2009a), human breast cancer cell line MDA-MB-231 (Goode et al., 2013), mouse keratinocyte

(Carvajal-Gonzalez et al., 2009b), mouse melanoma cells (María et al., 2013) and nematodes neurons (Qin and Powell-Coffman, 2004).

3.1.5 Objectives

The present study was attempted to elucidate the effects of prenatal TCDD exposure on cerebral cortex development, and investigate whether the AhR is involved in TCDD-induced toxicity as well as physiological function during cerebral cortex development.

3.2 Methods

3.2.1 Chemicals

TCDD (50μg/ml in Nonane) were purchased from Wako Pure Chemical Industries (Osaka, Japan).

3.2.2 Animals and exposure

Pregnant C57BL/6J mice (abbreviation: B6 mice) and DBA/2JJcl mice (abbreviation: D2 mice) were obtained from CLEA Japan (Tokyo, Japan). B6.D2N-*Ahr*^{d}/J mice (abbreviation: B6D2 mice) and B6.129 (FVB)-*Ahr*$^{tm3.1Bra}$/J mice (abbreviation: Ahr^{fx} mice) were purchased from the Jackson laboratory (Bar Harbor, Maine, USA). B6D2

strain is B6 genetic background mouse possessed D2-derived d variant of Ahr ($AhR^{d/d}$). AhR^{fx} strain carries loxP sites in both sides of exon 2 of AhR gene. AhR^{fx} mice homozygous for the mutant allele are viable and fertile. Timed-pregnant AhR^{fx} mice were generated by homozygous-homozygous mating.

To study the effects of TCDD on neuronal migration and mRNA expression, the pregnant B6 mice were orally administered vehicle (corn oil) or TCDD dissolved in vehicle at a dose of 0.6 or 3.0 μg/kg b.w. at E12.5. The doses of 0.6 μg/kg b.w. and 3.0 μg/kg b.w. were selected because those doses were reported to affect higher brain function upon perinatal exposure in previous studies (Haijima et al., 2010, Endo et al., 2012). To study the role of AhR in developmental toxicity of TCDD, the pregnant B6, B6D2 and D2 mice were orally administered vehicle (corn oil) or TCDD in vehicle at a dose of 20μg/kg b.w. at E12.5. According to the doses given to the dams, the control and TCDD-exposed groups were named as Control, TCDD0.6, TCDD3.0, and TCDD20, respectively. All animal experiments were performed according to the guidelines of the Animal Care and Use Committee of the Graduate School of Medicine, the University of Tokyo.

3.2.3 Plasmids

pCAGGS-Cre was constructed by inserting the full length cDNAs of Cre (cloned

from pCAG-Cre, a kind gifts from Dr. K. Kubo at Department of Anatomy, School of Medicine, Keio University, Tokyo, Japan) into the pCAGGS vector. The sequences of reconstructed plasmids were confirmed by DNA sequencing analysis (Eurofins Operon, Japan). pCAGGS-GFP was kindly provided by Dr. K. Kubo (Keio University, Tokyo, Japan). pCAGGS-RFP was kindly provided by Dr. E. Kimura (the University of Tokyo, Tokyo, Japan). Plasmids were purified using the NucleoBondXtra (Macherey-Nagel, Dueren, Germany) according to the manufacture's protocol. Purified plasmids were diluted with PBS to a final concentration of 1-2.5 μg/μl before use.

3.2.4 *In utero* electroporation

In utero electroporation was performed as described in Section 2.2.4. The study of effects of prenatal TCDD (0, 0.6, 3.0μg/kg b.w.) exposure on neuronal migration was performed as a joint research, in which IUE was performed by Dr. Ken-ichiro Kubo (Department of Anatomy, School of Medicine, Keio University), and brain tissues were analyzed by Dr. Noriko Nishimura (Tohyama Lab at the University of Tokyo).

3.2.5 Conditional AhR knockout

Conditional AhR knockout was performed by utilizing the Cre/loxP system and IUE technique. The Cre expression vectors (encoding Cre recombinase) were electroporated

into progenitor cells of AhR^{fx} mice brain at E14.5. In each dam, half embryos were transfected with pCAGGS-GFP as control and the other half was co-transfected with pCAGGS-GFP and pCAGGS-Cre to delete AhR. Neuronal migration was analyzed at E17.5 or E18.5.

3.2.6 Brain sections preparation and staining

Brain sections preparation and staining were performed as described in Section 2.2.5.

3.2.7 Analysis of cell distribution

Cell distribution was evaluated using a bin analysis as described previously (Tomita et al., 2011). The cortex was equally divided into 5 or 10 bins. The deepest bin and the most upper one were defined as Bin 1 and Bin 5 or 10, respectively. In each bin the number of GFP or RFP positive cells was counted and estimated as a percentage of the total number in all bins. Relative distance was calculated to assign the GFP or RFP positive cells to each bin (TCDD 0, 0.6, 3.0 experiment: relative distance = the distance of the nucleus from the ventricle / the distance between the ventricle and the top of MZ; TCDD20 experiment : relative distance = the distance of the nucleus from the top of the SP / the distance between the top of SP and the top of MZ; AhR-KO experiment (analyzed at E17.5): relative distance = the distance of the nucleus from the inner

margin of the IZ / the distance between the inner margin of IZ and the top of MZ; AhR-KO experiment (analyzed at E18.5): relative distance = the distance of the nucleus from the inner margin of the CP / the distance between the inner margin of CP and the top of MZ). All measurement was determined by Image-J software (NIH, USA).

3.2.8 Measurement of cortical thickness

For the analysis of cortical thickness, one male pup whose body weight was at the median value in a litter was selected at P0. A total of 5 male pups in each dosed group were used. Pups' brains were fresh frozen on dry ice and then cut into 20μm on a cryostat (CM3050S, Leica Microsystems, Wetzlar, Germany) at -20˚C. To visualize the boundaries of cerebral cortex, the brain sections were immunostained with MAP2. Map2 immunostaining was performed as described in Section 2.2.5. Cortical thickness was defined as the distance between the SP and MZ. All measurements were performed on the somatosensory cortex using the Neurolucida software (MBF bioscience, Williston, VT, USA). Average of cortical thickness was calculated from 5 serial sections, every section three measurements.

3.2.9 Tissue preparation

For RNA analysis, one male fetus whose body weight was at the median value in a

litter was selected at E18.5. A total of 5 male pups in each dosed group were used. Brains were collected, snap frozen in liquid nitrogen, and stored at -80˚C until analysis.

3.2.10 RNA quantification

RNA quantification was performed as described in Section 2.2.9.

3.2.11 Statistical analysis

Statistical analysis was performed as described in Section 2.2.10.

3.3 Results

3.3.1 Litter size and body weight

Table 3 summarized the data on litter size and body weight of B6 mice at E18.5. No significant difference in little size or body weight was observed between the control group and TCDD exposed group.

3.3.2 Effects of *in utero* exposure to TCDD on neuronal migration

To examine whether neuronal migration was affected in the developing cortex upon *in utero* exposure to low doses of TCDD, pregnant B6 mice were exposed to 0, 0.6, or 3.0 μg TCDD/kg b.w. at E12.5. GFP expressing plasmids were introduced into the

progenitor cells by IUE at E14.5, and neuronal migration was analyzed at P0 (Fig. 7A). In control group, most of the GFP-labeled neurons were located in cortical layer II/III at P0. In contrast, a greater number of GFP-labeled neurons were located throughout the cortex in the TCDD treated group (Fig. 7B). A bin analysis was applied to quantify the neuronal migration. The number of GFP-positive neurons was counted in each bin and estimated as a percentage of the total number in all 5 bins. The percentage of GFP-positive neurons in Bin 5 of TCDD0.6 and TCDD3.0 groups were significantly decreased compared with the control group. And the percentages of GFP-positive neurons in Bin 3 and Bin 4 of TCDD0.6 group were significantly increased compared with the control group (Fig. 7C). These data suggest that neuronal migration was disrupted by *in utero* TCDD exposure.

3.3.3 Developmental toxicity of TCDD in cerebral cortex via AhR

To investigate whether TCDD affects cerebral cortex development via AhR signaling pathway, the toxic effects of TCDD on B6, D2 and B6D2 mice which possess diffident types of AhR were studied. Although B6 and D2 have naturally different AhR, they differ in many genes other than the AhR, it is preferable to study the mice having same genetic background only differing in the AhR, such as B6 mice ($AhR^{b\text{-}1/b\text{-}1}$, high ligand binding affinity) and B6D2 mice ($AhR^{d/d}$, B6 background mice possessing D2-derived d

variant of AhR, low ligand binding affinity). The pregnant B6, B6D2 and D2 mice were exposed to TCDD (20 μg/kg b.w.) or corn oil at E12.5. mRNA expression of TCDD-responsive genes (Cyp1a1, Cyp1b1 and AhRR) in mice brains were measured at P0. The mRNA expression of Cyp1a1, Cyp1b1 and AhRR were significantly increased in TCDD treated B6, B6D2 and D2 mice compared with the control mice (Fig. 8). The increasing levels of TCDD induced mRNA expression were similar between B6D2 and D2 mice, but different from B6 mice. These data suggest that TCDD-induced mRNA expression in mouse brain was AhR dependent.

Next, cortical thickness of somatosensory area was measured in B6 and B6D2 mice at P0 (Fig. 9A-C). Prenatal TCDD exposure significantly decreased cortical thickness in B6 mice (Fig. 9D), but no significant difference was found in B6D2 mice (Fig. 9E), suggesting that TCDD affected cerebral cortex development via AhR signaling pathway.

Furthermore, whether prenatal TCDD exposure affected neuronal migration in B6D2 mice was investigated. The pregnant B6D2 mice were exposed to TCDD (20 μg /kg b.w.) or corn oil at E12.5. RFP expressing vectors were transfected into neuronal progenitor cells by IUE at E14.5, and neuronal migration were analyzed at E19 (Fig. 10A). In the control group, most of the RFP-labeled neurons were located in the cortical layer II/III at E19, but more RFP-labeled neurons were observed throughout the cortex in the TCDD-treated group (Fig. 10B). In a bin analysis, the number of RFP-positive

neurons was calculated in equally divided 10 bins and the percentage of RFP-positive neurons in Bin 8 was significantly decreased in the TCDD-treated group compared with the control group (Fig. 10C).

3.3.4 Role of AhR in the cerebral cortex development

To test the hypothesis that AhR plays a role in neuronal migration, conditional AhR knockout mouse model (named as AhR-KO) was generated by Cre-loxP system combing with IUE technique. AhR gene was deleted in Ahr^{fx} mice by introducing Cre expressing vectors into the progenitor cells at E14.5 using IUE technique, and neuronal migration was analyzed at E17.5 or E18.5 (Fig. 11A and Fig. 12 A). The body weight of AhR-KO mice had no significant difference compared with the control mice at E17.5 or E18.5 (Table 4). At E17.5, in both the control and AhR-KO group, the GFP-labeled neurons were scattered throughout the CP and IZ, which were underway to migrate to their final destination (Fig. 11B). No significant difference was found in the distribution of GFP-labeled neurons in cerebral cortex between the control and AhR-KO group at E17.5 (Fig. 11C). One day later, at E18.5, most of the GFP-labeled neurons completed migration and localized in layer II/III in the control group, whereas some of the GFP-labeled neurons were abnormally localized in the CP in the AhR-KO group (Fig. 12B). In a bin analysis, the percentage of GFP-labeled neurons in Bin 8 was

significantly decreased in the AhR-KO group compared with the control group (Fig. 12C).

3.3.5 Effects of *in utero* exposure to TCDD on mRNA expression

To study further gene expression with regard to possible TCDD effects on cerebral cortex development, referring to a microarray data of TCDD experiment in our laboratory, 44 genes were selected and analyzed in male B6 mice brain by quantitative RT-PCR (Fig. 13). The mRNA expression of Cyp1a1, Cyp1b1 and AhRR were significantly increased in TCDD3.0 group compared with control and TCDD0.6 group, but there was no significant difference between TCDD0.6 group and control group. The mRNA expression of Cxcl4 (Pf4), a member of the CXC chemokine family, was significantly increased in TCDD3.0 group compared with control and TCDD0.6 group. The mRNA expression of Pitx3, a gene regulates dopaminergic neuron development, was significantly decreased in TCDD3.0 group compared with control group. The mRNA expression of Pitx3 was also significantly decreased in B6 or B6D2 mice prenatally exposed to 20 μg TCDD /kg b.w., compared with the control group (Fig. 14). The mRNA expression of Pisd-ps3 was significantly decreased in TCDD0.6 group compared with control group. The mRNA expression of Ncam1 was significantly increased in TCDD3.0 group compare with TCDD0.6 group. Among the three groups,

no significant difference was found in mRNA expression of other genes (Erdr1, G530011O06Rik, Aldh1a1, Apc, Atm, Atrx, Cdk4, Cdkl2, Cdkn1b, Comt, DISC1, Gad1, Gh, Grin2a, Hoxa5, Icam1, Kif2a, Malat1, Mapt, Mdga2, Xlr3b, Ralgapa1, Reln, Rhox4, Sema3g, Slc15a2, Slc6a3, Slc6a4, Stxbp1, Th, Tel3, Top2b, Trip11, Ube3a, Vav3, Mbnl1, Mbnl2).

3.4 Discussion

Epidemiological studies have reported that exposure to dioxin from the environment was associated with the incidence of abnormal brain function and behavior in children (Golub and Jacobson, 1995, Guo et al., 1995, Patandin et al., 1999). A plethora of experimental studies have demonstrated that laboratory animals born to dams exposed to TCDD developed abnormal behavioral phenotypes (Amin et al., 2000, Markowski et al., 2002, Negishi et al., 2006, Takeda et al., 2009, Nguyen et al., 2013). Our laboratory has also found that perinatal low doses of TCDD exposure affected higher brain functions such as fear memory (Haijima et al., 2010), behavioral flexibility, sociality (Endo et al., 2012), paired associate learning and memory (Kakeyama et al., 2013) in mice offspring examined later in adulthood. This thesis study challenged the question whether or not TCDD induced morphological abnormality in the developing brain, and found that prenatal low doses of TCDD exposure (0.6 and 3.0 μg /kg b.w.) impaired

neuronal migration, a key process during cerebral cortex development.

Neurons in layer II/III are commissural projection neurons which extend axons to the opposite hemisphere across the corpus callosum. Higher brain function relies on the neuronal network in and between various brain regions, and the inappropriate positioning of layer II/III projection neurons may lead to inaccurate projection and impairment of synaptogenesis that possibly result in abnormal higher brain functions in various neurological disease (Tomasi and Volkow, 2012, Maximo et al., 2014), and the abnormal animal behaviors described above (Haijima et al., 2010, Endo et al., 2012, Kakeyama et al., 2013).

According to our laboratory's findings, B6 pups born to dams given TCDD at a single dose of 0.6 μg TCDD /kg b.w. developed abnormal behavior, such as executive functions deficits and social-behavioral abnormality with some unbalanced neural activity in the medial prefrontal cortex and amygdala as shown by immediate early genes (Endo et al., 2012). In the fear conditioning test, TCDD-exposed pups developed abnormal memory and emotion at a single dose of 3.0 μg TCDD /kg b.w. (Haijima et al., 2010). The present study showed that TCDD-exposed pups developed abnormal neuronal migration in both TCDD0.6 and TCDD3.0 group. These observations suggest that TCDD induced toxicity on developing brain has the molecular underlying mechanisms to induce these behavioral alterations as well as disrupt neuronal migration.

In order to search for the genes, the expression of which was responsible for TCDD-disrupted neuronal migration, microarray was used to screen them, followed by a further quantitative analysis by qRT-PCR for mRNA abundance in the brain of B6 fetuses at E18.5. The well-known TCDD responsive genes Cyp1a1, Cyp1b1 and AhRR were significantly increased in TCDD3.0 group, but had no change in TCDD0.6 group, suggesting a dose-dependent response of these genes. The present study showed a significant reduction in Pitx3 expression in the TCDD3.0 group, with a decreasing tendency in the TCDD0.6 group, compared with the control group. This alteration was also observed in B6 and B6D2 pups' brain prenatally exposed to 20 μg TCDD /kg b.w.. Pitx3, a transcription factor, plays an important role in the differentiation and survival of midbrain dopaminergic neurons during development (Li et al., 2009). Our previous studies found that perinatal TCDD exposure significantly altered dopamine and its metabolites in several brain regions of adult mice (Zhang et al., Master's thesis work at Graduate School of Medicine, the University of Tokyo, 2010, unpublished). It can be conjectured that abnormal Pitx3 expression in TCDD-treated mice may lead to dopaminergic neuron development deficits in fetal period, the effect of which may last into adulthood. Accurate migration of neurons was modulated by neurotransmitters in the embryonic brain (Crandall et al., 2007, Manent and Represa, 2007, Vasudevan et al., 2012). In the Pitx3-deficient *aphakia* mice, migration of both DA neurons and GABA

neurons was significantly disturbed compared with wild-type mice (Vasudevan et al., 2012). Although the relationship between Pitx3 and the delayed migration of excitatory projection neurons observed in the present study is unclear, abnormal expression of Pitx3 may affect projection neuron migration by interrupting the molecular signaling of dopamine system. Pisd-ps3 (phosphatidylserine decarboxylase, pseudogene 3) expression was significantly reduced in TCDD0.6 group compared with the control group. In Pitx3-deficient dopamine neurons, the expression of MGC65558 (a gene similar to phosphatidylserine decarboxylase) was significantly decreased (Hwang et al., 2009). Both microarray and qRT-PCR data showed very significant alteration of Pisd-ps3 expression in developing brain upon *in utero* TCDD exposure, however, whether the pseudogene, Pisd-ps3 is involved in TCDD-induced toxicity needs further study.

Chemokines are small proteins well known as chemoattractants to guide cell migration in the immune system, and recent studies indicate that these proteins may also play roles in central nervous system (Reaux-Le Goazigo et al., 2013). For example, Cxcl12, its receptor Cxcr4 and Cxcr7, are expressed in the developing brain and regulate neuronal migration (Lopez-Bendito et al., 2008, Sanchez-Alcaniz et al., 2011, Yang et al., 2013). In the present study, Cxcl4 (Pf4), a member of the CXC chemokine family, was significantly increased in TCDD3.0 group. This result was consistent with a

previous study (Mitsui et al., 2011), which showed that pregnant C57BL/6N mice administered 5.0 μg TCDD /kg b.w. at E12.5 exhibited significantly increased Cxcl4 expression in embryonic brains at E18.5. However, the expression of Cxcl4 in brain tissue and its biological function are largely unknown. A study reported that Cxcl4 was expressed in brain microglia and induced microglia migration (de Jong et al., 2008). Patients with depression exhibited increased concentration of Cxcl4 in plasma compared with the control group similar in age and gender (Musselman et al., 2000). Further studies are needed to clarify the role of Cxcl4 in brain and the effects of TCDD induced Cxcl4 expression in developing brain.

Most of other genes detected in the present study did not have significant changes in whole brain compared with the control group. Gene expression in specific brain regions in a particular reference to different cortical layers, hippocampus and substantia nigra should be examined in future studies using technique such as fluorescence laser microdissection combined with qRT-PCR (Yoshioka et al., 2012).

The majority of TCDD-induced toxicity, such as cleft palate and hydronephrosis, is mediated through AhR, which was proved using AhR-null mice (Mimura et al., 1997, Peters et al., 1999). A large strain difference in susceptibility to dioxin toxicity can be accounted for, albeit not in all the cases, by the dissociation constant of AhR alleles. AhR has been established to be comprised of four different forms of alleles in inbred

mice: AhR^{b-1} alleles, AhR^{b-2} alleles, AhR^{b-3} alleles, and AhR^{d} alleles (Poland and Glover, 1980, Okey et al., 1989). The dissociation constant of AhR to TCDD differs between AhR alleles: DBA/2, a TCDD less responsive strain, harbors an AhR allele, named $AhR^{d/d}$, whereas C57BL/6, TCDD high responsive strains, possess AhR allele, named $AhR^{b-1/b-1}$ respectively (Ema et al., 1994, Poland et al., 1994). To investigate whether TCDD-induced developmental toxicity in cerebral cortex via AhR, I compared toxic effects of TCDD in the two mouse strains, B6 and B6D2, possessing high affinity b-type allele and low affinity d-type allele, respectively. Cortical thickness was utilized as an endpoint of the TCDD toxicity on the cerebral cortex development. In B6 mice, cortical thickness was significantly decreased in TCDD-exposed group compared with control group, which was consistent with a previous study (Mitsuhashi et al., 2010), but no significant difference was observed in B6D2 mice. *In utero* exposure to TCDD significantly enhanced the expression of Cyp1a1 and Cyp1b1 in the brain of B6 mice than that of B6D2 mice. These data suggest that developmental toxicity of TCDD in brain is in AhR-dependent manner. Because the interrupted neuronal migration was observed in the B6 mice exposed to 0.6, 3.0 μg TCDD /kg b.w., and the AhR-b and AhR-d alleles have approximately 10-fold difference in susceptibility to TCDD, I tested whether or not the neuronal migration was affected in the less sensitive B6D2 mice exposed to a relative high dose of TCDD (20 μg /kg b.w.), and found a subtle but

significant alteration in neuronal migration in TCDD-exposed B6D2 mice, which suggested the TCDD induced developmental toxicity was possibly mediated through AhR.

Recent studies indicate that AhR is implicated in playing important roles in the neuronal development. For example, AhR participates in proliferation and differentiation of neural progenitor cells in hippocampus (Latchney et al., 2013), and guiding maturation and maintenance of granule neuron precursors in cerebellum (Collins et al., 2008). In C. elegans, ahr-1, the caenorhabditis elegans aryl hydrocarbon receptor homolog, regulates neuronal migration and differentiation (Huang et al., 2004, Qin and Powell-Coffman, 2004). To address the hypothesis that AhR plays a role in neuronal migration, I established a conditional AhR knockout mice model using Cre-loxP system combined with IUE technique. The AhR^{fx} mouse was utilized in the present study in which AhR gene was flanked by a pair of lox sites. Cre recombinase expressing constructs were transfected into the progenitor cells by IUE, which can recognize the lox sites and remove the AhR gene from the genome. This research strategy is thought to have several advantages over utilizing AhR-null mice. Firstly, Ahr^{fx} mice were born normally in size and did not display any gross physical or behavior abnormities, whereas morbidity during or by the end of weaning was significantly higher in AhR-null mice made by homologous recombination than in wild

type mice (Gonzalez and Fernandez-Salguero, 1998). Secondly, the conditional ablation of AhR gene expression in a specific brain region at a designated time may provide a clue to study the direct actions of AhR and rule out the effects of AhR deficiency in other tissues, whereas AhR null mice by homologous recombination exhibit AhR deficiency in the entire body at the very beginning of embryonic development. In the present study, AhR gene was deleted from projection neurons in the cerebral cortex at E14.5, a critical time for the birth and migration of the cortical neurons in layer II/III. It was interesting to find that neuronal migration in the conditional AhR-KO mice at E18.5 was significantly delayed compared with the control group, suggesting AhR may play a role in neuronal migration even in the absence of an exogenous ligand.

The conditional AhR-KO model was generated by electroporating the Cre-expressing plasmids into the progenitor cells of AhR^{fx} mice, and only the transfected cells labeled with GFP were considered as AhR deficiency. Thus, in this mouse model, it was difficult to quantify the AhR protein level in AhR-KO neurons by Western blot analysis. Immunostaining is useful to identify the AhR protein in cells. However, an appropriate antibody to AhR that can be used to detect AhR in brain tissue is not currently available, and the efficacy of Cre-induced AhR knockout by IUE in the cerebral cortex of AhR^{fx} mice remains to be studied. To elucidate the physiologic function of AhR in neuronal migration, other AhR loss-of-function experiments can be used, such as making a

genetic AhR knockout mouse in which AhR is specifically deleted in neurons by Cre/loxP recombination system or Flp/Frt recombination system, silencing AhR expression using RNA interference technology by means of delivering small interfering RNA (siRNA) into wild-type embryonic brain, or interfering normal AhR function in neurons utilizing dominant negative method. Rescue experiment performed by introducing the wild-type AhR expressing vectors into the AhR deficient cells to compensate the effect of lacking AhR protein during biological process is another useful method to demonstrate the physiological role of AhR. The work of this thesis explored and showed the possibility that AhR is involved in neuronal migration, but additional works are needed to fully clarify the role of AhR in the physiological processes.

In the present study, either TCDD exposure or deletion of AhR exhibited deficits in neuronal migration. One possibility is that persistent activation AhR by exogenous ligand like TCDD interrupts endogenous ligand binding with AhR in cytoplasm, and consequently interfere its normal physiologic function. Similar phenomena also reported in other studies. For example, both AhR activation and deletion adversely impacted hippocampal neurogenesis, and reduced hippocampal-dependent memory function (Latchney et al., 2013). Activation, knockdown, or inhibition of AhR, either of them blocks cardiomyocyte lineage differentiation (Wang et al., 2013b). Future studies

are needed to ascertain the molecular mechanism of TCDD induced toxicity and AhR mediated signaling pathway in neuronal migration.

Chapter 4 Perspectives

The present studies show that prenatal exposure to low doses of BPA or TCDD affected the neuronal migration, an essential process during cerebral cortex formation, which possibly results in abnormal higher brain function later in adulthood, suggesting the association between chemical exposure and abnormal behavior may have its origins at the early stage of brain development. Future studies need to answer the key question how the abnormal morphological phenotype of neuronal migration affects the higher brain function, such as learning, memory, and emotion in adulthood.

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Table 1 Primer sequences for real-time RT-PCR

Gene Name		**Sequence (5'->3')**
AhRR	Forward	GGAAGTCCCTATGTTCTCTGCC
	Reverse	AGTGTCCACAAAGCCTGACC
Aldh1a1	Forward	TGGTTTAGCAGCAGGACTCTTCAC
	Reverse	GGGCACTGGGCTGACAACATC
Apc	Forward	GGCAGAGTCCCTCACAGAATGAAA
	Reverse	GGATAACTGGTGTTCTGGCTTCTTG
Atm	Forward	AGCTCCAGACAGAAGAAGATGCAAG
	Reverse	CAGTCAGCTCCATACGTTAGACC
Atrx	Forward	TATGAGTTTCCTGTCTCCTGGCAC
	Reverse	GCAACAGGATGAAGCACCAGCA
BDNF	Forward	CGGCTTCACAGGAGACATCAG
	Reverse	TCAGGTCAACATAAACCACCAAC
Cdk4	Forward	GGCCCTCAAGAGTGTGAGAG
	Reverse	CATCAGCCGTACAACATTGG
Cdk5	Forward	TAGGGACACCGACTGAGGA
	Reverse	CGTTCACCAAGGATGTTGTAG
Cdkl2	Forward	TGAGGTCAAAGATGCAGAAGCAG
	Reverse	GAAAGAAGTCATGGCGCAGAAG
Cdkl3	Forward	CCAGAGTATGAAGGCGACCA
	Reverse	GAGAGGGTTTGTCAGTTCCAAG
Cdkn1b	Forward	GAAGAAGCGAGTCAGCGCAA
	Reverse	CACCTCCTGCCACTCGTATC
c-fos	Forward	GAA GGGAACGGAATAAGATGG
	Reverse	CTGTCTCCGCTTGGAGTGTA
Comt	Forward	GACCGCTACCTTCCAGACAC
	Reverse	CCTCACATACGCCAGGAAGT
Cux2	Forward	GCTGCTCTCCTTCCAACTCAA
	Reverse	GTCCCCTCCACCAGCATTTC
Cyp1a1	Forward	CACCGTATTCTGCCTTGGA
	Reverse	CAGCATGTGACCAATGAAGG
Cyp1b1	Forward	GGACAAGGACGGCTTCATTA
	Reverse	GCGAGGATGGAGATGAAGAG

Table 1 (continued)

Gene Name		**Sequence (5'->3')**
DISC1	Forward	TGTGGAAAGCAGACTTGGAGA
	Reverse	GAGGGACAGCCAGAGCAG
Drd1a	Forward	AGGCTGATGTGGTGGTTCC
	Reverse	AGGGGCAGAGCATTGGTAG
Drd4	Forward	GCCTTCCTGGTGTGTTGGAC
	Reverse	AAGATGGTGTAGATGATGGGGTTG
Erdr1	Forward	TGATGTCACCCACGAAAGCA
	Reverse	GTGAGAATCGCTCCGTCCTG
G530011O06Rik	Forward	TTAGGCACAGGCATCGGAAC
	Reverse	CAGCATTTCGGTGAAGCAGG
Gad1	Forward	CAGAGACCGACTTCTCCAAC
	Reverse	TTGGTGGAGCGATCAAATGT
GAPDH	Forward	AACTTTGGCATTGTGGAAGG
	Reverse	ACACATTGGGGGTAGGAACA
Gh	Forward	AAAGAGTTCGAGCGTGCCTAC
	Reverse	AGCAATTCCATGTCGGTTCTCTG
Grin2a	Forward	CAGCAGGACTGGTCACAGAA
	Reverse	GAGAGGGCCTGCTAAGGTCT
Hoxa5	Forward	GACCCTGGAGCTGGAGAAAGAA
	Reverse	TCGGAGAGGCAAAGGGCATGAG
Icam1	Forward	TTCCAGCTACCATCCCAAAG
	Reverse	GCTTCAGAGGCAGGAAACAG
Itga3	Forward	TGTGGTTGGTGCTTGTGG
	Reverse	ACGGCTGGCTCTTCATCTC
Kif2a	Forward	GTCCACGAACCCAAACAGAAAG
	Reverse	GCCTAGCAGTAAACCTGTAAACCA
Kitl	Forward	ATGTTCCCCGCTCTCTTTGG
	Reverse	CATAAGGGCTCACTCCTGAAGAC
Lhx2	Forward	AGCACGCCTACAACCCTCACA
	Reverse	GTGGCGAGTCATTAGAAAAGGTTGG
Lhx5	Forward	CGGTGAATGGTGGCTTTG
	Reverse	ATGGAGGAGCAGGGAGTTG

Table 1 (continued)

Gene Name		**Sequence (5'->3')**
Malat1	Forward	CGGTGCGGTAACATTCCTTGA
	Reverse	CTTTACACAGAAGCCTACAACTCCC
Mapt	Forward	AGGGAACATCCATCACAAGC
	Reverse	GCCAATCTTCGACTGGACTC
Mbnl1	Forward	CCCTCAGCCACAAGACATCC
	Reverse	ATCACCTCAGCACAAATACCAACT
Mbnl2	Forward	TCCAGCGAGGAAACTGTGCC
	Reverse	CGGTTACGGTGTTGTCGTTTG
Mdga2	Forward	GCCTTTAATGAGCCCAATGACC
	Reverse	GCAAAGCATCAGTATCAAACGCA
Ncam1	Forward	TAACCTGTGTGGCAAAGCTG
	Reverse	TGGTTTGGAGTCCGTTCTTC
Otx2	Forward	AGGCTATGCTGGCTCAACTTC
	Reverse	GGGGACTGATTGAGATGGCTG
Pf4	Forward	CTGGTCCCGAAGAAAGCGATG
	Reverse	ACAGTGGCGTCCTGCCTTGA
Pisd-ps3	Forward	GGTGGAACAGGTAAAGGGCGA
	Reverse	AGTGGTAGAGCGCATTCCCTTCTTG
Pitx3	Forward	ATGGAGTTTGGGCTGCTTGGTGA
	Reverse	CGGAGGGTGTGGAGTGCCTG
Ralgapa1	Forward	GCTGCTAGTTTGGTTTCCAGAGAAGA
	Reverse	GCTCCGTGAGAGTGCTTGTG
Reelin	Forward	CAACCCATCCTTCCACCTC
	Reverse	GCTTCACAACCCACCACAA
Rhox4	Forward	TCCAGCAGAATCACTTCATCCGT
	Reverse	CATAACTCTGGCTTCACTCACACC
Robo1	Forward	GTGTTGCTGATTGATTGCCTAACC
	Reverse	TCTGTTCTTCCTTGCTGGCTG
Sema3g	Forward	CTCAAGAGCCAAGGTGACAT
	Reverse	TGACCACCCTCAGTAGAGAC
Slc15a2	Forward	GCTCTGGGAACAGGAGGTATCAAA
	Reverse	ATGGAGAGGTAGAAGACCGAGAA

Table 1 (continued)

Gene Name		**Sequence (5'->3')**
Slc6a3	Forward	ATGTCCCTGTCCCTAATGGGTG
	Reverse	CAGTATGACCTGTTCTCCCAGAGTG
Slc6a4	Forward	TGCGGCTCAGATCTTTTTCT
	Reverse	GACAAAGCCAGAGACGAAGC
Slitrk1	Forward	CGGTCCAAGAGAAGGGATG
	Reverse	TGCCAGTAGGAAGAGTCACAGA
Stxbp1	Forward	GGAGCGGAAGGAGCGTATCA
	Reverse	GCGGGTAGAGATGTATGGGTAG
Th	Forward	CTTCAGTGATGCCAAGGACA
	Reverse	CAGGGTGTACGGGTCAAACT
Tle3	Forward	GGCAGATGGACAGACAAGATG
	Reverse	GCAAGGCAAGAGACAGATGG
Top2b	Forward	ATGATGCTGGTGGCAAACATTC
	Reverse	AGACTCCATATCTGTCCCTACCAA
Trip11	Forward	TGACAGAGATAAGTCGGCGGCA
	Reverse	ACCGTAACTCCTGAGCCACCTTG
TrkB	Forward	GCATTCTGCCTTCATCTTGG
	Reverse	GCATTCAGTCGCACACTCTC
Ube3a	Forward	CGGAAAGTCAAACAGCACACA
	Reverse	TCTTCCATAGCAGCAGCAGAAC
Vav3	Forward	GGAGTGGAGTCAGCCATCTC
	Reverse	ATTGGAACGACCAGCAAATC
Wnt1	Forward	GACCCAGCAGCAAAACCCTAC
	Reverse	CAGTGATGAGGAGGCAGGACA
Xlr3b	Forward	CTAACAGAGAAGTCCTTGATGCTGG
	Reverse	CTTCGAATTCTTGGACCAAGTCCTG

Table 2 Litter size and body weight of ICR mice upon *in utero* BPA exposure

Groups	Litter size			Body weight (g)		
	All pups	Male	Female	All pups	Male	Female
Control	14.8±0.8	6.67±0.61	8.17±0.79	1.63±0.04	1.67±0.05	1.60±0.04
BPA-40	13.7±1.5	5.83±1.10	7.83±1.01	1.76±0.05	1.81±0.05	1.71±0.05
BPA-400	15.0±0.7	8.50±0.80	6.50±1.09	1.73±0.03	1.77±0.03	1.69±0.03

Data are shown as mean ± SEM, n=6 dam per treatment.

Table 3 Litter size and body weight of C57BL/6J (B6) mice upon *in utero* TCDD exposure

Groups	Litter size			Body weight (g)		
	All pups	Male	Female	All pups	Male	Female
Control	8.43±0.81	3.71±0.64	4.71±0.81	1.22±0.02	1.25±0.03	1.18±0.02
TCDD-0.6	8.63±0.86	5.00±0.80	3.63±0.68	1.23±0.03	1.24±0.03	1.22±0.03
TCDD-3.0	9.13±0.52	4.13±0.44	5.00±0.50	1.23±0.02	1.25±0.03	1.22±0.01

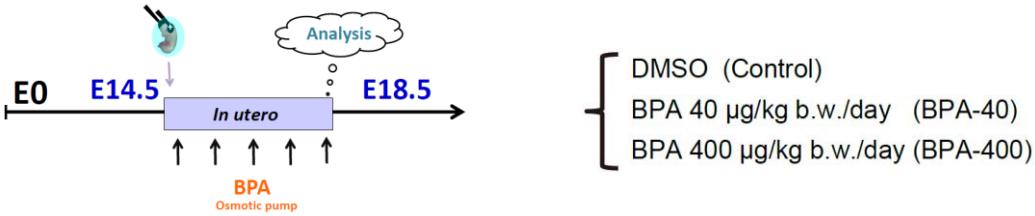
Data are shown as mean ± SEM, n=7 dam (Control), n=8 dam (TCDD0.6), n=8 dam (TCDD3.0).

Table 4 Body weight of B6.129 (FVB)-*Ahr*$^{tm3.1Bra}$/J (AhR^{fx}) mice in control and conditional AhR knockout (AhR KO) group

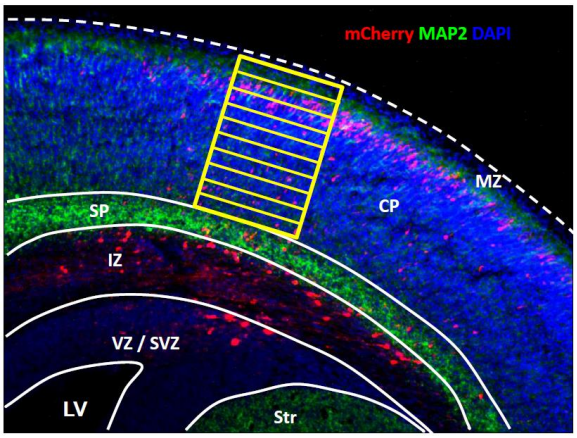
Group	Body Weight (g)	
	E17.5	E18.5
Control	0.73±0.03	1.05±0.06
AhR KO	0.72±0.01	1.09±0.04

Data are shown as mean ± SEM, n=5 dam (E17.5), n=7 dam (E18.5).

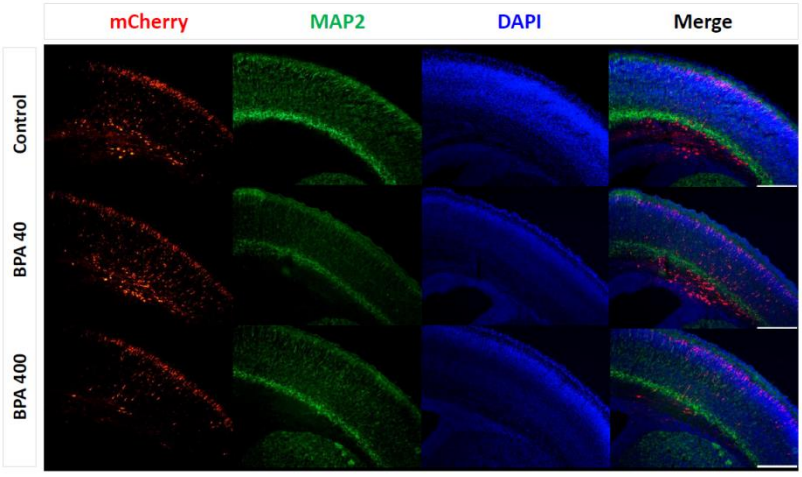
(A)


Analysis
E0
E14.5
In utero
E18.5
BPA
Osmotic pump
DMSO (Control)
BPA 40 μg/kg b.w./day (BPA-40)
BPA 400 μg/kg b.w./day (BPA-400)

(B)


mCherry MAP2 DAPI
MZ
CP
SP
IZ
VZ / SVZ
LV
Str

(C)


mCherry
MAP2
DAPI
Merge
Control
BPA 40
BPA 400

(D)

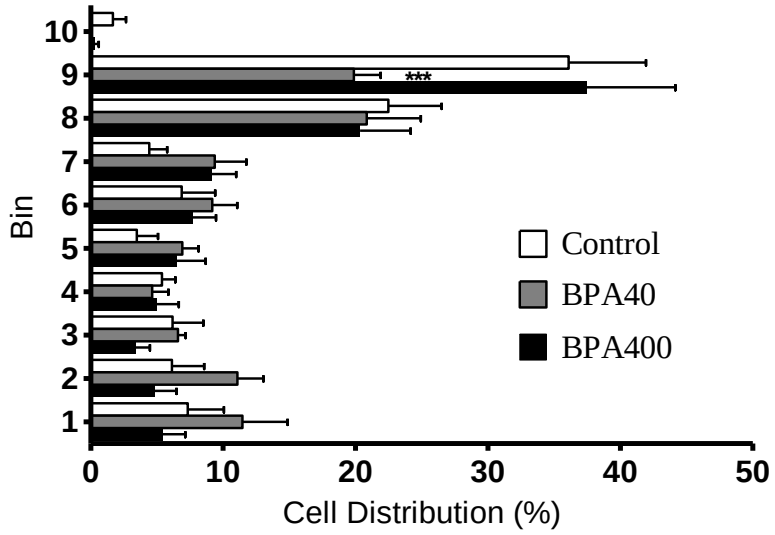


Figure 1 Effects of prenatal BPA exposure on neuronal migration in the developing cerebral cortex. (A) A scheme of the experiment design: pregnant ICR mice were exposed to BPA by implanting osmotic pump at a dose equivalent to 0, 40 or 400 μg/kg b.w./day from E14.5 to E18.5. Fluorescent protein expression plasmids (pCAG-mCherry) were transfected into neuronal progenitor cells at E14.5 by *in utero* electroporation (IUE), and embryonic brains were analyzed at E18.5. (B) Representative photograph of brain section transfected with mCherry plasmids (red), and immunostained with MAP2 (green) and DAPI (blue). A bin analysis was performed to evaluate cell distribution. The cortical plate was equally divided into 10 bins. The deepest and most upper bins were designated as Bin 1 and Bin 10, respectively. In each bin, the number of mCherry positive cells was calculated as a percentage of the total cell number in all 10 bins. MZ: marginal zone, CP: cortical plate, SP: subplate, IZ: intermediate zone, SVZ: subventricular zone, VZ: ventricular zone, LV: lateral ventricle, Str: striatum. Scale bars: 500 μm. (C) Representative photographs of brain sections in Control, BPA40 and BPA400 group. (D) Distribution of mCherry positive neurons in CP at E18.5. In a bin analysis, the percentage of mCherry positive neurons in Bin 9 of BPA40 group was significantly decreased compared with the control group and BPA400 group. Data are shown as mean ± SEM, n=4 (control), n=5 (BPA40), n=5 (BPA400), (***P<0.01, two-way ANOVA followed by Bonferroni's *post hoc* test).

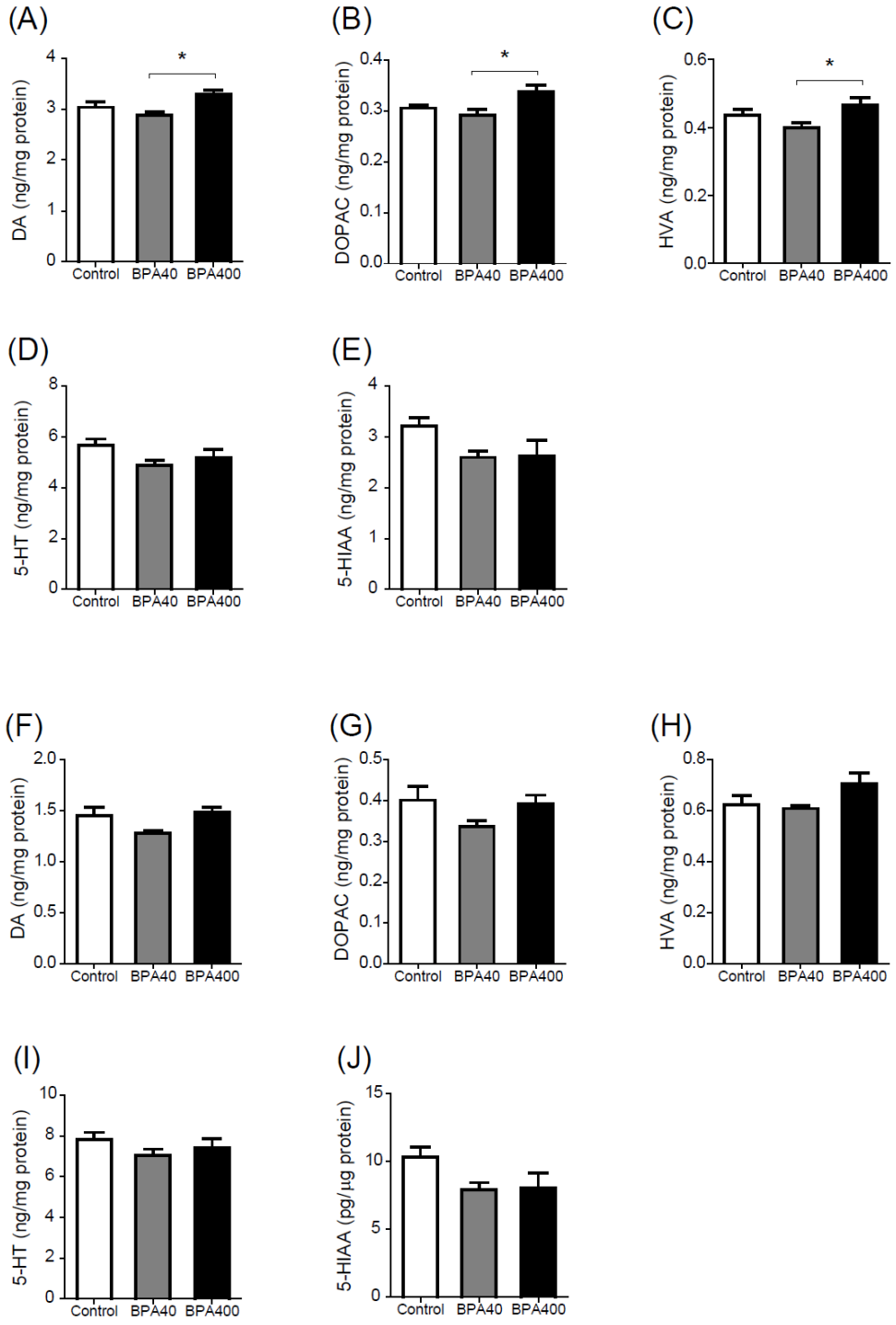


Figure 2 Monoamine concentrations in male mice brains. Concentrations of dopamine (DA), and its metabolites DOPAC and HVA in the forebrain (A-C) or mid-hindbrain (F-H). Concentrations of serotonin (5-HT) and its metabolite 5-HIAA in the forebrain (D-E) or mid-hindbrain (I-J). Concentrations of DA, DOPAC and HVA in the forebrain were significantly increased in BPA400 group compared with the BPA40 group. Data are shown as mean ± SEM, n=6 per treatment, (*P<0.05, One-way ANOVA followed by Tukey-Kramer's *post hoc* test).

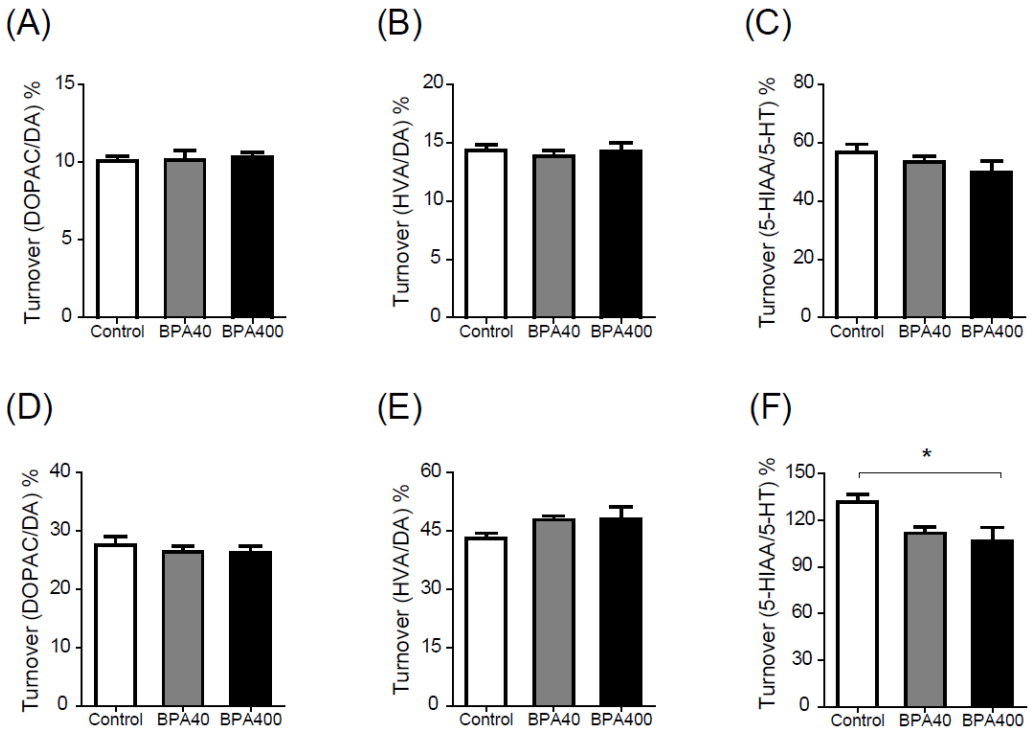


Figure 3 Dopamine and serotonin turnover in male mice brains. Dopamine turnover (i.e., ratio of DOPAC to DA, and HVA to DA) (A, B) and serotonin turnover (i.e., ratio of 5-HIAA to 5-HT) (C) in forebrain. Dopamine turnover (D, E) and serotonin turnover (F) in mid-hindbrain. Serotonin turnover in the hindbrain was significantly decreased in BPA400 group compared with the control group. Data are shown as mean ± SEM, n=6 per treatment, (*P<0.05, One-way ANOVA followed by Tukey-Kramer's *post hoc* test).

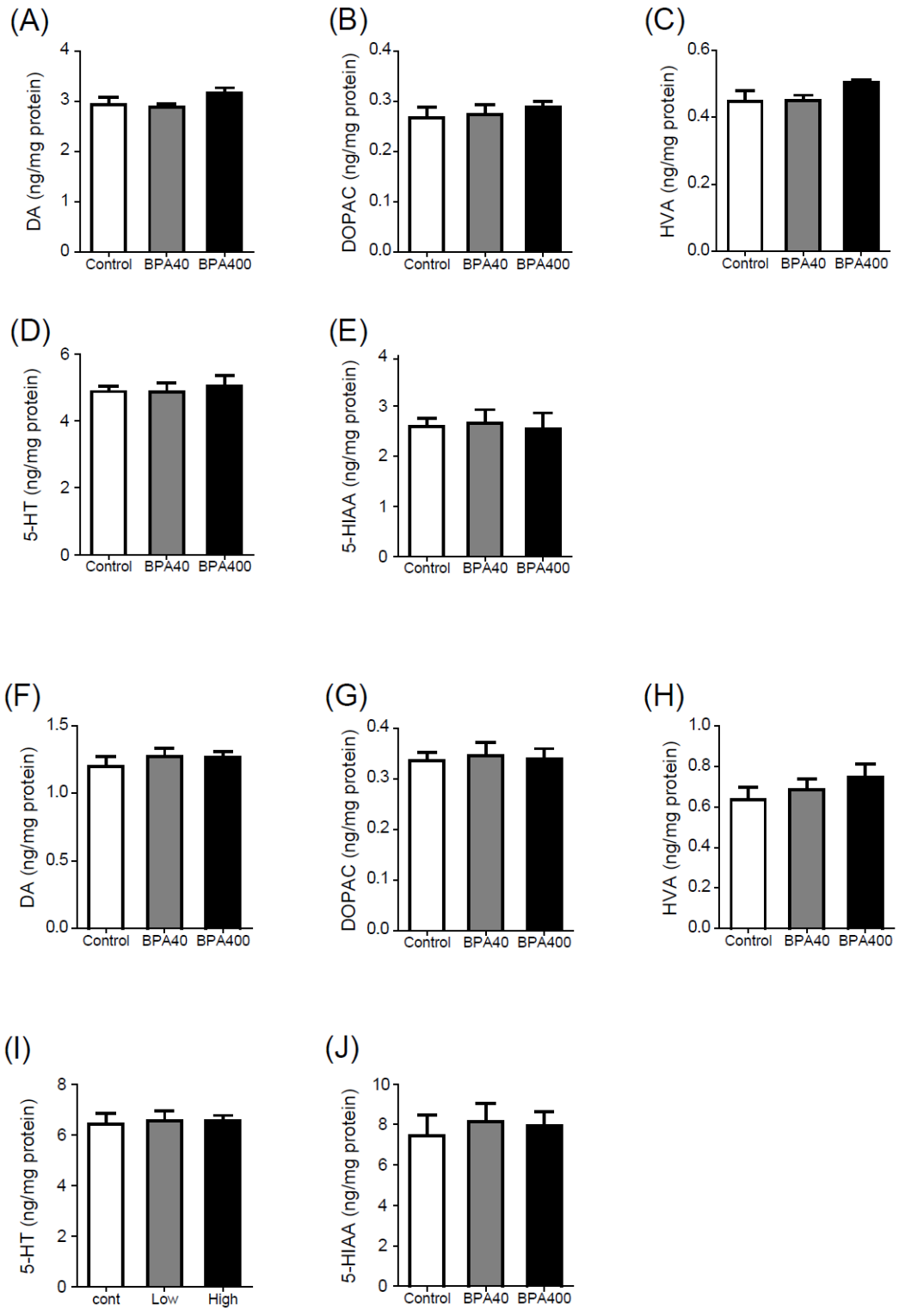


Figure 4 Monoamine concentrations in female mice brains. Concentrations of dopamine (DA), and its metabolites DOPAC and HVA in the forebrain (A-C) or mid-hindbrain (F-H). Concentrations of serotonin (5-HT) and its metabolite 5-HIAA in the forebrain (D-E) or mid-hindbrain (I-J). Data are shown as mean ± SEM, n=6 per treatment.

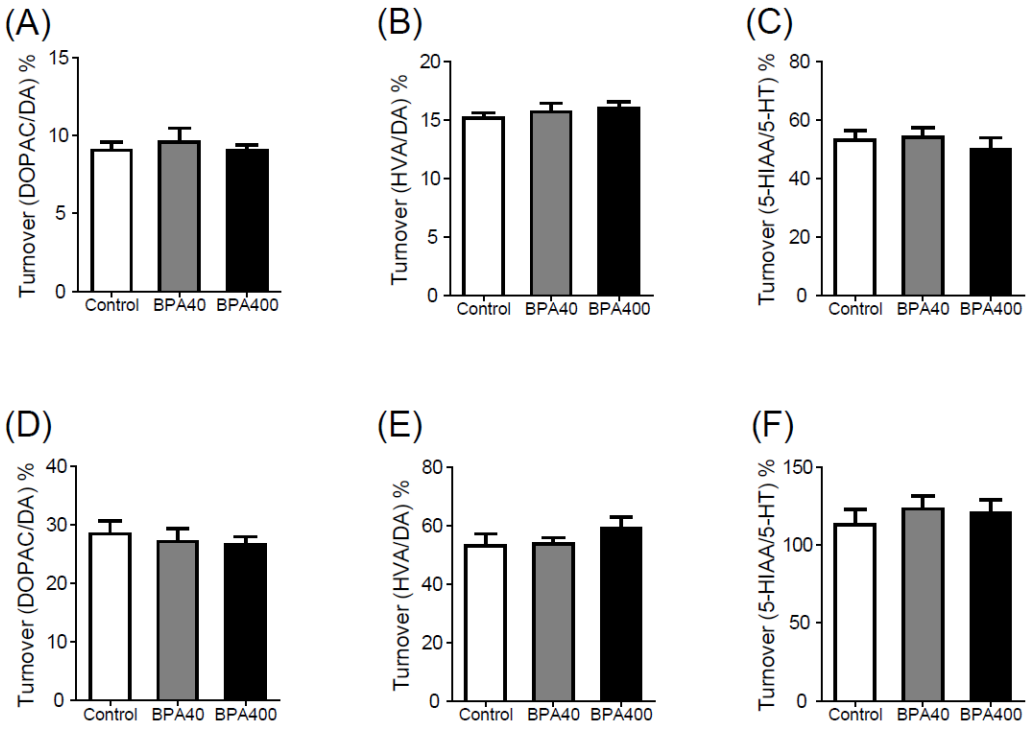
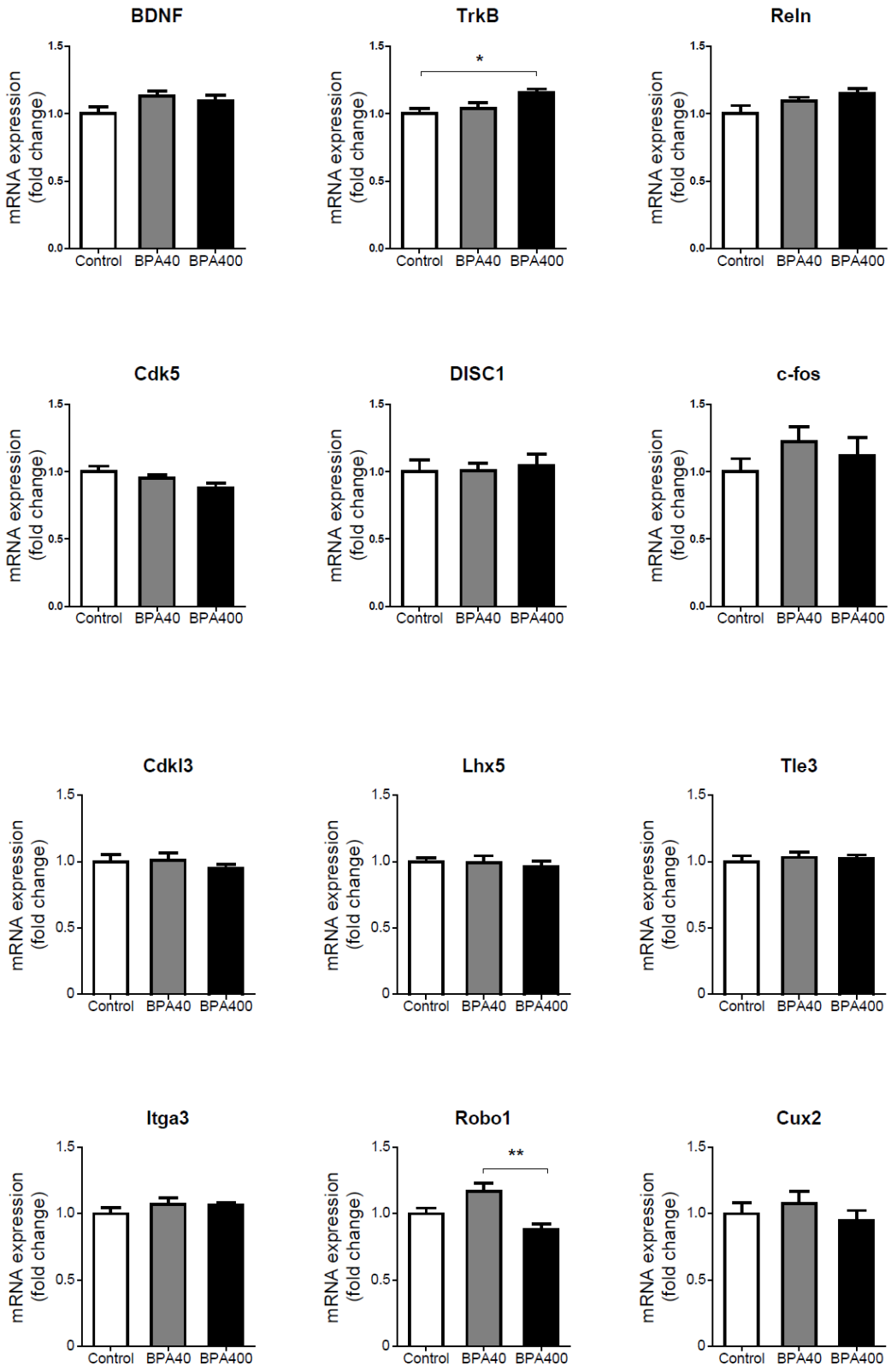


Figure 5 Dopamine and serotonin turnover in female mice. Dopamine turnover (i.e., ratio of DOPAC to DA, and HVA to DA) (A, B) and serotonin turnover (i.e., ratio of 5-HIAA to 5-HT) (C) in forebrain. Dopamine turnover (D, E) and serotonin turnover (F) in mid-hindbrain. Data are shown as mean ± SEM, n=6 per treatment.


BDNF
TrkB
Reln
Cdk5
DISC1
c-fos
Cdkl3
Lhx5
Tle3
Itga3
Robo1
Cux2
mRNA expression (fold change)
Control
BPA40
BPA400
*
**

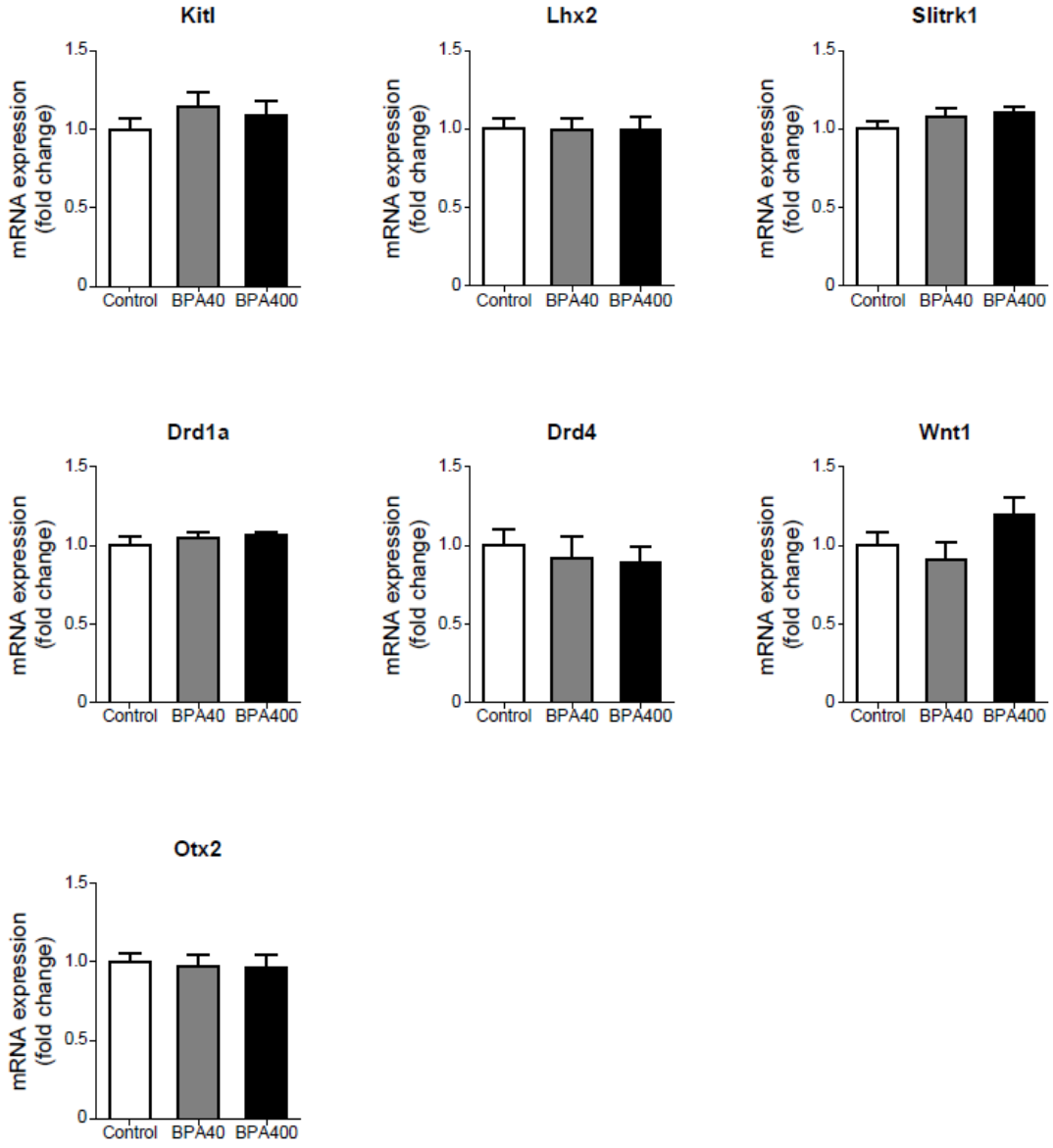


Figure 6 mRNA expression in the male mice forebrain. TrkB was significantly increased in BPA400 group compared with the control group. Robo1 was significantly increased in BPA40 group compared with BPA400 group. Data are shown as mean ± SEM, n=6 per treatment, (*P<0.05, **P<0.01, One-way ANOVA followed by Tukey-Kramer's *post hoc* test).

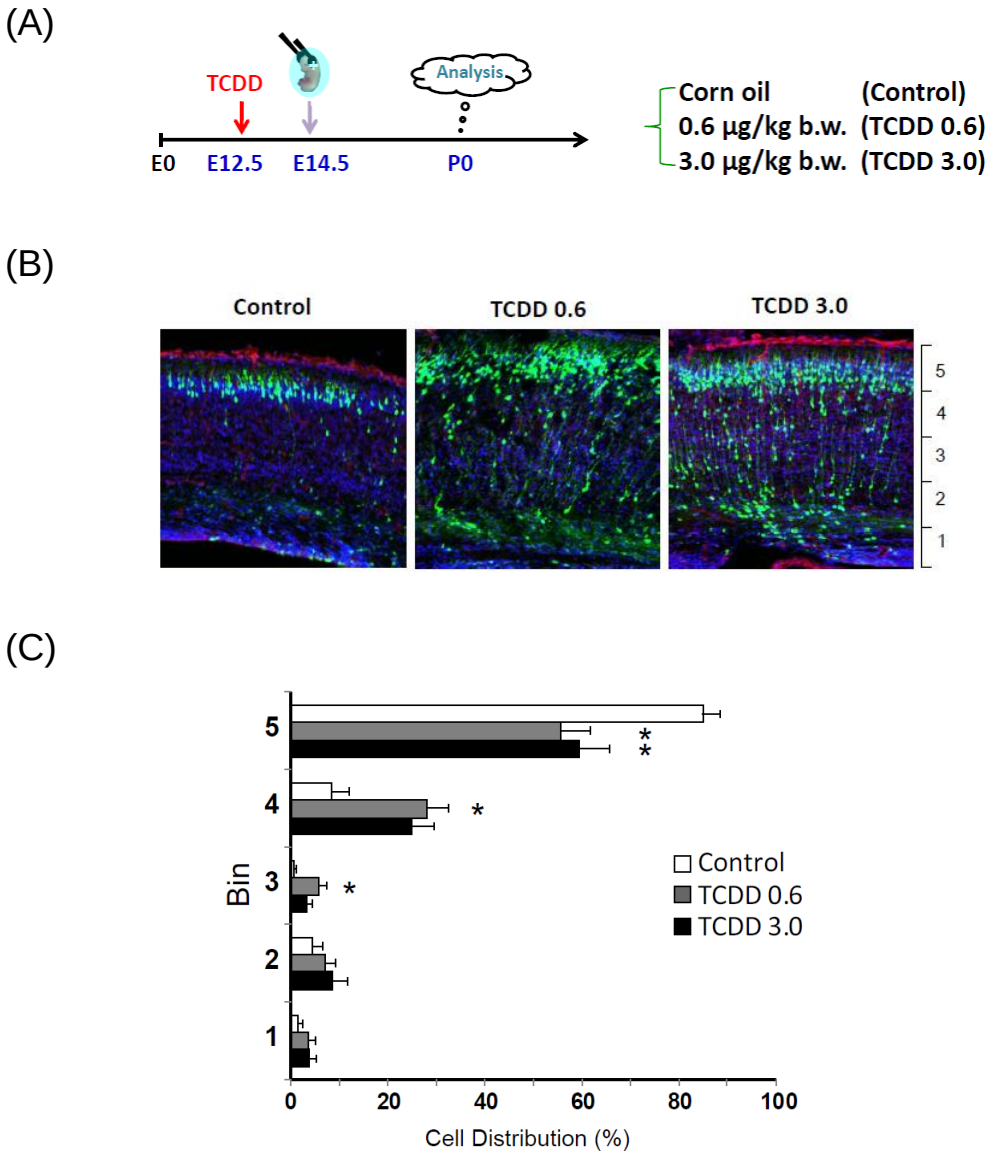


Figure 7 Effects of prenatal TCDD exposure on neuronal migration in the developing cerebral cortex. (A) A scheme of the experiment design: pregnant B6 mice were exposed to 0, 0.6 or 3.0 μg/kg b.w. TCDD at E12.5. GFP expressing plasmids (pCAGGS-GFP) were transfected into neuronal progenitor cells at E14.5 by IUE, and neuronal migration was analyzed at P0. (B) Representative photographs of brain sections transfected with GFP plasmid (green), and stained with DAPI (blue). (C) Distribution of GFP-positive neurons in cerebral cortex at P0. In a bin analysis, the percentage of GFP-positive neurons in Bin 5 of TCDD0.6 group and TCDD3.0 group were significantly decreased compared with the control group. The percentage of GFP-positive neurons in Bin 3 and Bin 4 of TCDD0.6 group were significantly increased compared with control group. Data are shown as mean ± SEM, n=6 per treatment, (*P<0.05, two-way ANOVA followed by Tukey-Kramer's *post hoc* test).

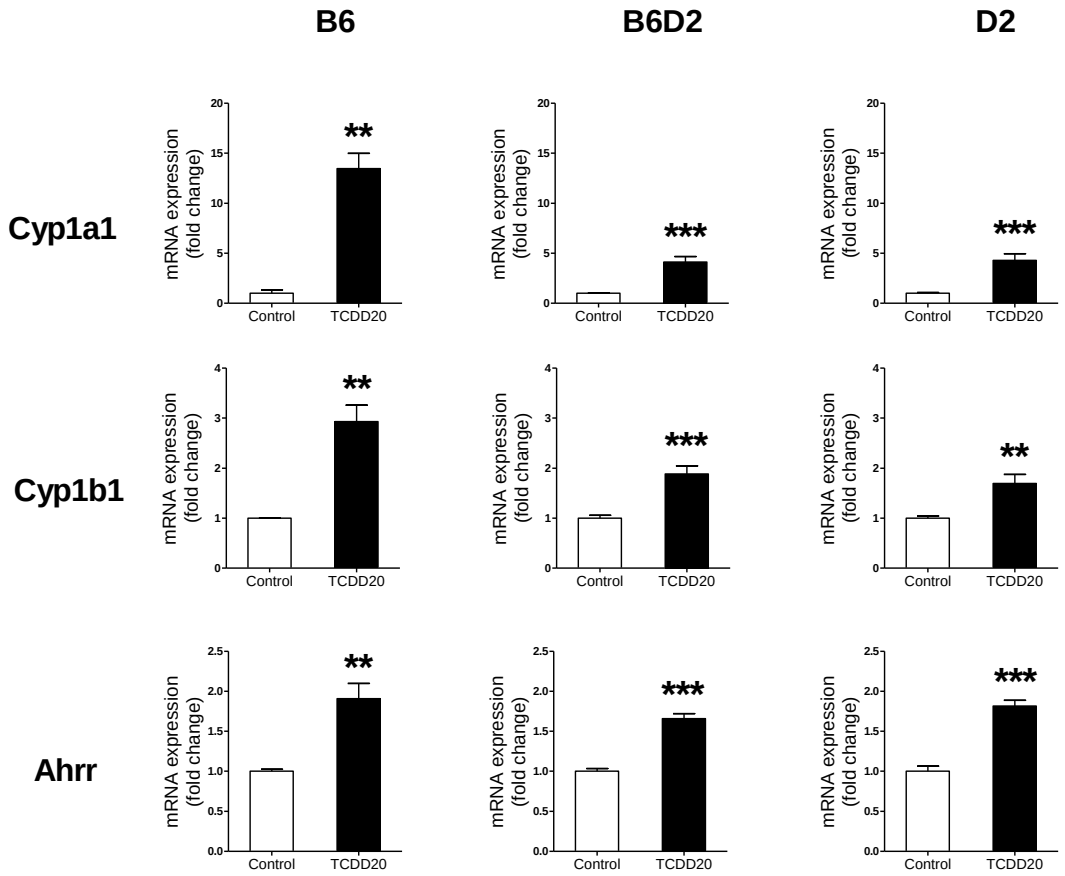


Figure 8 mRNA expression of Cyp1a1, Cyp1b1 and AhRR in B6, B6D2, and D2 mice brains at P0. In B6, B6D2, and D2 mice brains, mRNA expression of Cyp1a1, Cyp1b1 and AhRR were significantly increased in TCDD treated group compared with control group. Data are shown as mean ± SEM. B6 mice: n=3 per treatment; B6D2 mice: n=6 per treatment; D2 mice: n=7 (control), n=5 (TCDD20), (**P<0.01, ***P<0.001, Student's *t* test).

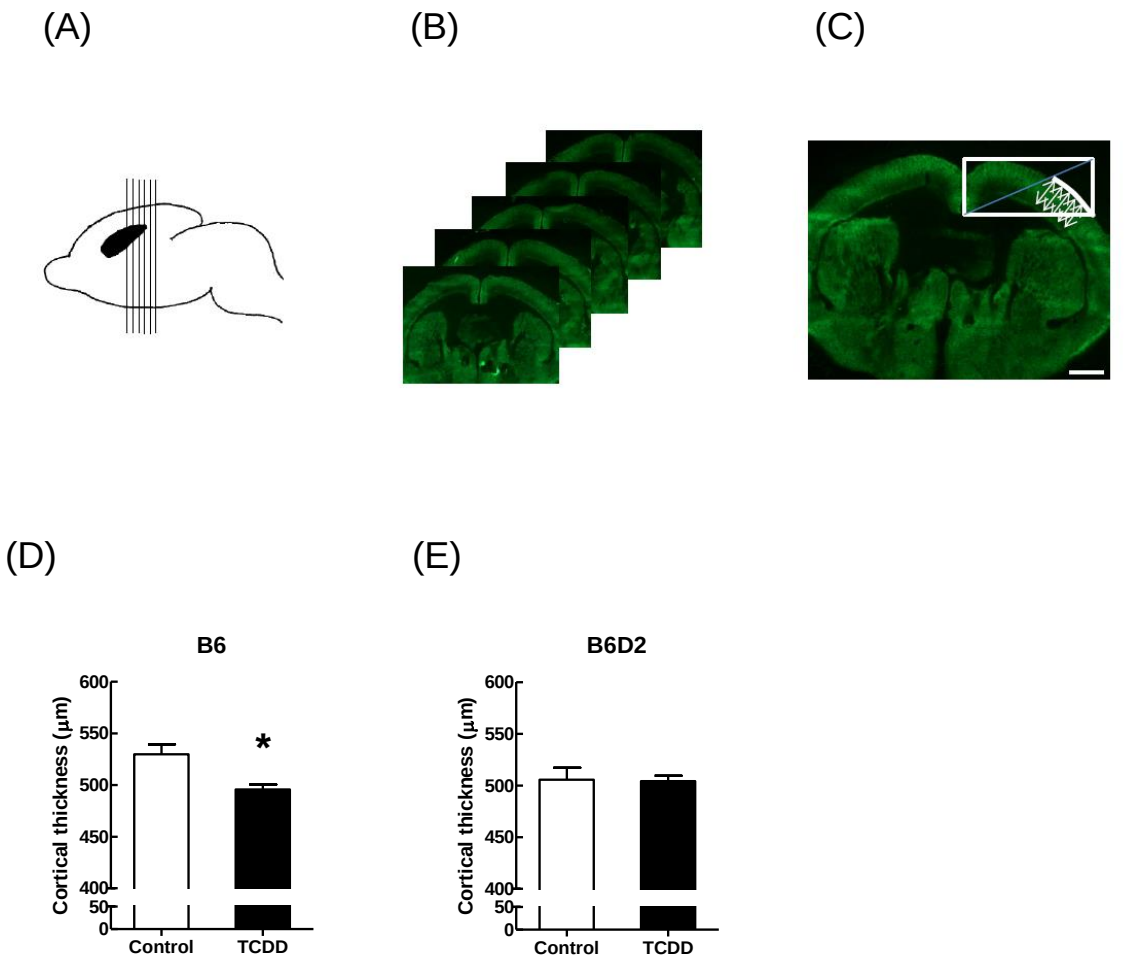


Figure 9 Effects of prenatal TCDD exposure on cortical thickness in B6 or B6D2 mice at P0. (A) Frozen brain was cut into a serial of coronal sections. (B) Bain sections were immunostained with MAP2, and the cortical thickness was determined using 5 serial brain sections. (C) Cortical thickness was defined as the distance between the marginal zone and subplate. All measurements were performed on the somatosensory cortex. Scale bar: 500 μm. (D, E) Cortical thickness of B6 and B6D2 mice upon *in utero* exposure to corn oil or TCDD (20 μg/kg b.w). The cortical thickness of B6 mice was significantly decreased in TCDD group compared with the control group. Data are shown as mean ± SEM, n=5 per treatment, (*P<0.05, Student's *t* test).

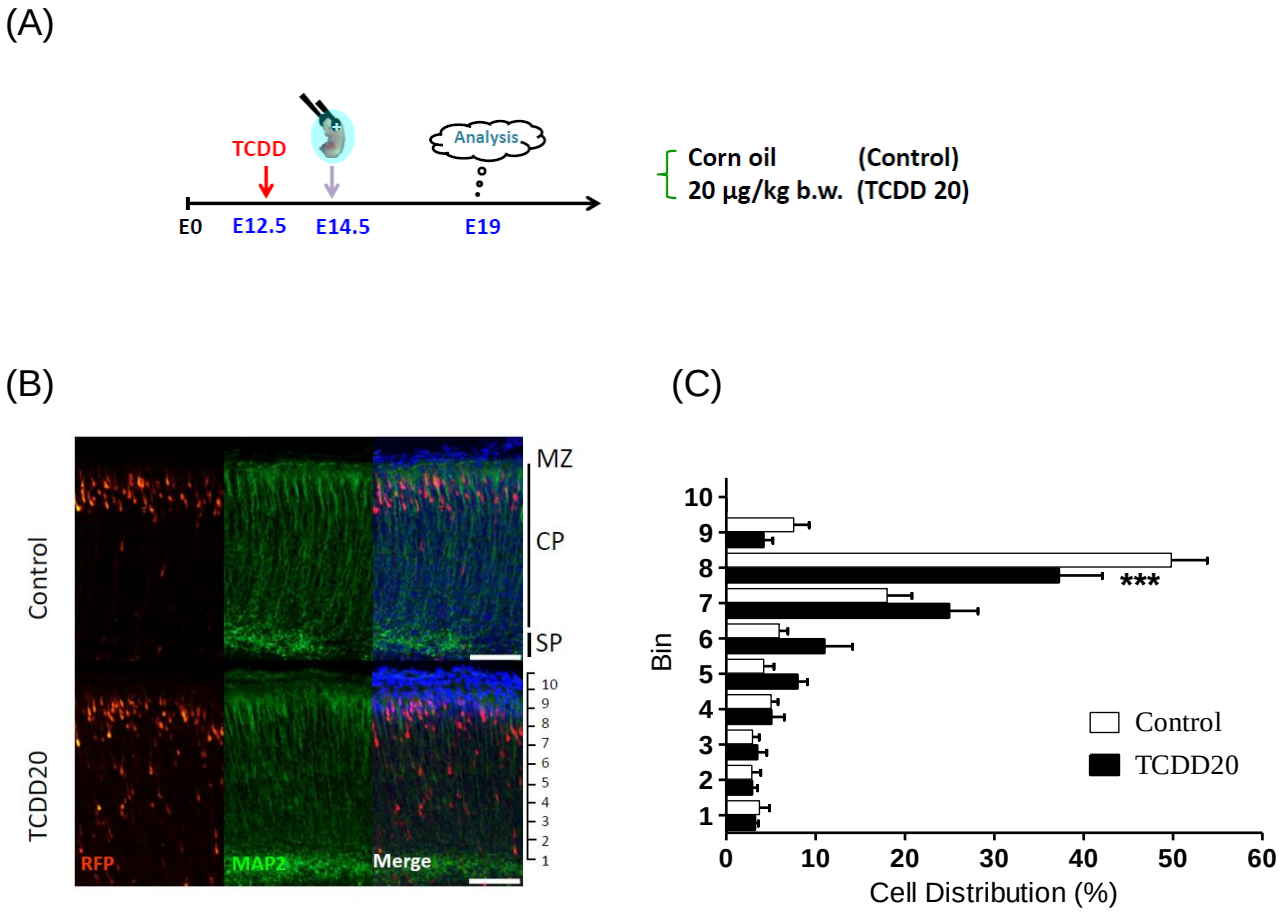


Figure 10 Effects of prenatal TCDD exposure on neuronal migration in the developing cerebral cortex. (A) A scheme of the experiment design: pregnant B6D2 mice were exposed to 0 or 20 μg/kg b.w. TCDD at E12.5. RFP expressing plasmids (pCAGGS-RFP) were transfected into neuronal progenitor cells at E14.5 through *in utero* electroporation, and neuronal migration was analyzed at E19. (B) Representative photographs of brain sections transfected with RFP plasmids (red), and immunostained with MAP2 (green) and DAPI (blue). MZ: marginal zone, CP: cortical plate, SP: subplate. Scale bars: 100 μm. (C) Distribution of RFP-positive neurons in cerebral cortex at E19. In a bin analysis, the percentage of RFP-positive neurons in Bin 8 of TCDD20 group was significantly decreased compared with the control group. Data are shown as mean ± SEM, n=5 per treatment, (***P<0.001, two-way ANOVA, with Bonferroni's *post hoc* test).

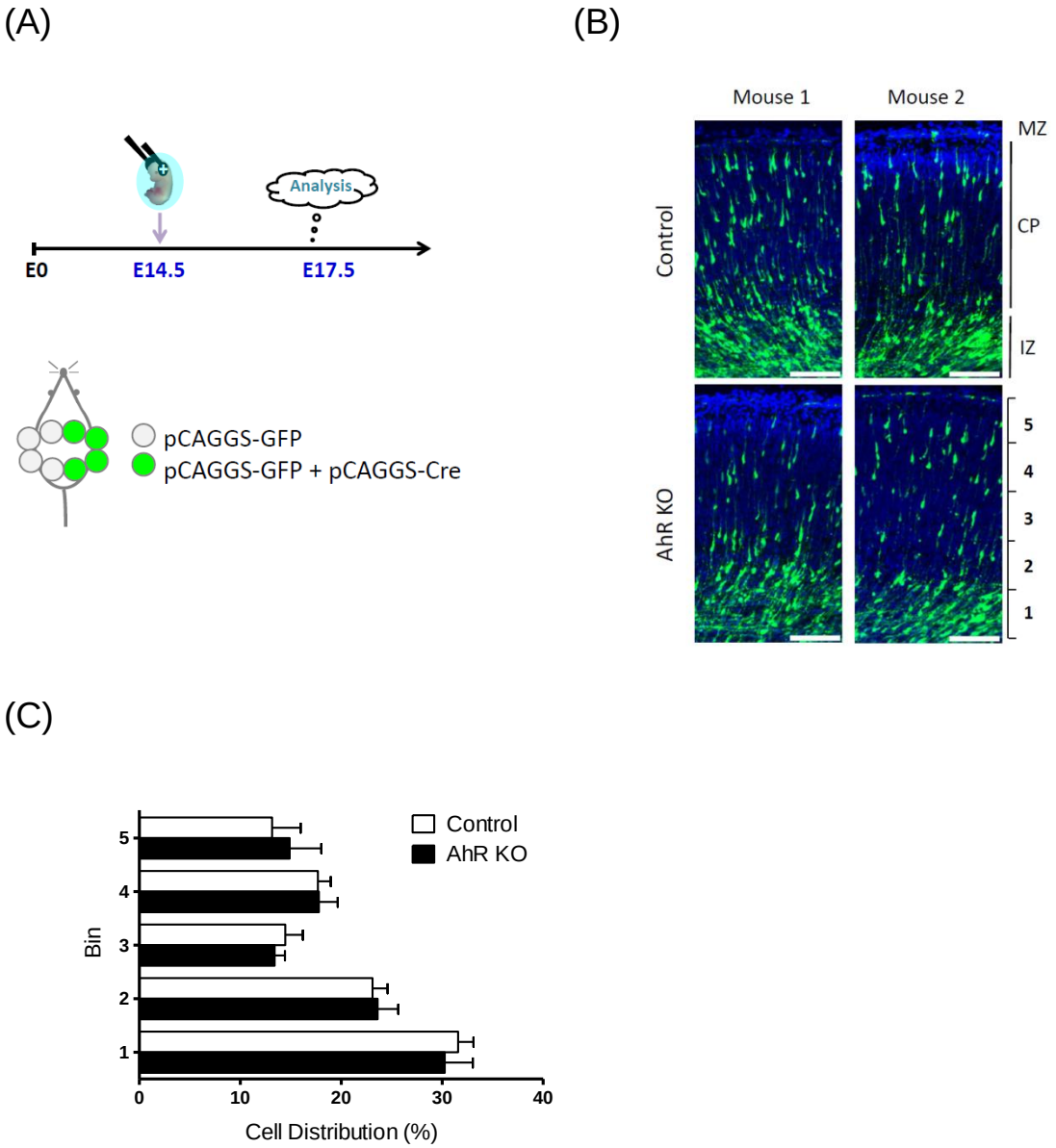


Figure 11 Effects of conditional AhR knockout on neuronal migration in the developing cerebral cortex at E17.5. (A) A scheme of the experiment design: pregnant Ahr^{fx} mice were performed IUE at E14.5. In each dam, half embryos were transfected with pCAGGS-GFP as control and the other half was co-transfected with pCAGGS-GFP and pCAGGS-Cre to delete AhR. Neuronal migration was analyzed at E17.5. (B) Representative photographs of brain sections transfected with GFP (green), and stained with DAPI (blue). MZ: marginal zone, CP: cortical plate, IZ: intermediate zone. Scale bars: 100 μm. (C) Distribution of GFP-positive neurons in cerebral cortex at E17.5. No significant difference was found between control and AhR-KO group at E17.5. Data are shown as mean ± SEM, n=5 (Control), n=7 (AhR KO).

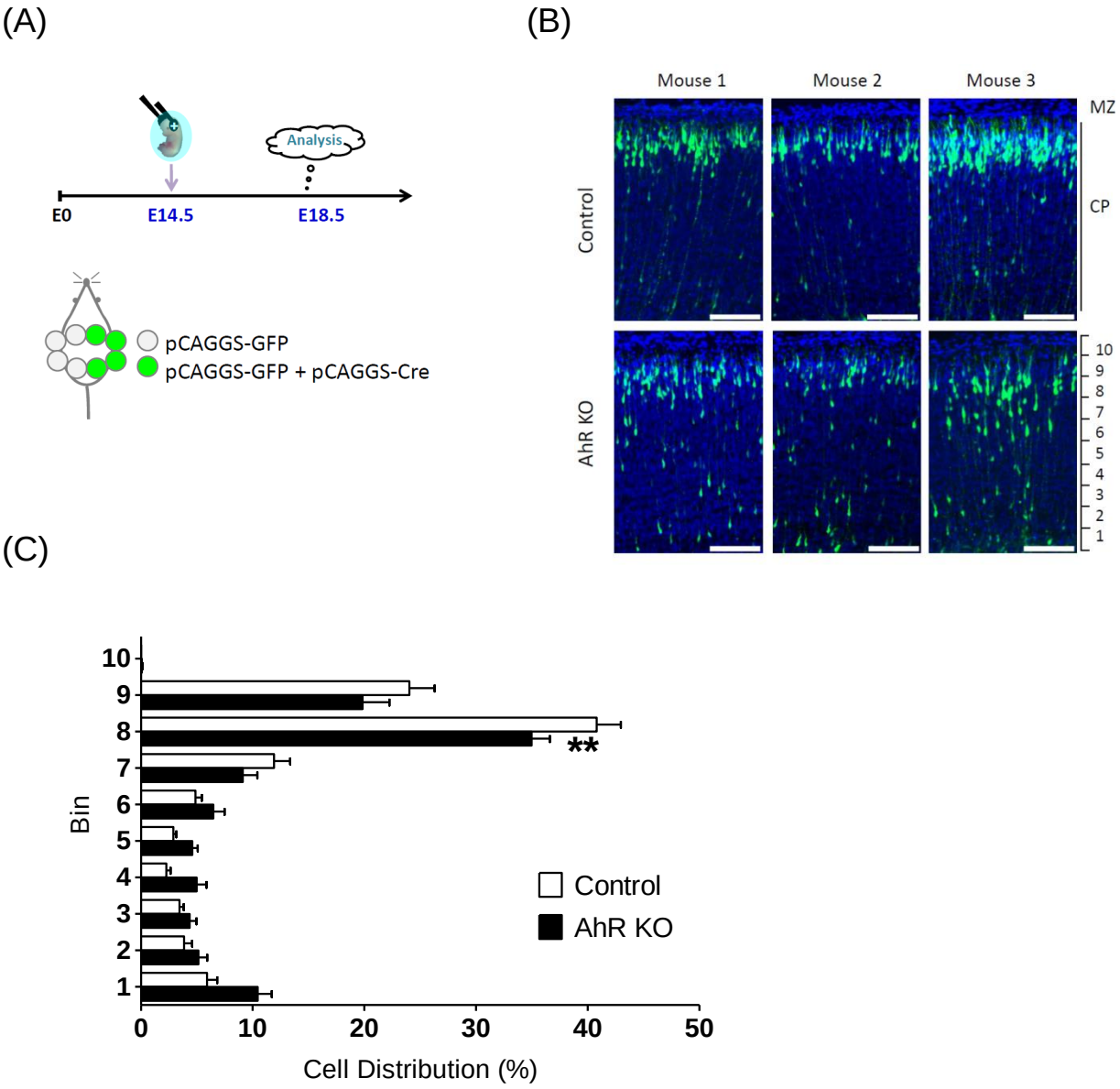


Figure 12 Effects of conditional AhR knockout on neuronal migration in the developing cerebral cortex at E18.5. (A) A scheme of the experiment design: pregnant Ahr^{fx} mice were performed IUE at E14.5. In each dam, half embryos were transfected with pCAGGS-GFP as control and the other half was co-transfected with pCAGGS-GFP and pCAGGS-Cre to delete AhR. Neuronal migration was analyzed at E18.5. (B) Representative photographs of brain sections transfected with GFP (green), and stained with DAPI (blue). Scale bars: 100 μm. (C) Distribution of GFP-positive neurons in cerebral cortex at E18.5. In a bin analysis, the percentage of GFP-positive neurons in Bin 8 was significantly decreased in AhR-KO group compared with the control group at E18.5. Data are shown as mean ± SEM, n=11 per group, (**P<0.01, two-way ANOVA followed by Bonferroni's *post hoc* test).

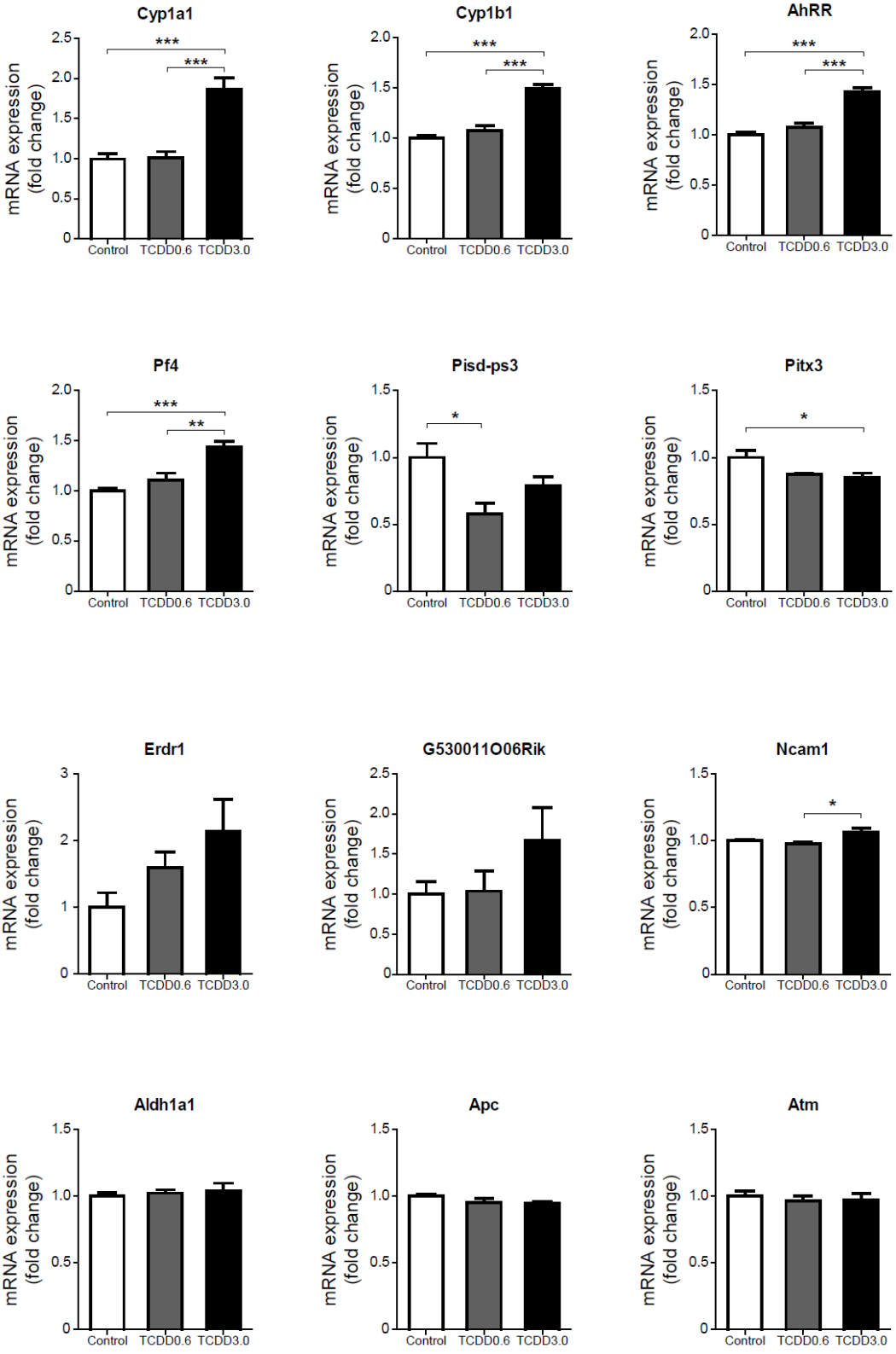
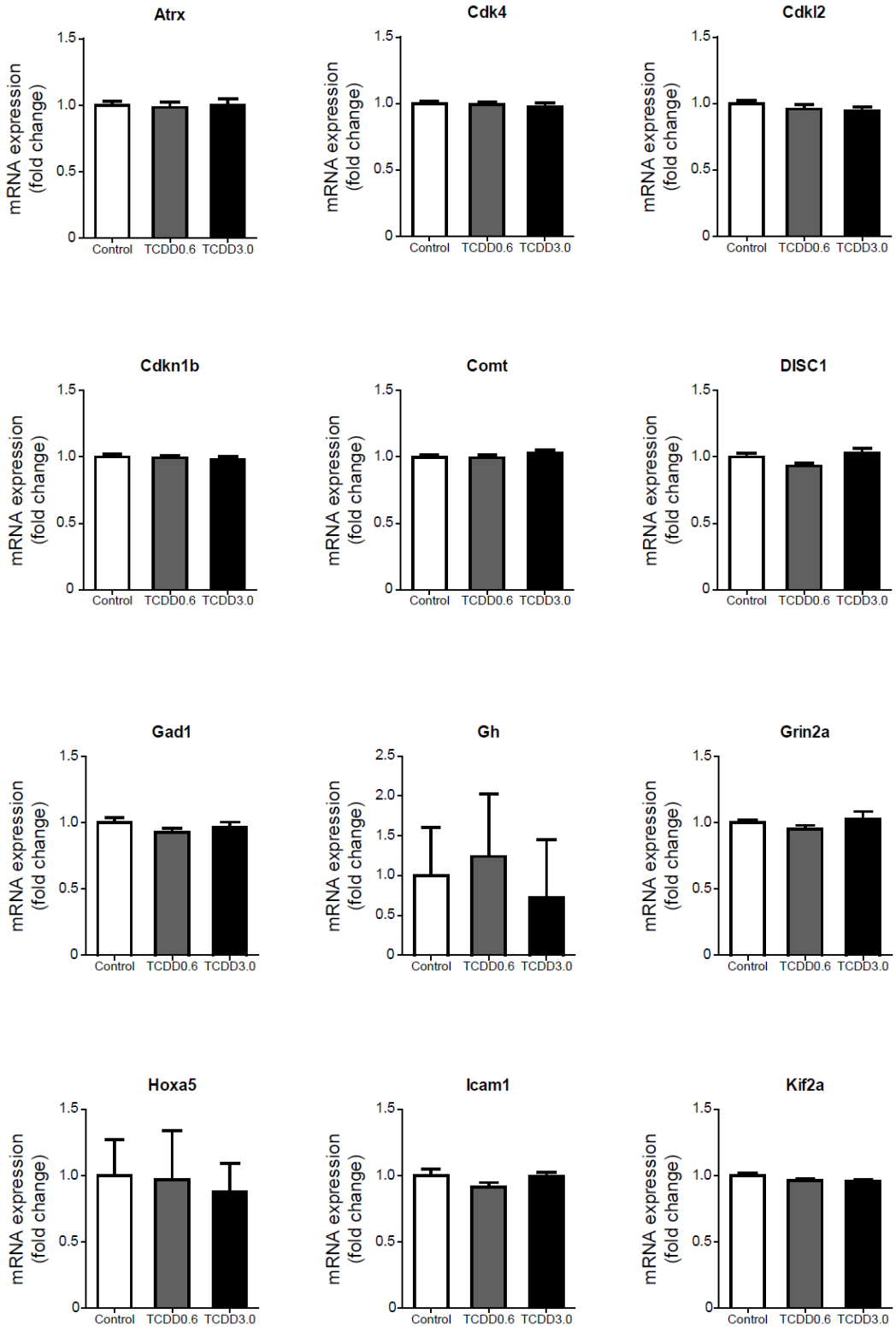

Cyp1a1
Cyp1b1
AhRR
Pf4
Pisd-ps3
Pitx3
Erdr1
G530011O06Rik
Ncam1
Aldh1a1
Apc
Atm
mRNA expression (fold change)
Control
TCDD0.6
TCDD3.0

Figure 13 (continued)



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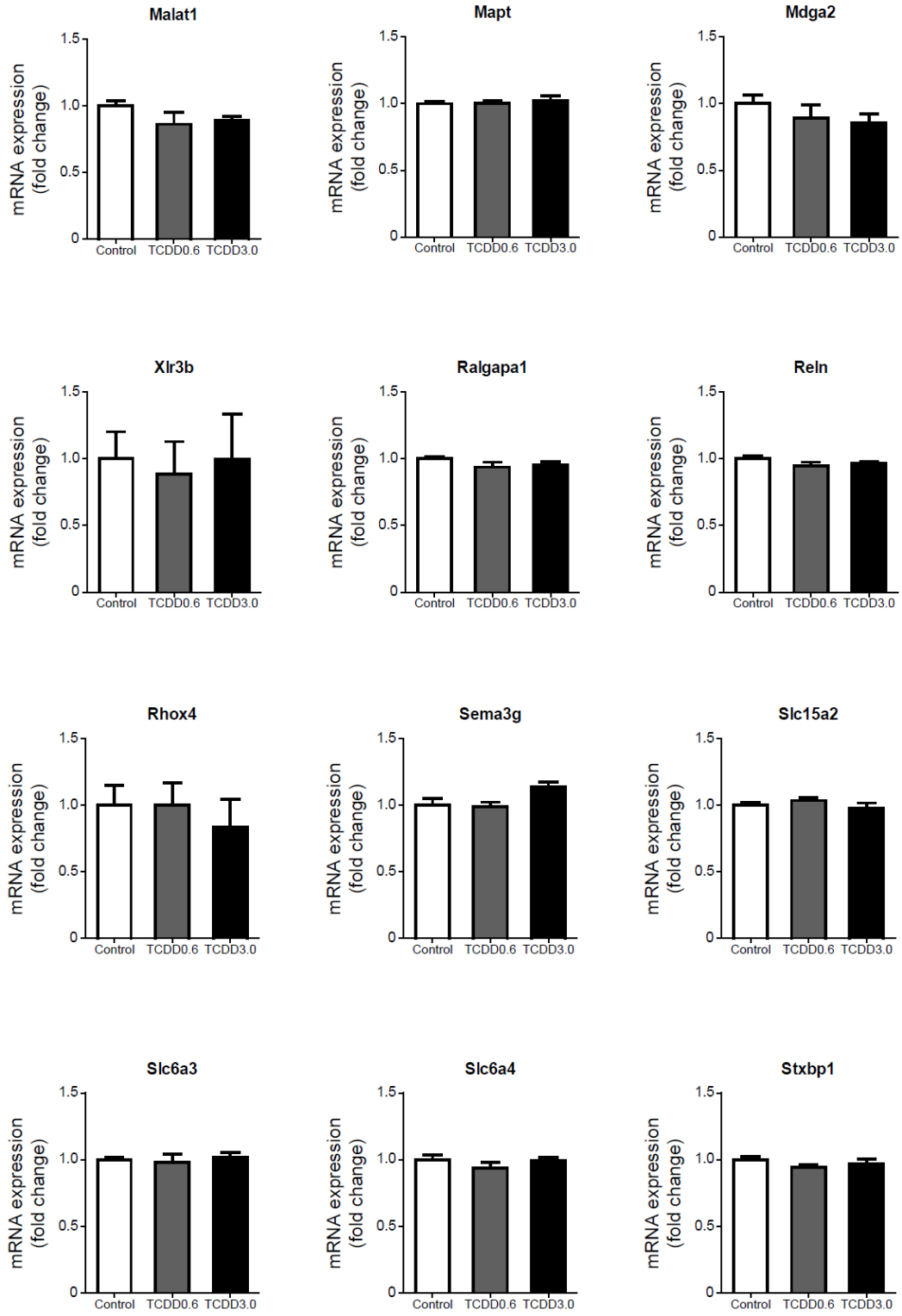

Figure 13 (continued)

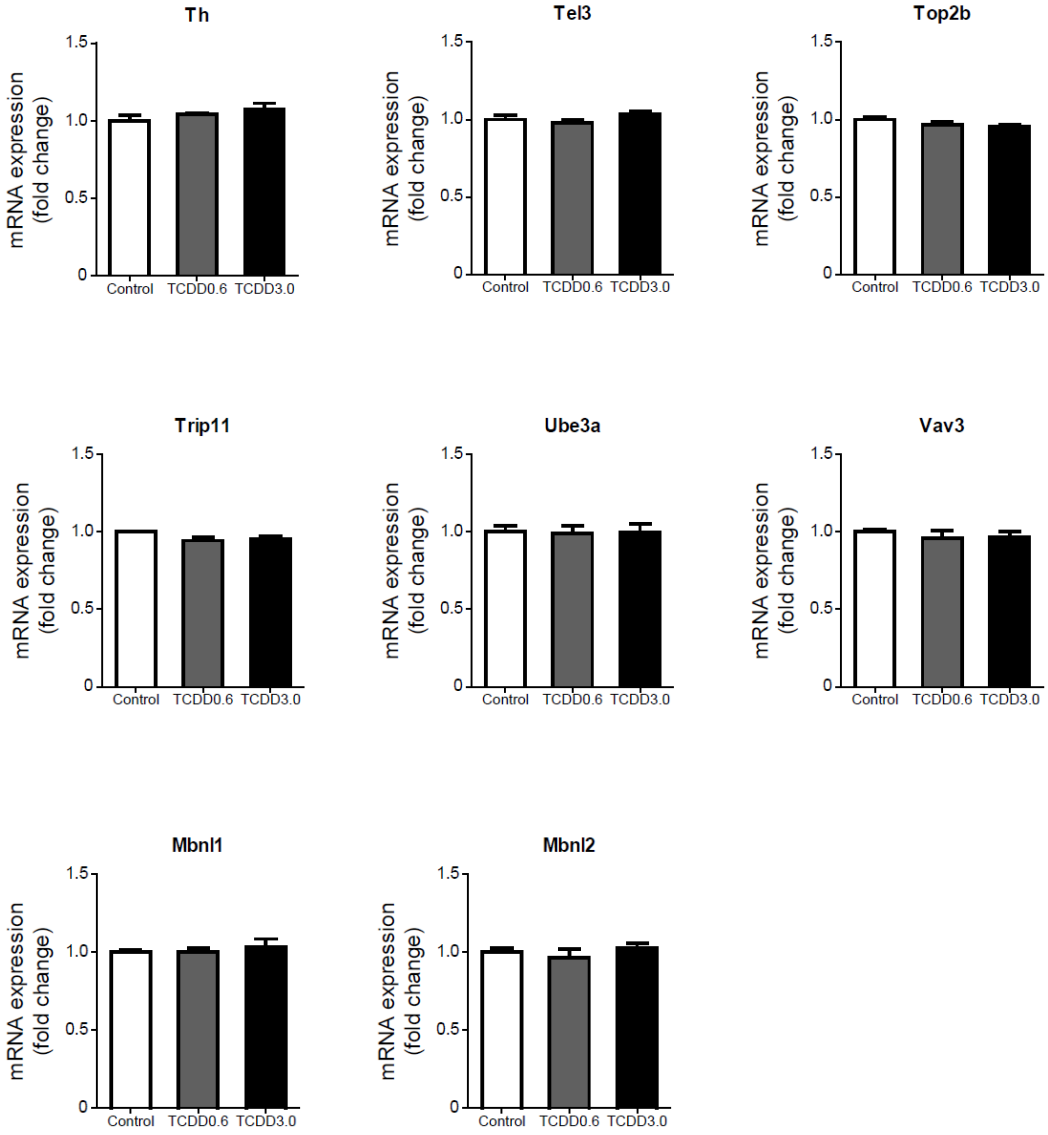

Figure 13 mRNA expression in B6 embryonic brains prenatally treated with TCDD (0, 0.6 or 3.0 μg/kg b.w.). Data are shown as mean ± SEM. n=5 per treatment, (*P<0.05, **P<0.01, ***P<0.001, One-way ANOVA, with Tukey-Kramer's *post hoc* test).

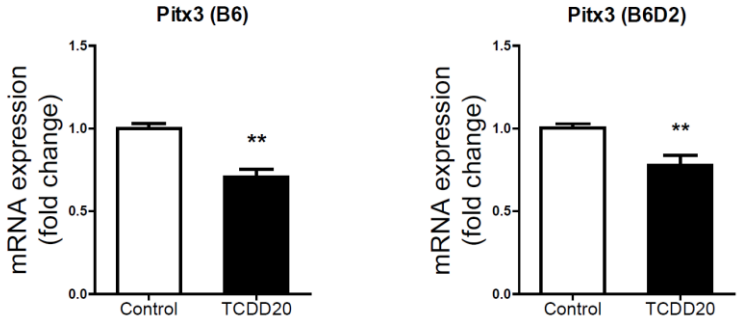

Figure 14 mRNA expression of Pitx3 in B6 or B6D2 mice brains prenatally treated with TCDD (0 or 20 μg/kg b.w.). Data are shown as mean ± SEM. n=3 per treatment (B6), n= 5 per treatment (B6D2), (**P<0.01, Student's *t* test).