CHEMICALS IN THE ENVIRONMENT AND BRAIN DEVELOPMENT: IMPORTANCE OF NEUROENDOCRINOLOGICAL APPROACHES

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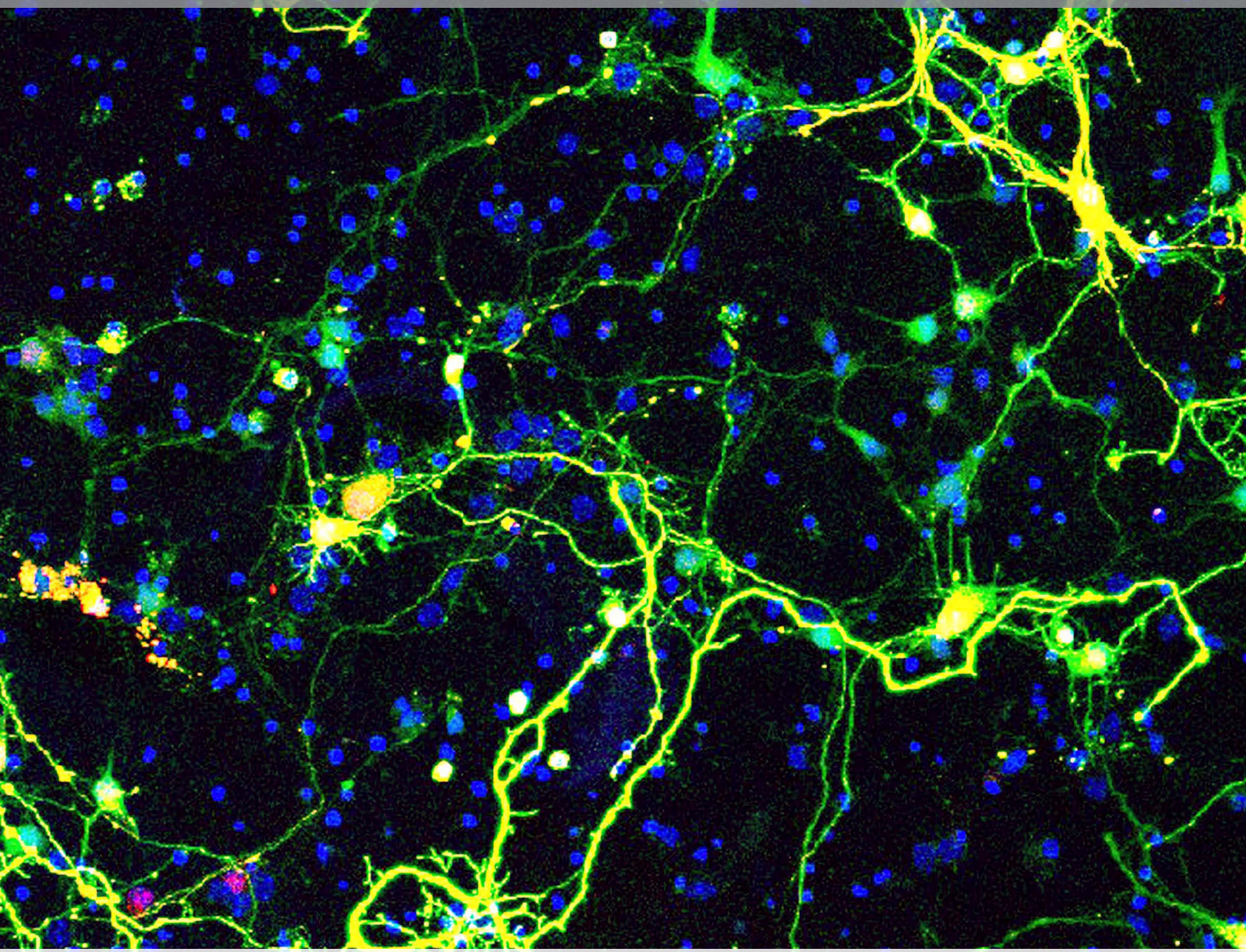

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CHEMICALS IN THE ENVIRONMENT AND BRAIN DEVELOPMENT: IMPORTANCE OF NEUROENDOCRINOLOGICAL APPROACHES

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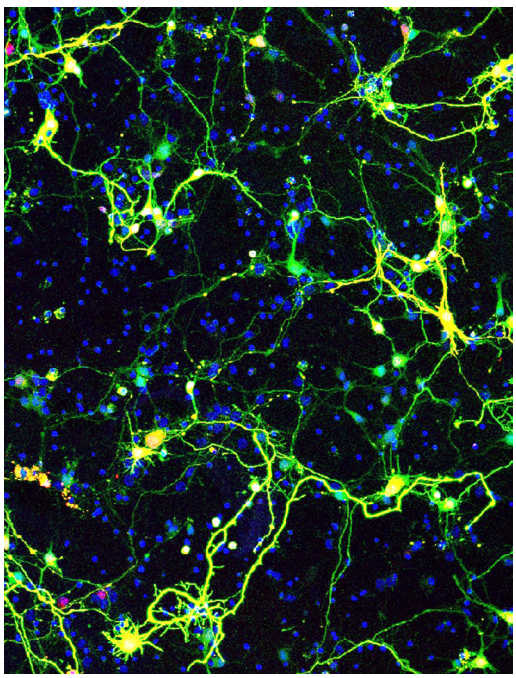

Primary cultured mouse cortical neurons expressing green fluorescent protein (green) and monomeric strawberry protein (red) with a nuclear marker, DAPI (blue), for developmental neurotoxicity testing in vitro. Photo by Fumihiko Maekawa

Mounting evidence shows that increasing numbers of children are being diagnosed with neurodevelopmental disorders, and it is clear that this increase cannot be explained by genetic background alone. A number of studies, including epidemiological studies, have found an association between in-utero and childhood exposure to certain chemicals, such as endocrine disruptors, psychoactive pharmaceuticals, volatile organic chemicals, persistent organic compounds and heavy metals, and children's brain development. Yet, the mechanisms by which these chemicals

impair brain development and function are not fully understood. In addition, little is known about how these chemicals enter and accumulate in the brain. Experimental approaches are essential to understand how those harmful chemicals enter children's brain and pose discrete effects on specific brain sites. These approaches include the following: improvement of technologies for the detection and measurement of neuroendocrinological and behavioral changes in animal models: development of analytical methods for the identification and quantification of chemicals and their metabolites in the brain; development of in vitro cell line assays; and imaging technologies to illustrate cellular functions.

In this research topic, we collected articles that provide state-of-the-art science and technologies that can help us identify environmental chemicals that influence brain development. We also included articles that lead to a better understanding of the actions and dynamics of these chemicals. The articles in this research topics supplied novel information about harmful endpoints of environmental chemicals. The reviews demonstrated the typical and novel interactions between environmental chemicals and the developing brain. We believe that these studies would lead to further understanding of neurodevelopmental disorders caused by environmental factors.

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EDITORIAL
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Editorial: Chemicals in the Environment and Brain Development: Importance of Neuroendocrinological Approaches

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Editorial on the Research Topic

Chemicals in the Environment and Brain Development: Importance of Neuroendocrinological Approaches

In the past three decades, a sharp increase in the number of children diagnosed with neurodevelopmental disorders has been observed; the reason for this is not well-explained (Weintraub, 2011). The human genome does not change rapidly; this suggests that non-genetic factors are the driving forces of this dramatic surge. Several reports, including epidemiological studies, have found an association between *in utero* and childhood exposure to certain environmental chemicals and children's brain development. Yet, the mechanisms by which these chemicals impair brain development and function are not fully understood. In addition, how these chemicals enter and accumulate in the brain are still unknown. Experimental approaches are essential to understand how those harmful chemicals enter children's brain and pose discrete effects on specific brain sites. These approaches include the following: improvement of technologies for the detection and measurement of neuroendocrinological and behavioral changes in animal models: development of analytical methods for the identification and quantification of chemicals and their metabolites in the brain; development of *in vitro* cell line assays; and imaging technologies to illustrate cellular functions.

In this Research Topic, we collected articles that provide state-of-the-art science and technologies that can help us identify environmental chemicals that influence brain development. We also included articles that lead to a better understanding of the actions and dynamics of these chemicals. As summarized in the review by Fujiwara et al. certain chemical exposures such as atmospherically released chemicals (volatile organic chemicals and pesticides), metals, endocrine disruptors, and psychoactive pharmaceuticals are associated with an increased risk of autism spectrum disorder, a neurodevelopmental disorder. Thus, we especially encouraged researchers to submit their works that are related to fetal and early childhood (i.e., early-life) exposure to these chemicals.

Among volatile organic chemicals, Win-Shwe et al. revealed that early-life exposure to secondary organic aerosol, a component of particulate matter (PM), especially $PM_{2.5}$, impairs social memory in adulthood; this was demonstrated using the murine three-chamber test. As for pesticides,

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recent studies have suggested the possibility that neonicotinoids, which are known to overstimulate insect nicotinic acetylcholine receptors and kill insects, also impair neuronal transmission in the mammalian brain. Sano et al. elucidated the effect of developmental exposure to acetamiprid, a neonicotinoid, on murine behavioral profiles in adulthood; their study confirmed the transfer of acetamiprid to the developing brain. They revealed that exposure to acetamiprid induces abnormalities in socio-sexual and anxiety-related behaviors in sex- and dose-dependent manners.

Lead, mercury, and arsenic are infamous as neurotoxic chemicals. Early-life exposures to these metals might be related to the increased risk in neurodevelopmental disorders. Since more than 200 million people worldwide have been estimated to be exposed to arsenic from drinking water and food, experimental studies on its effects on the developing brain are required to evaluate whether early-life exposure to arsenic at environmentally relevant doses causes neurodevelopmental disorders. Aung et al. found that mice exposed to arsenic *in utero* displayed an impaired adaptation to repetitive reversal tasks, one of the typical features of autistic spectrum disorder. They also found that the neurite length of neurons in the prelimbic cortex is significantly reduced in the mice exposed to arsenic *in utero*; this suggests the possibility that impaired formation of neural connections in the prelimbic cortex is one of the causes of the observed behavioral abnormality. In the brain, astrocytes could also be affected by arsenic neurotoxicity. Htike et al. developed a method to evaluate the effect of arsenic exposure on the cell cycle of primary cultured cortical astrocytes using transgenic mice expressing a fluorescence protein indicator, which enabled the visualization of the cell cycle. Using this method, they found that arsenic exposure led to early entry to mitotic S-phase and subsequently induced cell death. The mechanisms of the transport into the brain and the neurotoxicity of metals are not yet fully understood. Ximenes-da-Silva reviewed the biological route through which metal ions is transported and focused on a metal transporter, aquaporin-4. On the toxicity of metals, Harada et al. suggested that gliotransmitter release from astrocytes needs to be investigated as a new target of metal ions.

Among endocrine-disrupting chemicals, bisphenol A has been demonstrated to possess an estrogenic activity in many experimental models. Cano-Nicolau et al. developed a method to detect Cyp19a1 and Cyp19b1 promoter activities by *in vivo* imaging of transgenic zebrafish expressing the green fluorescent protein (GFP) under the control of either Cyp19a1 or Cyp19b1 promoters. They revealed that bisphenol A and its substitutes have strong estrogenic activities. Apart from exhibiting estrogenic activity, bisphenol A and related compounds also have broad toxicological effects. Ling et al. established a method to visualize the neuronal migration in the cerebral cortex using *in utero* electroporation of a plasmid expressing a fluorescent protein. They demonstrated that exposure to bisphenol A during the late embryonic period in mice disturbed neuronal migration and impaired gene expression of neurotrophic factor receptor tropomyosin receptor kinase B (TrkB). The mode of action for endocrine-disrupting chemicals includes epigenetic effects. Derghal et al. reviewed how exposure to certain endocrine-disrupting chemicals changed the expression of microRNA and thereby caused endocrine diseases and disorders.

Exposure to psychoactive pharmaceuticals could affect brain development. Furukawa et al. revealed that the administration of benzodiazepines to mice during the juvenile period caused irreversible learning and memory deficits; this suggests that an extraordinary amount of care is required for prescription of benzodiazepines to juveniles. Steinberg and Moreira reviewed the risk of treatment of pregnant women diagnosed with acute ischemic stroke with recombinant tissue plasminogen activator, which has been shown to have neuroendocrine effects in vasopressin secretion. Although tissue plasminogen activator has been generally avoided in pregnant women, the authors claimed that the treatment risk must be balanced against the potential of maternal health risk of ischemic stroke.

To elaborate the precise mechanism of the effect of early-life exposure to environmental chemicals and use the knowledge for prevention and intervention, the development of alternative animal models that enable the detection of subtle physiological, anatomical, and functional alterations is an urgent matter. Kawashima et al. reviewed how avian models could be used to evaluate developmental abnormalities of the neurologic and reproductive systems. Animal models could be also used to detect compounds that counteract the harmful effects of environmental factors. Ge et al. established the rat model of subclinical hypothyroidism using partial thyroid electrocauterization and found that resveratrol ameliorated the anxiety- and depression-like behaviors. Goto et al. reviewed the murine depression model for future detection of antidepressant-like effects of chemicals.

The articles in this Research Topic, by applying newly established methods, supplied novel information about harmful endpoints of environmental chemicals such as secondary organic aerosol, neonicotinoid, arsenic, bisphenol A, and psychoactive pharmaceuticals. The reviews demonstrated the typical and novel interactions between environmental chemicals and the developing brain. We believe that these studies would lead to further understanding of neurodevelopmental disorders caused by environmental factors.

AUTHOR CONTRIBUTIONS

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MINI REVIEW
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Chemicals, Nutrition, and Autism Spectrum Disorder: A Mini-Review

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The rapid increase of the prevalence of autism spectrum disorder (ASD) suggests that exposure to chemicals may impact the development of ASD. Therefore, we reviewed literature on the following chemicals, nutrient to investigate their association with ASD: (1) smoke/tobacco, (2) alcohol, (3) air pollution, (4) pesticides, (5) endocrine-disrupting chemicals, (6) heavy metals, (7) micronutrients, (8) fatty acid, and (9) parental obesity as a proxy of accumulation of specific chemicals or nutritional status. Several chemical exposures such as air pollution (e.g., particular matter 2.5), pesticides, bisphenol A, phthalates, mercury, and nutrition deficiency such as folic acid, vitamin D, or fatty acid may possibly be associated with an increased risk of ASD, whereas other traditional risk factors such as smoking/tobacco, alcohol, or polychlorinated biphenyls are less likely to be associated with ASD. Further research is needed to accumulate evidence on the association between chemical exposure and nutrient deficiencies and ASD in various doses and populations.

Keywords: autism spectrum disorder, air pollution, chemicals, pesticide, fatty acid, micronutrients, heavy metal, environment

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INTRODUCTION

Autism spectrum disorder (ASD) is a developmental disorder typified by impaired communication and social skills (Grabrucker, 2012). A recent increase in cases of ASD from 4–5 of 10,000 persons in 1966 to 100 cases of 10,000 persons currently (Fombonne, 2009) may not solely be explained by genetic factors (Abrahams and Geschwind, 2010). Thus, it needs to be determined whether environmental factors play a role in the onset of ASD (Grabrucker, 2012), and a recent study using twin samples reported that around 50% of cases of ASD can be explained by environmental factors (Hallmayer et al., 2011).

In the present mini-review, we report several relatively new studies that have evaluated the association between ASD and environmental factors by focusing on chemical or nutritional exposures because these are modifiable factors. These exposures included smoking/tobacco, alcohol, air pollution, pesticides, endocrine-disrupting chemicals, heavy metals, micronutrients, and fatty acid. Parental obesity was also included as an exposure because maternal obesity can be an indicator of exposure to chemicals or nutrition.

SMOKE OR TOBACCO

Although not consistent, most recent population-based studies have suggested that maternal smoking during pregnancy is not directly associated with ASD after adjusting for socioeconomic status (Burstyn et al., 2010; Kalkbrenner et al., 2012; Lee et al., 2012; Tran et al., 2013). For example,

Lee et al. (2012) performed a population-based nested case-control study of 3958 cases of ASD and 38,983 controls in a longitudinal register-based study consisting of individuals aged 4–17 years, and found that maternal smoking during 8–12 weeks of gestation was significantly associated with an increased odds of high-functioning autism in an unadjusted model (odds ratio [OR] = 1.22, 95% confidence interval [CI]: 1.09, 1.36); however, this finding was no longer statistically significant after adjusting for parental socioeconomic status. Additionally, Tran et al. (2013) conducted a population-based nested case-control study comprising 16,185 samples, including 4020 cases of ASD, based on the Finnish National Birth Cohort. Maternal smoking during all pregnancies was not associated with the offsprings' ASD status after adjusting for confounding factors.

Considering that these studies were conducted mostly among Caucasians, the impact of smoking on the development of ASD may differ by race. Zhang et al. (2010) conducted a case-control study using 190 Han children aged 3–21 years with and without autism in China, and found that maternal second-hand smoke exposure during pregnancy, was significantly associated with autism (OR = 3.53, 95% CI: 1.30, 9.56), suggesting that maternal smoking may be associated with ASD among Asians.

ALCOHOL

Few studies have evaluated the impact of maternal alcohol use on the onset of ASD among offspring. Two population-based nested control studies in North European countries reported that maternal alcohol intake during pregnancy was not associated with ASD (Daniels et al., 2008; Eliasen et al., 2010).

AIR POLLUTION

In the last decade, literature on the effect of air pollution exposure during pregnancy on the risk of ASD has grown immensely. Although a large study using direct person-based air sampling is needed, the analytical models used to calculate residence-based effects have become increasingly complex to correctly estimate exposure during a specific time. Regardless of this change in measuring the effect of exposures, most studies have shown a positive association between air pollution exposure and ASD (Suades-Gonzalez et al., 2015). Recent findings support that exposure to particulate matter (PM) <2.5 μm in diameter ($PM_{2.5}$) during the third trimester causes the most detrimental effect on the development of ASD (Kalkbrenner et al., 2015; Raz et al., 2015; Talbott et al., 2015; Weisskopf et al., 2015).

One of the earlier studies that measured air pollution during pregnancy was conducted in 2011 using the distance of one's residence to major roadways as its proxy. Comparing 304 cases of ASD and 259 controls, Volk et al. (2011) reported that mothers of children with ASD were more likely to have lived near a freeway during their third trimester (OR 2.22, CI: 1.16, 4.42) or at the time of delivery (OR 1.86, CI: 1.04, 4.42). This study raised another research question: which type of air pollution has the most effect on the onset of ASD? Evidence of the effect of ozone or nitro-oxides on ASD has been inconclusive (Becerra et al., 2013; Gong T. et al., 2014; Guxens et al., 2016), but maternal exposure to small particles such as diesel PM (Windham et al., 2006; Roberts et al., 2013), PM 2.5 (Becerra et al., 2013; Volk et al., 2013; Raz et al., 2015; Talbott et al., 2015), and PM <10 μm in diameter (PM_{10}) (Volk et al., 2013; Kalkbrenner et al., 2015) has been most consistently reported with an increased risk of ASD. Several case-control studies have investigated the effect of individual hazardous air pollutants (HAP) such as metals and volatile organics on ASD (Windham et al., 2006; Kalkbrenner et al., 2010; Roberts et al., 2013; von Ehrenstein et al., 2014) with all studies conducted in the United States showing that cases of ASD have an elevated exposure of HAP by 1.3–2.0 times. However, two reports from European countries (Guxens et al., 2016) and a separate twin study in Sweden (Gong T. et al., 2014) showed no association of maternal exposure to air pollution and ASD.

Most studies about the effect of air pollution on ASD have been prone to residual confounders such as a low socioeconomic status, which is related to both worse living environments and an increased risk of ASD (Bell and Ebisu, 2012; Shmool et al., 2014).

PESTICIDES

Evidence from previous studies has suggested a strong relationship between pesticide exposure and ASD. Despite the quick turnover in commercial product names, organophosphates (OP) and organochlorines (OC) are still in use despite their neurotoxicity (Kalkbrenner et al., 2014). The association between ASD and pesticides has been observed across studies that measured exposures from residential exposure to agricultural drift (Roberts et al., 2007; Roberts and English, 2013), administered questionnaires on the use of insecticides (Keil et al., 2014), and assessed bio-specimens to detect metabolites (Rauh et al., 2006; Eskenazi et al., 2007; Cheslack-Postava et al., 2013) and numerous pesticides, including but not limited to OC (Roberts et al., 2007; Cheslack-Postava et al., 2013; Roberts and English, 2013; Braun et al., 2014) and OP (Rauh et al., 2006; Eskenazi et al., 2007; Shelton et al., 2014) pesticides.

Shelton et al. (2014) compared 486 cases of ASD and 316 controls, and found an association with OP exposure and ASD, which strengthened later in pregnancy for mothers living within 1.75 km from the agricultural use of OP during their third trimester. They also found increased exposure to pyrethroids in patients with ASD. Eskenazi et al. (2007) and Rauh et al. (2006) reported that cases of ASD had higher OP metabolites during early- to mid-pregnancy. Other case-control studies reported that exposure to imidacloprid through the consistent use of flea/tick pet treatment throughout pregnancy period was associated with ASD (Kalkbrenner et al., 2014; Keil et al., 2014).

ENDOCRINE-DISRUPTING CHEMICALS

Although, polychlorinated biphenyl (PCB) and several dioxins such as tetrachlorodibenzodioxin were banned by the Stockholm Convention in 2001, they are still detected in humans due to their long half-life in the environment, as well as the consumption of predatory fish in which such chemicals tend to accumulate. Other chemicals are still used, such as bisphenol A (BPA), in many

canned foods, receipts, toys, and medical equipment, and some chemicals such as polybrominated diphenyl ethers and phthalates may have even increased body burden (Zota et al., 2008, 2014). Studies on these chemicals are sparse with mixed findings.

Associations between ASD and PCB are inconsistent (Kim et al., 2010; Cheslack-Postava et al., 2013; Braun et al., 2014), and the seemingly elevated risks in a pilot study (Cheslack-Postava et al., 2013) have been criticized for possible bias due to lack of adjustment for birth order (Kalkbrenner et al., 2014). Kardas et al. reported higher serum BPA concentrations in a case-control study of 48 cases of ASD and 41 controls, but no measurement of prenatal exposure was reported (Kardas et al., 2016). Braun et al. (2014) and Miodovnik et al. (2011) failed to find any association with the score of Social Responsiveness Scale (SRS), measurement of ASD traits, and maternal BPA serum or urine concentration and in their cohort studies; however, Braun et al. (2009) found that mid-pregnancy BPA concentrations were associated with an increase in externalizing problem behaviors in early childhood.

However, studies on phthalates mostly suggest an association between ASD and phthalates. Miodovnik et al. (2011) studied 137 children and found that higher phthalate metabolites in maternal urine in the third trimester were associated with a lower score on several of the SRS subscales at 7–9 years old (Miodovnik et al., 2011). Larsson et al. (2009) followed 4779 children and reported that those at 1–6 years old living in homes with polyvinyl chloride flooring (a significant source of phthalates) were 2.4 times more likely to be diagnosed with ASD (Larsson et al., 2009). Kardas et al. (2016) also reported higher serum phthalates concentrations in cases of ASD (Kardas et al., 2016). Braun et al. (2014) failed to detect an association between phthalates in maternal urine and ASD, and Phillipat et al. (Philippat et al., 2015) also failed to detect an association between house dust levels of phthalates and ASD; however, Phillipat et al. explained that the lack of association may be due to fact that the measured exposure may have only poorly reflected the actual exposure of phthalates.

HEAVY METALS

There is sufficient evidence that maternal exposure to heavy metals such as lead, mercury, cadmium, and arsenic cause an increase in neurodevelopmental disorders, and restrict fetal and infant growth even at low-level exposures (De Palma et al., 2012; Ornoy et al., 2015). However, less research has been conducted on heavy metals in relation to ASD. Recently, Rossignol et al. (2014) systematically reviewed literature on environmental toxicants and summarized 40 case-control studies that compared a variety of heavy metal concentrations (i.e., lead, mercury, arsenic, cadmium, aluminum, fluoride, manganese, chromium, nickel, uranium, and tin) in blood, hair, brain, teeth, or urine in children with ASD compared to controls, as well as seven similar studies on urinary porphyrin, which is considered to have a heavy metal burden (Rossignol et al., 2014). The most studied metals were mercury (29 studies) and lead (25 studies). Although the urinary porphyrin studies collectively suggest a higher heavy metal burden among children with ASD, a recent study by Dickerson et al. (2015) found that among 2489 children the prevalence of ASD was higher when mothers were living closer to industrial facilities that released arsenic, lead, or mercury.

A meta-analysis of seven studies on the mean hair level of mercury in a total of 343 cases of ASD and 317 controls did not show any significant association between mercury and ASD (De Palma et al., 2012) and neither did a recent cohort study by van Wijngaarden et al. (2013) on 1784 children and young adults. Some studies that assessed blood have found an association between mercury and ASD (Ip et al., 2004; Desoto and Hitlan, 2007; Geier et al., 2010), whereas others have not (Hertz-Picciotto et al., 2010; Stamova et al., 2011; Albizzati et al., 2012; Adams et al., 2013; Rahbar et al., 2013). However, the lack of adjusting for strong protective factors such as fish oil that are ingested concomitantly in many of the studies (Karagas et al., 2012) and the possible conflict of interest with industries (Kern et al., 2015) may be masking existing associations, as studies on air-borne mercury consistently report an association between mercury exposure and ASD (Windham et al., 2006; Roberts et al., 2013).

MICRONUTRIENTS

Micronutrients are essential for neurogenesis and the development of the neuro-network (Curtis and Patel, 2008). Lower levels of magnesium (Strambi et al., 2006), zinc (Adams and Vogelaar, 2005), selenium (Adams and Vogelaar, 2005), vitamin A (Adams and Vogelaar, 2005), vitamin B complex (Adams and Vogelaar, 2005; Pineles et al., 2010), vitamin D (Adams and Vogelaar, 2005; Gong Z. L. et al., 2014; Kocovska et al., 2014), vitamin E (Adams and Vogelaar, 2005), and carnitine (Filipek et al., 2004) in blood, hair, or other tissue among children with ASD have been reported. Further, the association between a deficiency of micronutrients during pregnancy, such as folic acid (Schmidt et al., 2011, 2012; Suren et al., 2013) and vitamin D (Cannell, 2008; Grant and Soles, 2009), have been reported as a risk for offspring developing ASD.

These previous studies advanced to intervention studies to confirm the causality or possibility of using nutrients to treat ASD. Several studies have reported that nutritional intervention showed a trend toward improvement in patients with ASD. For example, a double-blind study on 20 children (age 3–8 years) with ASD who took a broad-based multi-vitamin and mineral supplement suggested the possible benefit of improving general behavior and receptive language, although this finding was not significant (Adams and Holloway, 2004). Another double-blind study reported that supplementing L-carnosine to children (age 3–12 years) with ASD showed statistically significant improvements in the symptoms on ASD (Chez et al., 2002). In another study, it was also reported that oral magnesium and vitamin B6 supplements led to improvements in social interactions, communication, stereotyped restricted behavior, and abnormal/delayed functioning among children (age 1–10 years) with ASD (Mousain-Bosc et al., 2006).

Several studies have reported the association between gender and ASD in the relationship with micronutrients. For example, a study conducted in the Faroe Islands (Kocovska et al., 2014) noted the trend for ASD males having lower levels of vitamin

D and 25(OH)D3. Similarly, another study suggested that the differenteial effects of estrogen and testosterone on vitamin D metabolism might explain the gender difference of ASD (Cannell, 2008).

FATTY ACIDS

As neural development requires essential fatty acids, particularly long-chain omega-3 fatty acids during critical growth periods, and inflammation may be associated with ASD (Ornoy et al., 2015), the fatty acid level may play an important role in the development of ASD. Several studies have shown that both red blood cell and plasma fatty acid composition among cases of ASD differ from those of non-ASD people. Specifically, the levels of omega-3 fatty acids (Vancassel et al., 2001; Bell et al., 2004; Brigandi et al., 2015), docosahexaenoic acid (DHA) (Meguid et al., 2008; Wiest et al., 2009; El-Ansary et al., 2011; Al-Farsi et al., 2013; Brigandi et al., 2015), and arachidonic acid (AA) (Meguid et al., 2008; El-Ansary et al., 2011; Brigandi et al., 2015; Yui et al., 2016) were significantly lower in the red blood cell or plasma of cases of ASD compared to controls, although some studies did not support these claims (Bu et al., 2006; Bell et al., 2010). To date, only one study has examined maternal fatty acid intake during pregnancy is association with ASD (Lyall et al., 2013). Women with higher intake of polyunsaturated fatty acids (PUFA) before and during pregnancy had a reduced risk of having a child with ASD than those with lower PUFA intake. Analysis on specific PUFAs showed that women in the highest quartile of intake of omega-6 fatty acids had a 34% reduction in the risk of having a child with ASD compared with those in the lowest quartile, with similar results for linoleic acid intake. In concern with omega-3 fatty acids, women with very low intakes (i.e., the lowest 5% of the distribution) of had a significantly increased risk of having a child with ASD compared with those in the middle 90% of the distribution.

Reports on the benefits of fatty acid supplementation in children with ASD are inconclusive. Recently, Mankad et al. (2015) conducted a randomized controlled 6-month trial of 1.5 g/day of omega-3 fatty acids or a placebo in 38 children aged 2–5 years with ASD, and found no evidence for the efficacy of omega-3 fatty acids on improving core symptoms (Mankad et al., 2015). However, Ooi et al. (2015) conducted a 12-week open-label study of 1 g/day of omega-3 fatty acids in 41 children aged 7–18 years with ASD, and found significant improvements in the core symptoms and attention problems (Ooi et al., 2015). Yui et al. (2012) conducted a randomized controlled 16-week trial of AA and DHA supplementation or a placebo in 13 individuals aged 6–28 years with autism, and found significant improvements in social withdrawal and communication (Yui et al., 2012). These studies were relatively small, thus the findings may be by coincidental so a further larger randomized controlled trial is needed.

PARENTAL OBESITY

Maternal obesity can be associated with having offspring with ASD due to the accumulation of the aforementioned chemicals, or it can serve as a proxy of poor nutrition (Dodds et al., 2011; Kawicka and Regulska-Ilow, 2013; Ornoy et al., 2015). According to a Swedish cohort study of 333,057 participants, which included 6420 individuals with ASD, maternal overweight or obesity evaluated at the first antenatal visit was associated with having an offspring with ASD (Gardner et al., 2015). However, the association between an elevated maternal body mass index and the risk of ASD was not clear in matched sibling analyses.

In a population-based prospective cohort study of 92,909 children (age 4–13 years), Suren et al. (2014) investigated the association between ASD and paternal obesity recorded in the questionnaires answered by the fathers. They found that paternal obesity was associated with an increasing risk of ASD (adjusted OR: 1.73, 95% CI: 1.07, 2.82), whereas maternal obesity showed only a weak association with ASD (Suren et al., 2014).

SUMMARY AND FUTURE DIRECTIONS

In summary, several chemical exposures such as air pollution (e.g., PM 2.5), pesticides, BPA, phthalates, mercury or lead, and nutrition deficiencies such as folic acid, vitamin D, or fatty acid are possibly associated with the onset of ASD, whereas other traditional risk factors such as smoke/tobacco, alcohol, or PCB are less likely to be associated with ASD. Apparently, no single environmental factor can explain the development of ASD, suggesting that upstream environmental factors such as socioeconomic status need to be considered as risk factors for ASD, which have not been as rigorously investigated (Fujiwara, 2014). Further, few studies have investigated the accumulative or synergistic effect of the different chemical exposures and nutrition deficiencies simultaneously. The impact of multiple exposures to chemicals and nutrient deficiencies, which are suggestive of association with ASD, need to be studied together to assess whether effect is additive or multiplicative. Moreover, not all children exposed to these chemicals or nutrients may have risk of developing ASD, suggesting that some genetic polymorphism related to ASD, such as *CD38* (Higashida et al., 2012), may have an interaction effect with these environmental exposures during the onset of ASD, as studied in the exposure of heavy mental and genetic polymorphism related to metabolism (Rossignol et al., 2014). Moreover, few chemical or nutritional exposures were investigated to elucidate the mechanism of gender difference of ASD prevalence. These uncovered topics need to be investigated in future research.

AUTHOR CONTRIBUTIONS

TF conceived the review focus, conducted literature review, summarized, and finalized the manuscript. NM, YH, MS, and YT reviewed literature, wrote first draft, and finalized the manuscript. All authors approved final version of manuscript.

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Exposure of BALB/c Mice to Diesel Engine Exhaust Origin Secondary Organic Aerosol (DE-SOA) during the Developmental Stages Impairs the Social Behavior in Adult Life of the Males

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Secondary organic aerosol (SOA) is a component of particulate matter (PM) 2.5 and formed in the atmosphere by oxidation of volatile organic compounds. Recently, we have reported that inhalation exposure to diesel engine exhaust (DE) originated SOA (DE-SOA) affect novel object recognition ability and impair maternal behavior in adult mice. However, it is not clear whether early life exposure to SOA during the developmental stages affect social behavior in adult life or not. In the present study, to investigate the effects of early life exposure to DE-SOA during the gestational and lactation stages on the social behavior in the adult life, BALB/c mice were exposed to clean air (control), DE, DE-SOA and gas without any PM in the inhalation chambers from gestational day 14 to postnatal day 21 for 5 h a day and 5 days per week. Then adult mice were examined for changes in their social behavior at the age of 13 week by a sociability and social novelty preference, social interaction with a juvenile mouse and light-dark transition test, hypothalamic mRNA expression levels of social behavior-related genes, estrogen receptor-alpha and oxytocin receptor as well as of the oxidative stress marker gene, heme oxygenase (HO)-1 by real-time RT-PCR method. In addition, hypothalamic level of neuronal excitatory marker, glutamate was determined by ELISA method. We observed that sociability and social novelty preference as well as social interaction were remarkably impaired, expression levels of estrogen receptor-alpha, oxytocin receptor mRNAs were significantly decreased, expression levels of HO-1 mRNAs and glutamate levels were significantly increased in adult male mice exposed to DE-SOA compared to the control ones. Findings of this study indicate early life exposure of BALB/c mice to DE-SOA may affect their late-onset hypothalamic expression of social behavior related genes, trigger neurotoxicity and impair social behavior in the males.

Keywords: diesel exhaust, secondary organic aerosol, brain, social behavior, mice

INTRODUCTION

Current epidemiological studies have indicated that inhalation of high levels of particulate matter (PM) is associated with damage to the central nervous system (Block and Calderón-Garcidueñas, 2009; Win-Shwe and Fujimaki, 2011; Block et al., 2012; Genc et al., 2012; Weisskopf et al., 2015). Ambient PM consists of primary particles emitted directly from sources, and secondary particles formed by photo-oxidation reactions of volatile organic compounds and gases in the atmosphere, which are known as secondary organic aerosols (SOAs) (Robinson et al., 2007). Diesel exhaust (DE) is a major component of PM and a major precursor of SOA (Kanakidou et al., 2005; Virtanen et al., 2012). Nowadays, the importance of SOA formation in urban areas is well-recognized, not only in the atmosphere but also in indoor environments (Wang et al., 2012; Youssefi and Waring, 2012). It has been reported that exposure to SOA emitted from coal-fired power plants may be associated with an increased risk of heart disease in susceptible animals (Wellenius et al., 2011). However, data showing the effects of SOA on central nervous system and neurobehavioral functions are very limited.

Human epidemiological studies and animal studies suggest that exposure to air pollution may lead to neurotoxicity (Costa et al., 2014). Recent review report indicates that the constituents of air fresheners can react with ozone to produce SOA and these pollutants adversely affect human health such as damage to the central nervous system and respiratory system and alteration of hormone secretion and immune responses (Kim et al., 2015). Previously, our research group has shown that the effects of primary particles such as carbon black nanoparticles and nanoparticle-rich diesel exhaust on brain inflammatory mediators, neurotransmitter system, memory function-related gene expression and learning performance in adult mice (Win-Shwe and Fujimaki, 2011). We have demonstrated that neuroinflammatory effects and neurotoxic effects of carbon black nanoparticle exposure by measuring inflammatory mediators and excitatory amino acid neurotransmitter levels in the hippocampus of BALB/c adult mice (Win-Shwe et al., 2006, 2008a). Furthermore, we have also shown that the effects of nanoparticle-rich diesel exhaust exposure on brain neurotransmitter, inflammatory biomarkers and learning ability in adult mice (Win-Shwe et al., 2008a,b, 2009, 2012a,b). We have generated SOA by adding ozone to diesel exhaust particles and established SOA inhalation chamber in our Research Institute. Using SOA inhalation chambers, we have shown that exposure to SOA for 3 months caused learning and memory impairment in adult male mice and SOA exposure for 1 month in female mice may cause changes in maternal behavior (Win-Shwe et al., 2014). Moreover, we have established the neonatal animal model for early detection of environmental pollutant-induced learning disability and reported that the diesel engine exhaust-derived secondary organic aerosol (DE-SOA) impairs olfactory-based spatial learning activity in preweaning mice (Win-Shwe et al., 2015). In that study, we have also shown that learning impairment was associated with modulation of N-methyl-D-aspartate (NMDA) receptor, signaling pathway gene CaMKII and inflammatory markers in the hippocampi of preweaning mice.

The purpose of the study was to investigate the early life exposure to DE-SOA during the gestational stages and lactation impairs the hypothalamic expression of social behavior-related genes and social behavior in adult life using a mouse model. We hypothesized that the potential toxic substances contained in DE-SOA may reach the brain via the olfactory nerve route or via the systemic circulation and cause social behavioral impairment in later life. Our study is the first report to show that exposure to DE-SOA during the developmental stage affects social performance and the related gene expressions in the hypothalamus of mature mice.

MATERIALS AND METHODS

Animals

Timed pregnant BALB/c mice (gestational day; GD 13) purchased from SLC Japan, Inc. (Tokyo, Japan) were exposed to clean air, diesel engine exhaust (DE), diesel engine exhaust origin secondary organic aerosol (DE-SOA) and gas only without diesel exhaust particles (Gas) from GD 14 to postnatal day (PND) 21 in the whole body exposure chambers. Food and water were given *ad libitum*. The day of the birth was recorded as PND 0 and the offspring were housed in cages with mothers under controlled environmental condition (temperature, 22 ± 0.5°C; humidity, 50 ± 5%; lights on 07:00–19:00 h). The pups were weaned at PND 21 and 5∼6 pups of same sex were housed in a plastic cage. Social behavioral tests were started at approximately 13-week-old. Our social behavioral test consisted of sociability and social novelty preference task, social interaction with a juvenile mouse, and light-dark test. Behavioral testing was performed between 09:00 and 17:00 h. Before performing each test, the apparatus to be used was cleaned with 50% ethanol. After completing social behavioral test, these mice were sacrificed for brain sampling. The experimental protocols were approved by the Ethics Committee of the Animal Care and Experimentation Council of the National Institute for Environmental Studies (NIES), Japan.

Generation of DE-SOA

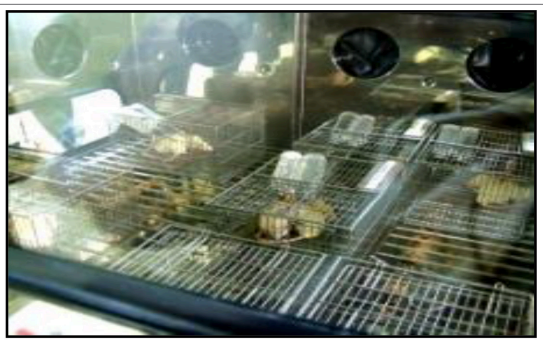
DE-SOA was generated at the National Institute for Environmental Studies, Japan as described previously (Fujitani et al., 2009; Win-Shwe et al., 2014). An 81-diesel engine (J08C; Hino Motors Ltd., Hino, Japan) was used to generate diesel exhaust. The engine was operated under a steady-state condition for 5 h per day. In the present study, our driving condition of diesel engine was not simulated to any special condition as in the real world. The engine operating condition (2000 rpm engine speed and 0 Nm engine torque) in this study permits suppression of the generation of soot particles of relatively large size as well as the generation of high concentrations of nanoparticles. There are four chambers: a control chamber receiving clean air filtered through a HEPA filter and a charcoal filter (referred to as "clean air"), the diluted exhaust (DE which was without mixing O_3), DE-SOA which was generated by mixing DE with ozone at 0.6 ppm after secondary dilution and

gas without diesel exhaust particles. Secondary dilution ratio in DE and DE-SOA chambers were the same which resulted in the same particle and gaseous concentrations when O_3 was not mixed. Actually, the concentrations of particles in DE-SOA was higher when O_3 was mixed and concentrations of DE and DE-SOA were 113.19 ± 19.5 μg/m^3 and 130.90 ± 31.2 μg/m^3, respectively. The increased mass concentration was due to the generation of secondary particles. The temperature and relative humidity inside each chamber were adjusted to approximately 22 ± 0.5°C and 50 ± 5%, respectively. The particle characteristics were evaluated from the sample air taken from inside of the exposure chamber and presented in **Table 1**. In detail, sample air was taken from the breeding space of the inhalation chamber (2.25 m^3) using stainless steel tubing. The gas concentrations (CO, CO_2, NO, NO_2, and SO_2) were monitored using a gas analyzer (Horiba, Kyoto, Japan). CO and NOx concentrations in both chambers were similar, but NO and NO_2 are different each other because NO was oxidized to NO_2 by reacted with O_3. The particle size distributions were measured using a scanning mobility particle sizer (SMPS 3034; TSI, MN). The sizes of the particles used in the present study were 25.42 ± 1.6 nm for DE and 28.30 ± 1.3 nm for DE-SOA. The particles were collected using a Teflon filter (FP-500; Sumitomo Electric, Osaka, Japan) and a Quartz fiber filter (2500 QAT-UP; Pall, Pine Bush, NY, USA), and the particle mass concentrations were measured using a Teflon filter. The particle weights were measured using an electrical microbalance (UMX 2, Mettler- Toledo, Columbus; OH, USA; readability 0.1 μg) in an air-conditioned chamber (CHAM-1000; Horiba) under constant temperature and relative humidity conditions (21.5°C, 35%). For the Quartz fiber filter, the quantities of elemental carbon (EC) and organic carbon (OC) were determined using a carbon analyzer (Desert Research Institute, NV, USA). EC to OC ratio in the present study were 0.14 ± 0.05 for the control chamber, 0.33 ± 0.02 for DE-SOA chamber and 0.32 ± 0.03 for DE exposure chamber. An analysis of the particle composition (DE and DE-SOA) showed that the percentage of OC relative to the total carbon in diluted exhaust was about 60% and the DE and DE-SOA was nearly same carbon composition.

Experimental Schedule

Pregnant mice were exposed to clean air, DE, DE-SOA or gas in the whole-body exposure chamber (Shibata) for 5 h per day (from 22:00 h to 03:00 h) on 5 days of the week till PND 21. The male offspring mice at PND 21 were allocated into four different groups (n = 12 per group) as follows: (1) mice exposed to clean filtered air; (2) mice exposed to DE; (3) mice exposed to DE-SOA, and (4) mice exposed to gas only without diesel exhaust particles. On the day after the final exposure, the social performance of each mouse was examined using a three chamber sociability and social novelty preference performance, social interaction and

TABLE 1 | Characteristics of diesel exhaust particles and gaseous compounds in the exposure chambers.



	Diesel exhaust particles			Temperature	Relative humidity		
	Size (nm)	Particle number (cm^{-3})	Concentration (μg/m^3)	(°C)	(%)	EC/OC	WSOC/OC
Clean air	–	0.87 ± 0.57	13.20 ± 2.78	23.58 ± 0.27	48.07 ± 0.77	0.14 ± 0.05	0.03 ± 0.04
DE-SOA	28.30 ± 1.28	$2.74 \times 10^6 \pm 8.69 \times 10^4$	130.90 ± 31.17	23.76 ± 0.19	48.40 ± 0.85	0.33 ± 0.02	0.11 ± 0.05
DE	25.42 ± 1.63	$2.85 \times 10^6 \pm 6.10 \times 10^4$	113.19 ± 19.46	22.97 ± 0.21	49.32 ± 0.87	0.32 ± 0.03	0.17 ± 0.11
Gas	–	5.55 ± 1.12	13.30 ± 1.45	23.67 ± 0.24	48.56 ± 1.08	0.14 ± 0.04	0.12 ± 0.06
GASEOUS COMPOUNDS							
	CO (ppm)	SO_2 (ppm)	NO_x(ppm)	NO_2 (ppm)	NO (ppm)	O_3 (ppm)	CO_2 (%)
Clean air	0.21 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	–	0.05 ± 0.00
DE-SOA	2.51 ± 0.07	0.00 ± 0.00	1.14 ± 0.03	0.99 ± 0.03	0.15 ± 0.03	0.07 ± 0.00	0.07 ± 0.00
DE	2.52 ± 0.07	0.01 ± 0.00	1.21 ± 0.03	0.43 ± 0.02	0.78 ± 0.02	–	0.07 ± 0.00
Gas	2.50 ± 0.06	0.00 ± 0.00	1.14 ± 0.03	0.99 ± 0.03	0.14 ± 0.03	0.06 ± 0.00	0.07 ± 0.00

Data were expressed as mean ± SD.

light-dark test with Any-maze software video-assisted tracking system (Muromachi Kikai Co. Ltd., Japan).

Behavioral Tasks

All behavioral procedures were video-recorded, and data were analyzed by an experimental blind to the conditions.

Sociability and Social Novelty Preference

The apparatus is a Plexiglas rectangular, three-chambered box (60 × 40 × 22 cm). The size of three chambers is equal. Dividing partition was made by clear Plexiglas, with small doorways (5 × 8 cm) allowing free access to each chamber. Small Plexiglas cups (diameter 8 cm; height, 10 cm) were used to house strangers and placed in each side chamber. Stranger mouse was placed on the left or right side. For habituation, subject mice from four different groups were first placed in center of the middle chamber and allowed to explore for 5 min. During habituation phase, each of two side chambers contained an empty Plexiglas cup. Following habituation, for the sociability test, a novel mouse (stranger 1, age-matched male) was enclosed in one of the cup and placed in one of the side chambers; the subject mice were allowed to explore for 10 min. The social novelty preference test was performed immediately after the sociability test. Another novel mouse (stranger 2, age-matched mouse) was enclosed in the other cup. And the subject mice were allowed to explore the two strangers for 10 min. The time spent in each Plexiglas cup was measured. The subject mouse was considered to be spent in cup when its head was facing the cup from a distance of within 3 cm.

Social Interaction with a Juvenile Mouse

Male juvenile mice were used instead of adults to avoid any effect of mutual aggression (Moretti et al., 2005; Jung et al., 2013). A single male subject mouse was placed in a new cage which was identical to those in which the mice were normally housed and allowed to free for 10 min (habituation phase). Three to four week-old juvenile male mouse was introduced to the new cage and then allowed for 5 min (Test phase). Nose-to-nose sniffing, direct contact and close following (within <1 cm) were recorded as social interaction parameters (Jung et al., 2013). The total time of social interaction with juvenile mouse was measured.

Light-Dark Test

The test apparatus consisted of a clear plastic box (40 × 20 × 25 cm) with a dark compartment (20 × 25 × 25 cm) and a light compartment (20 × 25 × 25 cm). The dark compartment had an open doorway (2 × 5 cm) that led to the light side of the apparatus, which was illuminated by a 40-W bulb (about 350 lux on the floor). Mice were moved from the living room to the testing room at least 1 h before the test. At the beginning of the tests, mice were removed from their cages, gently placed to the corner of the dark side of black box away from the doorway. Any-maze software were used to collect and store data. For each mouse, the following measurements were recorded for 10 min: total moving time, total time spent in the dark compartment, total time in the light compartment, number of transitions between the dark and light compartments, and the latency to the first emergence from the dark to the light compartment. Between the tests, the apparatus was thoroughly wiped to clean with 50% alcohol.

Quantification of the Expression Levels of mRNAs

Twenty-four hours after the completion of the social behavioral tests, the mice from each group were sacrificed under deep pentobarbital anesthesia and the hypothalami were collected for mRNA analyses. Hypothalami samples were frozen quickly in liquid nitrogen then stored at –80°C until the total RNA was extracted. Briefly, total RNA extraction from the hypothalami samples was performed using the BioRobot EZ-1 and EZ-1 RNA tissue mini kits (Qiagen GmbH, Hilden, Germany). Then, the purity of the total RNA was examined, and the quantity was estimated using the ND-1000 NanoDrop RNA Assay protocol (NanoDrop, USA), as described previously (Win-Shwe et al., 2006, 2008a,b). Next, we performed first-strand cDNA synthesis from the total RNA using SuperScript RNase H⁻ Reverse Transcriptase II (Invitrogen, Carlsbad, USA), according to the Manufacturer's protocol. Next, we examined the expression levels of 18S, estrogen receptor (ER) α and oxytocin receptor (OTR), cyclo-oxygenase (COX)-2, heme-oxygenase (HO)-1, interleukin (IL)1 β, tumor necrosis factor (TNF) α mRNAs by a quantitative real-time RT-PCR method using the Applied Biosystems (ABI) Prism 7000 Sequence Detection System (Applied Biosystems Inc., Foskr City, CA, USA). The tissue 18S rRNA level was used as an internal control. Primers (ER-α, NM_007956; OTR, NM_001081147, IL-1β NM_008361; COX2, NM_011198; HO1, NM_010442) were purchased from Qiagen, Sample & Assay Technologies. TNF-α primer (forward: 5′-GGTTCCTTTGTGGCACTTG-3′, reverse: 5′-TTCTCTTGGTGACCGGGAG-3′) was purchased from Hokkaido System Science (Hokkaido System Science, Hokkaido, Japan). Data were analyzed using the comparative threshold cycle method. Then, the relative expression levels of memory function-related genes and the related transduction pathway molecule mRNAs were individually normalized to the 18S rRNA content in the respective samples and expressed as mRNA signals per unit of 18S rRNA expression.

Measurement of Glutamate Concentration

Glutamate concentration in the right hypothalamus of mice was measured using glutamate research ELISA assay kit (Ref: BA E-2300, Neuroscience. Inc., Tokyo, Japan) according to the manufacturer's instructions.

Statistical Analysis

All the data were expressed as the mean ± standard error (S.E.). The statistical analysis was performed using the StatMate II statistical analysis system for Microsoft Excel, Version 5.0 (Nankodo Inc., Tokyo, Japan). Paired *t* test was used to analyze the time approach to the empty cup and stranger 1, then stranger 1 and stranger 2. Messenger RNA data and glutamate concentration were analyzed by a one-way analysis of variance with a *post-hoc* analysis using the Bonferroni/Dunn method. Differences were considered significant at $P < 0.05$.

RESULTS

Body and Brain Weight of Adult Mice Exposed to Clean Air, DE, DE-SOA and Gas during Developmental Period

To detect the general toxicity, body and brain weight were measured in adult male mice exposed to clean air, DE, DE-SOA and gas without particles at the time of sampling (**Table 2**). We did not find any significant changes between the control and exposure groups.

Effects of DE or DE-SOA on Social Behavior

Sociability

The control mice spent more exploring time with the stranger 1 than the empty cup (**Figure 1A**, $^*P < 0.05$). In contrast, DE, DE-SOA, or gas exposed mice showed no preference for stranger 1, which could reflect decreased sociability.

Social Novelty Preference

The control mice spent more exploring time with the stranger 2 than the known mouse (stranger 1) (**Figure 1B**, $^*P < 0.05$). However, DE, DE-SOA or gas exposed mice showed no preference for novel one (stranger 2). DE-SOA exposed mice preferred old one (stranger 1) significantly compared to the novel one (stranger 2) (**Figure 1B**, $^*P < 0.05$), which may indicate that they may have poor social novelty preference.

Social Interaction with a Juvenile Mouse

Nose-to-nose sniffing, direct contact and close following (within <1 cm) were recorded as social interaction parameters. The total time of social interaction with juvenile mouse was measured. We found that DE or DE-SOA exposed mice showed significantly decreased interaction time with juvenile mouse compared to the control mice (**Figure 1C**, $^*P < 0.05$).

Light-Dark Test

Light-dark box is a characteristic tool used in the assessment of anxiety. In the present study, total moving time, total time spent in the dark compartment, total time in the light compartment, number of transitions between the dark and light compartments, and the latency to the first emergence from the dark to the light compartment. However, we did not find any significant difference between the control and exposure groups. Time spent in the light compartment was shown in **Figure 1D**.

TABLE 2 | Body and brain weight of adult mice exposed to clean air, DE, DE-SOA, and gas during developmental period.

Exposure groups	Control	DE	DE-SOA	Gas
Body weight (g)	28.22 ± 0.39	28.99 ± 0.97	28.03 ± 0.57	29.07 ± 0.53
Whole brain (mg)	462.11 ± 5.19	469.75 ± 5.14	468.67 ± 4.57	472.33 ± 4.90

Effects of DE or DE-SOA on the Hypothalamic Expression of Social Behavior-Related Genes

Recently, Ervin and colleagues have demonstrated that estrogens are involved in various social behavior such as social preferences, aggression and dominance, and learning and memory (Ervin et al., 2015). Moreover, it was reported that the ERα in the medial amygdala and ventromedial nucleus of the hypothalamus palys a role in social recognition, anxiety and aggression (Spiteri et al., 2010). Our present study has shown that expression of ERα was decreased significantly in DE-SOA exposed group compared to the control group (**Figure 2A**, $^*P < 0.05$).

The role of the OT in the normal processing of socially relevant clues has been extensively investigated in genetically-modified rodent models. Social deficit has been observed in OT receptor knockout mice (Winslow and Insel, 2002; Crawley et al., 2007). In the present study, expression level of OTR in the hypothalamus was examined and found that OTR mRNA was decreased significantly in mouse exposed to DE-SOA compared to DE group (**Figure 2B**, $^*P < 0.05$) and tended to decrease compared to the control group.

Effects of DE or DE-SOA on the Hypothalamic Expression of Inflammatory and Oxidative Stress Marker Genes

To detect the inflammatory response in the brain, we investigated the expression level of potent inflammatory cytokines such as IL-1 β, TNF-α and potent inflammatory marker COX2. The expression levels of IL-1 β and TNF-α were not different between the control and the exposure groups (data not shown). COX is the enzyme responsible for the conversion of arachidonic acid to prostaglandin, which is involved in the inflammatory response. COX2 is an inducible form and is released at the site of inflammation. In the present study, COX2 mRNA was tended to increase in the DE-SOA-exposed group compared with the control ones (**Figure 3A**). To understand the mechanism underlying the inflammatory response in the hypothalamus of mice exposed to DE-SOA, we also examined the expression of the oxidative stress marker HO1 and found that HO1 mRNA was significantly upregulated in the DE-SOA-exposed group compared with the control group (**Figure 3B**, $^*P < 0.05$).

Effects of DE or DE-SOA on the Hypothalamic Level of Neuronal Excitatory Marker

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (Fonnum, 1984) and the excessive increase in extracellular glutamate level, known as excitotoxicity, triggers the death of neurons (Choi and Rothman, 1990). We detected glutamate concentration in the mouse hypothalamus and found that glutamate secretion was increased remarkably in mice exposed to DE or DE-SOA during fetal and neonatal developmental period (**Figure 4**, $^{**}P < 0.01$).

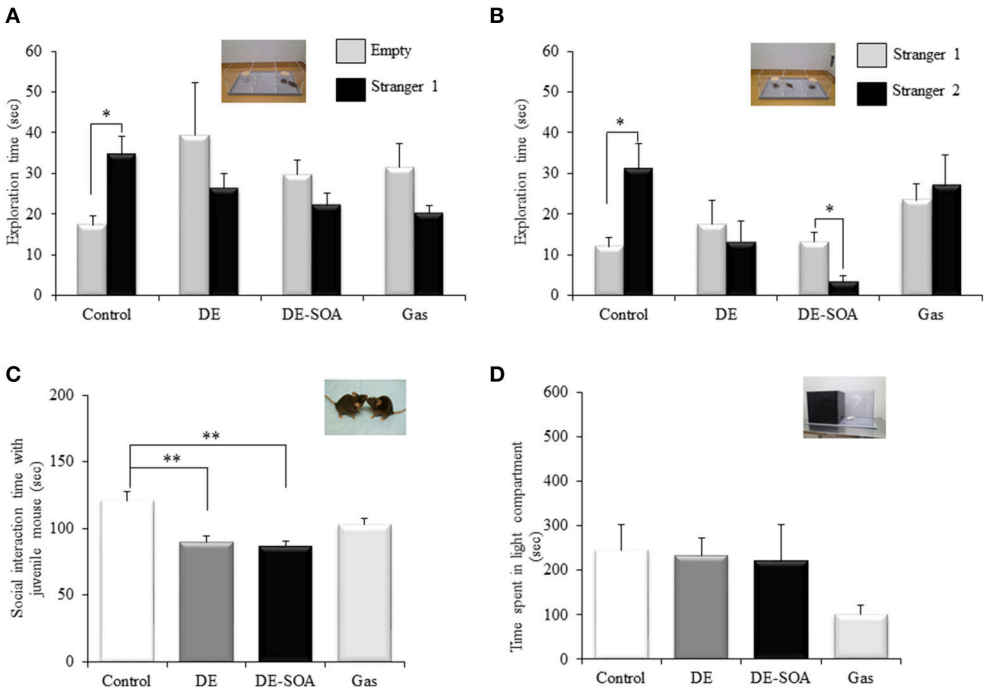


FIGURE 1 | Scores of the social behavioral tests. (A) sociability test, **(B)** social novelty preference test, **(C)** social interaction test with juvenile mouse and **(D)** light dark test in 13 week-old male mice after developmental period exposure to clean air, DE, DE-SOA or gas without particles. Each bar represents the mean ± SE ($n = 12$, $^{**}P < 0.01$, $^{*}P < 0.05$).

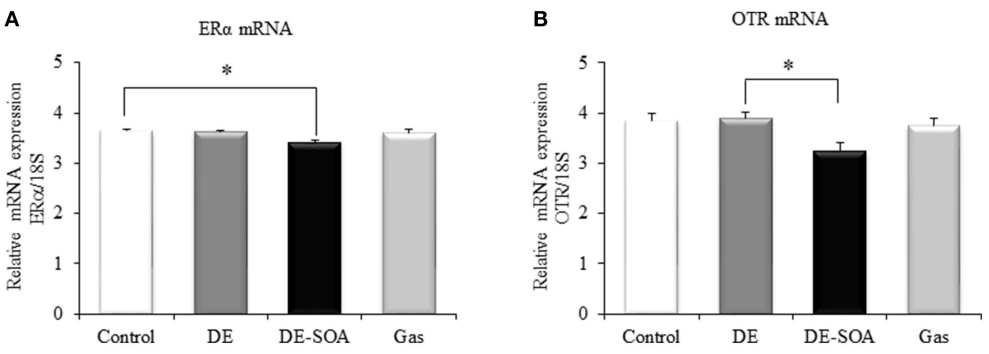


FIGURE 2 | Hypothalamic expression of social behavior-related genes. Messenger RNA expression level of **(A)** ERα and **(B)** OTR in the hypothalami of 13 week-old male mice after developmental period exposure to clean air, DE, DE-SOA or gas without particles. Each bar represents the mean ± SE ($n = 12$, $^{*}P < 0.05$).

DISCUSSION

The major findings in the present study indicate that exposure to DE-SOA during brain developmental period may impair some social behaviors in adult male BALB/c mice accompanied with modulation of expression of ERα and OTR, inflammatory mediator COX2 and oxidative stress marker HO1 in the hypothalamus. We suggest that, although the potential toxic substances contained in DE-SOA have not yet been identified, they may reach the brain via placenta of pregnant mice during fetal period and via the olfactory nerve route or systemic circulation during neonatal period and induce neurotoxicity. These effects may be persistent because we exposed the mice during early brain developmental period and investigated the social behavior and related gene expression in later age.

Previous epidemiological evidences have shown the existence of positive associations between the inhalation of elevated levels of PM and pulmonary and cardiovascular morbidities and mortality in susceptible populations (Dockery, 1993; Peters et al., 1997; Penttinen et al., 2001; Pope et al., 2002). Recent reports indicate that the central nervous system is an important target for air pollution to cause adverse health effects such as neurodevelopmental and neurodegenerative disorders like autism spectrum disorder (ASD) and Alzheimer's disease

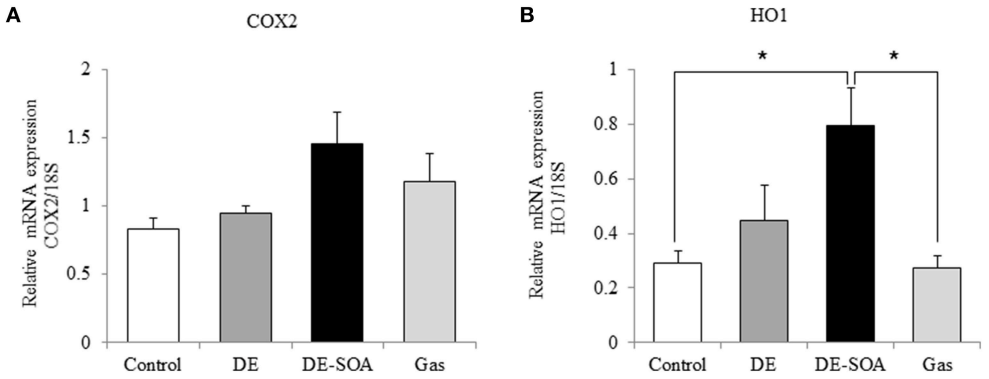


FIGURE 3 | Hypothalamic expression of inflammatory and oxidative stress markers. (A) COX2 and **(B)** HO1 mRNAs in 13 week-old male mice after developmental period exposure to clean air, DE, DE-SOA or gas without particles. Each bar represents the mean ± SE ($n = 12$, $^*P < 0.05$).

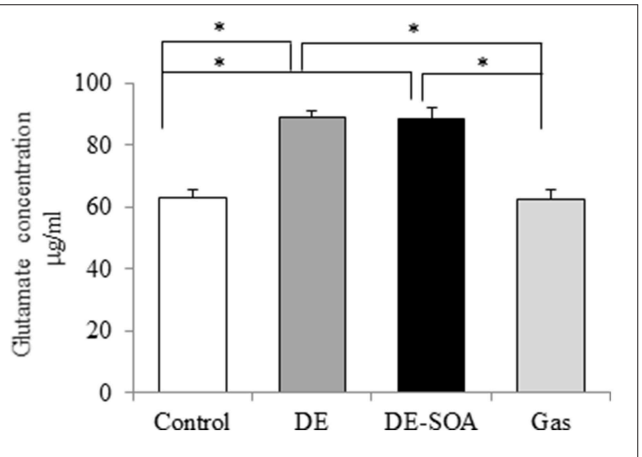


FIGURE 4 | Hypothalamic levels of neuronal excitatory marker. Glutamate concentration in the hypothalami of 13 week-old male mice after developmental period exposure to clean air, DE, DE-SOA or gas without particles. Each bar represents the mean ± SE ($n = 12$, $^*P < 0.05$).

(Calderón-Garcidueñas et al., 2004; Deth et al., 2008; Lee et al., 2010; Landrigan et al., 2012). DE is a major source of ambient PM and one of the major precursors for SOA formation. SOAs are formed in the atmosphere by oxidation of products originating from anthropogenic and biogenic volatile organic compounds (Virtanen et al., 2012). SOA formation may occur not only in the atmosphere, but also in indoor environments where laser printer, copiers were used (Wang et al., 2012; Youssefi and Waring, 2012).

First, we have shown that a single intranasal administration of SOA induces inflammatory responses in the lungs by modulating the expressions of proinflammatory cytokines, transcription factors, and inflammation-responsive neurotrophins (Win-Shwe et al., 2013). Currently, we have generated SOA by adding ozone to diesel exhaust particles and establish SOA inhalation chamber in our Research Institute. Using SOA inhalation chambers, we have shown that exposure to SOA for 3 months caused learning and memory impairment in adult male mice and SOA exposure for 1 month in female mice may cause changes in maternal behavior (Win-Shwe et al., 2014). Recently, we have established the neonatal animal model for early detection of environmental pollutant-induced learning disability and reported that DE-SOA impairs olfactory-based spatial learning activity in preweaning mice (Win-Shwe et al., 2015). In that study, we have also shown that learning impairment was associated with modulation of NMDA receptor, signaling pathway gene CaMKII and inflammatory markers in the hippocampus. From these findings, we suggest that glutamate, a ligand for NMDA receptor, may involve SOA induced neurobehavioral dysfunctions.

Human studies have reported that children from Mexico City with prefrontal lesions exposed to air pollution showed cognitive deficits (Calderón-Garcidueñas et al., 2008a, 2011). An association has also been shown between air pollution and cognitive impairment in healthy individuals, including adult and elderly women (Calderón-Garcidueñas et al., 2004, 2008b). An *in vitro* study indicated that decreased phagocytic activity was found in human macrophages exposed to SOA from alpha-pinene, and IL-8 production was increased in pig explants exposed to SOA from 1,3,5-trimethlbenzene with high particle numbers (Gaschen et al., 2010). However, it is not clear whether an association may exist between exposure to SOA derived from DE and higher functions of the brain such as social behavior.

In the present study, the control group only approached longer time to stranger 1 cup compared to empty cup in sociability test. In social novelty preference test, the control group approached longer time to stranger 2 cup compared to stranger 1 cup. These findings suggests that treatment groups such as DE, DE-SOA or gas without particles groups may have poor communication with new partner. We have also examined social behavioral related genes such as ERα and OTR and proinflammatory cytokines and potent inflammatory marker and oxidative stress marker and found that the expression level of COX2 and HO1 mRNA were increased in the hypothalamus of DE-SOA exposed mice. Components of DE-SOA may exert their deleterious effects directly on the central nervous system, the possibility and the extent of a peripheral contribution to the central effects should be considered. It was reported that high levels of circulating proinflammatory cytokines may negatively affect the he central nervous system (Block and Calderón-Garcidueñas, 2009; Calderón-Garcidueñas et al., 2013), and the

blood-brain barrier may represent an important site for air pollution induced neurotoxicity.

To detect the possible mechanism of the action of SOA in social impairment, we have examined glutamate concentration in hypothalamus. Glutamate is one of excitatory amino acid neurotransmitters. Neurotransmitters play many critical roles in the neuronal transmission and maintenance of many higher brain functions. Deviation of neurotransmitter from the normal physiological level may lead to certain malfunctions and pathological states of the brain. Under normal conditions, extracellular glutamate is maintained at safe physiological concentrations by a number of buffering mechanisms which include uptake of glutamate by glial cells and its conversion by glutamine synthetase or glutamate decarboxylase to the nontoxic glutamine (Bezzi et al., 1999; Rauen et al., 1999). In the present study, glutamate concentration was remarkably increased in DE or DE-SOA exposed mice. It is suggested that there may be association exists between increased glutamate neurotransmission and impaired social behavior. Amino acid transporters present in both the neurons and the glial cells are critically important for the normal function of glutamatergic transmission, as well as for the maintenance of extracellular glutamate levels below potentially excitotoxic concentrations (Kanai et al., 1993, 1995). The possible reasons for increased glutamate level in DE-SOA exposed mice are due to blockade of re-uptake by glutamate transporter in the presynaptic neurons and decreased downstream enzymes such as glutamic acid decarboxylase (GAD) 67 and GAD 65 for gamma amino butyric acid (GABA) synthesis. Further studies are needed to evaluate the role of glutamate transporters in SOA induced impaired behavior.

Recent report has indicated that estrogens are involved in various social behavior such as social preferences, aggression and dominance, and learning and memory (Ervin et al., 2015). It was also reported that the ERα in the medial amygdala and ventromedial nucleus of the hypothalamus palys a role in social recognition, anxiety and aggression. (Spiteri et al., 2010). Our present study has shown that expression of ERα was decreased in DE-SOA exposed group compared to the control group. Moreover, impaired social recognition has been observed in OT peptide and receptor knockout mice (Winslow and Insel, 2002; Takayanagi et al., 2005). Oxytocin can reduce repetitive behavior in subjects with autism (File et al., 1998) and promote social behavior in high functioning ASDs (Andari et al., 2010). Recent reports have indicated that intranasal oxytocin reduces psychotic symptoms and improves theory of mind and social performance in schizophrenia patients (Pedersen et al., 2011; Davis et al., 2013). In the present study, although statistically not significant, OTR mRNA expression tends to decrease in DE-SOA exposed mice compared to the other groups. Taken together, ERα and OTR, at least in part, may play a role in DE-SOA induced social behavioral disturbance.

Our present results indicate that brain developmental period exposure to diesel exhaust origin SOA may impair some social behavior in adult BALB/c mice. In the present study, not only the offspring, but also the dam were exposed to DE-SOA during gestational and lactational periods. Normal maternal behaviors such as nesting, licking, crouching and retrieving were observed during exposure period. Therefore, impairment of social behavior in adult male mice might not due to abnormal maternal caring during brain developing periods. Glutamate neurotransmission and ERα-OTR signaling pathway in the hypothalamus may take part in DE-SOA induced social behavioral impairment. Further studies are needed to explore the effects of DE-SOA on other brain targets such as amygdala and hippocampus and other social behavior such as sexual, aggressive or anxiety after early life exposure. Furthermore, animal experiments showed the same pattern of neurotoxic effects such as increased oxidative stress markers, increased neuroinflammatory mediators and age factor, as in humans, suggesting that animal studies would be useful predictors of human outcomes (Costa et al., 2014). Finally, *in vitro* studies are needed for better understanding of air pollution induced neurotoxicity and their consequences.

AUTHOR CONTRIBUTIONS

TW and SH designed this research; YF, arranged the exposure system; CK and YM performed the behavioral tests and molecular analyses; TW wrote the article; SH and ST critically revised the article.

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ORIGINAL RESEARCH
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In utero and Lactational Exposure to Acetamiprid Induces Abnormalities in Socio-Sexual and Anxiety-Related Behaviors of Male Mice

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Neonicotinoids, a widely used group of pesticides designed to selectively bind to insect nicotinic acetylcholine receptors, were considered relatively safe for mammalian species. However, they have been found to activate vertebrate nicotinic acetylcholine receptors and could be toxic to the mammalian brain. In the present study, we evaluated the developmental neurotoxicity of acetamiprid (ACE), one of the most widely used neonicotinoids, in C57BL/6J mice whose mothers were administered ACE via gavage at doses of either 0 mg/kg (control group), 1.0 mg/kg (low-dose group), or 10.0 mg/kg (high-dose group) from gestational day 6 to lactation day 21. The results of a battery of behavior tests for socio-sexual and anxiety-related behaviors, the numbers of vasopressin-immunoreactive cells in the paraventricular nucleus of the hypothalamus, and testosterone levels were used as endpoints. In addition, behavioral flexibility in mice was assessed in a group-housed environment using the IntelliCage, a fully automated mouse behavioral analysis system. In adult male mice exposed to ACE at both low and high doses, a significant reduction of anxiety level was found in the light-dark transition test. Males in the low-dose group also showed a significant increase in sexual and aggressive behaviors. In contrast, neither the anxiety levels nor the sexual behaviors of females were altered. No reductions in the testosterone level, the number of vasopressin-immunoreactive cells, or behavioral flexibility were detected in either sex. These results suggest the possibility that *in utero* and lactational ACE exposure interferes with the development of the neural circuits required for executing socio-sexual and anxiety-related behaviors in male mice specifically.

Keywords: neonicotinoids, acetamiprid, *In utero* and lactational exposure, sociosexual behavior, anxiety-related behavior

INTRODUCTION

There is a growing concern that exposure to environmental chemicals in early life may interfere with brain development (Júlvez et al., 2016). In particular, neonicotinoid pesticides have drawn considerable attention. As a pesticide class, neonicotinoids are designed to overstimulate insect nicotinic acetylcholine receptors (nAChRs). These pesticides were previously thought to be relatively harmless to mammalian species because of their low binding affinities to mammalian nAChRs (Tomizawa and Casida, 1999, 2005). However, recent *in vivo* and *in vitro* studies have reported that neonicotinoids possess sufficient binding affinity and agonistic potential for mammalian nAChRs to exert nicotine-like effects that are stronger than originally believed (de Oliveira et al., 2010; Rodrigues et al., 2010; Li et al., 2011; Kimura-Kuroda et al., 2012). Neonicotinoids such as acetamiprid (ACE), imidacloprid, and clothianidin can bind to the α_4 and β_2 subunits of mammalian nAChRs (Tomizawa and Casida, 1999; Li et al., 2011; Kimura-Kuroda et al., 2012). The $\alpha_4\beta_2$ nAChRs are present in various brain regions such as the amygdala, hypothalamus, substantia nigra, ventral tegmental area, raphe nuclei, hippocampus, and medial habenula (Cimino et al., 1995; Millar and Gotti, 2009), and regulate the development and functions of these regions (Dwyer et al., 2009; Takarada et al., 2012). These brain regions are involved in the regulation of socio-sexual behaviors, anxiety, depression, memory, and learning (Pfaff, 1989; Newman, 1999; Nelson and Trainor, 2007; Drevets et al., 2008; Gaskin and White, 2013; Russo and Nestler, 2013). Therefore, perinatal exposure to neonicotinoids is thought to impair specific behaviors by affecting the formation of neuronal circuits, including circuits involving these areas.

Since ACE has a higher affinity and potency for mammalian nAChRs compared to those of other neonicotinoids (Tomizawa and Casida, 1999; Kimura-Kuroda et al., 2012), we here studied effects of perinatal exposure to ACE on murine behaviors later in adulthood, focusing on socio-sexual and anxiety-related behaviors and behavioral flexibility. In addition to examining these adult behaviors, we evaluated the blood testosterone levels and the numbers of cells expressing arginine-vasopressin (AVP) in the hypothalamus because they are closely associated with socio-sexual and anxiety-related behaviors (Hull and Dominguez, 2007; Nelson and Trainor, 2007; Ho et al., 2010; Stevenson and Caldwell, 2012).

MATERIALS AND METHODS

Animals

Male and female C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan) and mated at the National Institute for Environmental Studies (NIES). Mice were housed in a room that was maintained at a constant temperature (24 ± 1°C) and humidity (50 ± 10%) with a 12/12-h light/dark cycle. Food and water were provided *ad libitum* unless otherwise specified. The presence of vaginal plugs was checked daily; gestational day (GD) 0 was defined as the day on which a vaginal plug was detected. The dams were administered ACE (Sigma-Aldrich, St. Louis, MO), dissolved in H_2O, at doses of 0 mg/kg (control group), 1.0 mg/kg (low-dose group), or 10.0 mg/kg (high-dose group) by oral gavage from GD 6 to postnatal day (PND) 21. The litters were weaned from their mothers 2–3 h after the last ACE administration on PND 21 and housed with same-sex littermates. All procedures were approved by the Animal Care and Use Committee at the NIES and were conducted strictly in accordance with the NIES guidelines.

Body Weights

The body weight (BW) was measured at birth, at weaning (PND 21), and at 23–26 weeks of age, and the average body weight within a litter (BW/litter) was compared between treatment groups.

Brain Weights

At the time of weaning (PND 21), randomly selected mice that would not be used for behavioral testing were deeply anesthetized with isoflurane and decapitated. The brains were rapidly removed and weighed.

ACE Analysis in the Brain

An ACE standard was obtained from Sigma-Aldrich. Acetamiprid-d3 (Fluka, Sigma-Aldrich, St. Louis, MO, USA) was used as an internal standard (IS). The standard and IS were diluted with acetonitrile and stored at −20°C. Acetonitrile, acetic acid (LC-MS grade), and ammonium acetate (JIS Special Grade) were purchased from Wako Pure Chemical. Purified water was prepared by MilliQ filtration (Millipore, Billerica, MA, USA). The brain levels of ACE were analyzed using previously reported analytical methods with some modifications (Seccia et al., 2008; Xiao et al., 2011; Zhang et al., 2012; Ueyama et al., 2014; Gbylik-Sikorska et al., 2015). A whole brain (0.34–0.49 g) was transferred to a 15 mL polypropylene (PP) tube and mixed with 500 μL of purified water and 50 μL of 50 ng/mL IS solution. The solution was homogenized using a Handy Ultrasonic Homogenizer (Microtec, Funabashi, Chiba, Japan) for 2 min. After adding 2 mL of acetonitrile, the sample solution was vortexed and ultrasonically extracted for 5 min. The extract was centrifuged at 3500 rpm for 5 min (Centrifugator, H-36, Kokusan, Saitama, Japan,), transferred to another 15 mL PP tube, and the solvent was evaporated with a centrifugal evaporator (CentriVap, Asahi Life Science, Saitama, Japan) for 90 min. The residue was re-dissolved in 0.1 mL of acetonitrile and diluted with purified water, up to 1 mL. The sample solution was passed through an Oasis HLB cartridge (1 mL/30 mg, Waters) that was pre-conditioned with 1 mL of acetonitrile and 1 mL of purified water. After rinsing with 1 mL of purified water, the ACE was eluted with 1 mL of acetonitrile/purified water (1:1, v/v). The eluates were evaporated with a centrifugal evaporator for 60 min and re-dissolved in 0.5 mL of acetonitrile/purified water (1:9, v/v) prior to the analysis. Quantification was performed using an ultra-high performance liquid chromatography (Nexera UHPLC, Shimadzu, Kyoto, Japan) coupled to a tandem mass spectrometry (LCMS8050, Shimadzu). The analytical column was Kinetex C18 (100 × 2.1 mm, 2.6 μm, Phenomenex, Torrance, CA, USA), and the injection volume was 10 μL. The mobile phase was (a) 17 mmol/L acetic acid and 5 mmol

ammonium acetate in acetonitrile and (b) 17 mmol/L acetic acid and 5 mmol ammonium acetate in purified water; the flow rate was maintained at 0.4 mL/min. The gradient parameters were as follows: the initial condition (phase ratio a:b = 10:90) for 5 min, 40:60 for 5.5 min, 100:0 for 1 min, the initial condition for 6 min. A multiple reaction monitoring (MRM; precursor ion: m/z = 222.7 and product ion: m/z = 126.0) transition was used for the ACE quantification, and another MRM transition (precursor ion: m/z = 222.7 and product ion: m/z = 56.2) was used for confirmation. Quantification was performed using the relative response to IS. The linearity of the calibration curve of the ACE standard solution was confirmed from 0.1 to 100 ng/mL, with $r^2 > 0.998$. The instrumental detection limit (IDL) of ACE was 0.024 ng/mL, which was calculated from the results of 7 replicate analyses of the standard solution. To calculate the method detection limit (MDL), 7 replicated analyses of a fortified blank sample were performed. The blank sample (0.4 mL of purified water) was spiked with 0.05 mL of 0.1 ng/mL standard solution and processed throughout the analytical procedure. The MDL was calculated to be 0.032 ng/g. The IS recovery during the sample analysis ranged from 63 to 98%. The extraction efficiency was evaluated by repeated extraction; the detected ACE level were below the IDL in the second and third extractions.

General Test Procedure

When the offspring were 9–12 weeks old, 1 or 2 mice of both sexes were randomly selected from each litter for a behavioral test battery for socio-sexual and anxiety-related behaviors, consisting of tests for male and female sexual behaviors, aggressive behaviors, and the light-dark transition (LDT) test. The behavioral tests were performed during the dark phase (starting more than 2 h after lights off) of the light/dark cycle under red light. After completing the behavioral tests, the mice were sacrificed, and blood and brain samples were collected for the enzyme immunoassays and immunohistochemistry.

Another 1–2 mice of both sexes from each litter (males: 13–20 weeks of age; females: 23–32 weeks of age) were assigned to the behavioral flexibility test using the IntelliCage apparatus (NewBehavior AG, Zurich, Switzerland). The experimental design for the tests and the number of animals for each group are shown in **Figure 1**.

Male Sexual Behavior

The male mice were separated from their littermates and individually housed in plastic cages (5 × 22 × 12 cm). Starting 12–14 days later, each mouse was tested in its home cage for sexual behavior toward ovariectomized and sex-hormone-treated C57BL/6J female mice once weekly for 3 weeks. The duration of each trial was 30 min. All female stimulus mice were primed with subcutaneous injections of estradiol benzoate (EB) in sesame oil (10 μg/0.1 mL) twice before testing (at 24 and 48 h). The mice were also administered progesterone (P) in sesame oil (500 μg/0.1 mL) 4−6 h before testing to ensure high sexual receptivity. All tests were video-recorded; the numbers of attempted mounts, successful mounts, intromissions, and ejaculations were scored for each mouse.

Male Aggressive Behavior

Five to Seven days after the last sexual behavior test, each male mouse was tested for its aggressive behavior against a gonadally intact, olfactory-bulbectomized C57BL/6J male intruder mouse using a resident-intruder paradigm. This test was performed weekly for 3 consecutive days, for a total of 9 trials. The duration of each trial was 15 min. All tests were video-recorded; the duration and number of aggressive bouts toward the intruder were scored for each mouse. The data for the 3 trials obtained each week were averaged for each mouse and used for statistical analysis. An aggressive bout was defined as a set of behavioral interactions that included at least one of the following behavioral actions toward the intruder: chasing, boxing, wrestling, biting, tail rattling, and offensive lateral attack. If the interval between 2 aggressive bouts did not exceed 3 s, the 2 bouts were considered to be continuous and scored as 1 bout.

Female Sexual Behavior

At 10–12 weeks of age, the female mice were separated from their littermates and housed individually in plastic cages (5 × 22 × 12 cm). Fourteen to Sixteen days after the isolation, all mice were ovariectomized under isoflurane inhalation anesthesia. Fourteen to Sixteen days after the ovariectomy, each female mouse was tested for sexual behavior toward a sexually experienced ICR/JCL male mouse (CLEA Japan, Tokyo, Japan) in the male's home cage. This test was performed weekly, for a total of 3 trials. The female mice were subcutaneously injected with EB (5 μg/0.1 mL dissolved in sesame oil) at 24 and 48 h before testing, and P (250 μg/0.1 mL dissolved in sesame oil) at 4–6 h before testing. Each test lasted until females received 15 mounts or intromissions. The number of lordosis responses to the male mounts or intromissions was scored for each mouse. A lordosis quotient was calculated by dividing the number of lordosis responses by 15 mounts or intromissions (Ogawa et al., 1999).

The LDT Test

Each mouse was tested once for its emotional behaviors in the LDT apparatus for 10 min. The test apparatus consisted of enclosed dark and open-top light compartments (30 × 30 × 30 cm each) connected by an inner door way (3 × 3 cm) located in the center of the partition at the floor level. The open-top light compartment was brightly illuminated with a white light (350 lux). The latency to enter the light compartment, the cumulative time spent in the light compartment, and the total distance traveled in the light compartment were measured by an automated video tracking system (ANY-maze, Stoelting, USA). The data from 2 male mice (1 in the control group and 1 in the low-dose group) were excluded from the analysis because of recording errors.

Sample Collection

After completing the behavioral tests, mice were deeply anesthetized with sodium pentobarbital (60 mg/kg), and blood was collected from the left ventricle of each mouse. The mice were subjected to transcardial perfusion with 0.1 M phosphate-buffered saline (PBS; pH 7.2), followed by 4% paraformaldehyde (PFA) in 0.1 M PBS. Brains were removed, post-fixed overnight

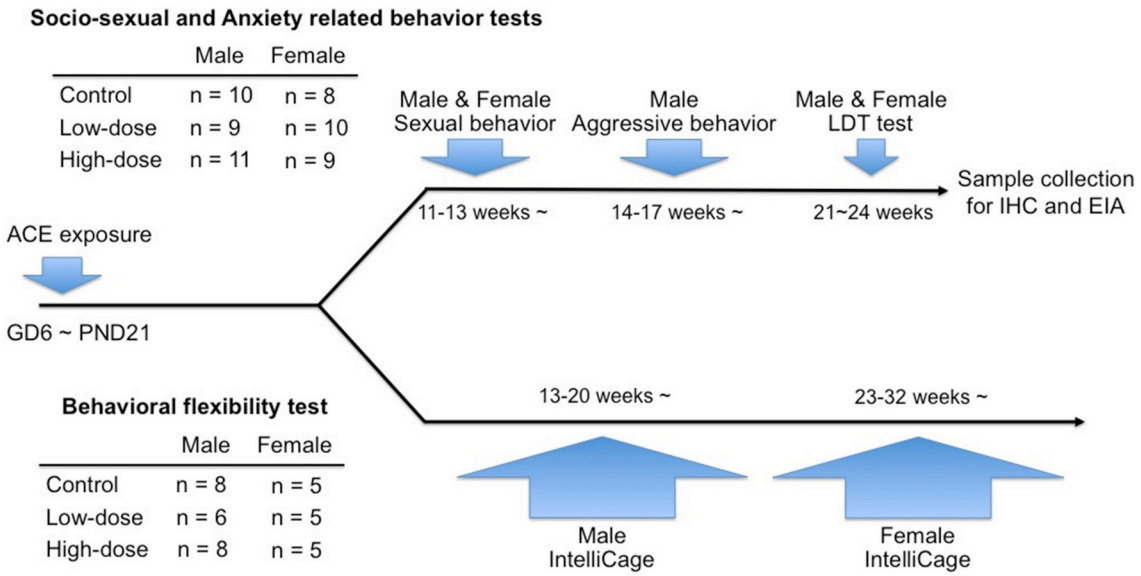

FIGURE 1 | Timeline of the behavioral tests in the study and information of the numbers of animals used in each cohort.

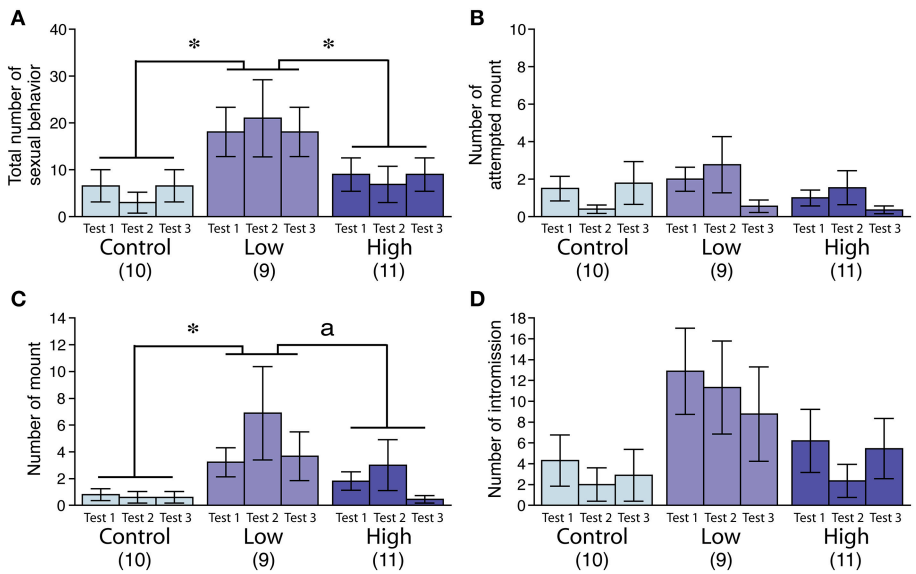

FIGURE 2 | Effects of developmental ACE exposure on male sexual behavior. Comparison of **(A)** the total number of sexual behaviors, as well as the numbers for each male sexual behavior component, including **(B)** attempted mounts, **(C)** mounts, and **(D)** intromissions across the treatment groups. The numbers of animals used are indicated in parentheses. The data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{a}P < 0.1$.

at 4°C with 4% PFA in 0.1 M PBS, and cryoprotected in 0.1M PBS containing 30% sucrose.

Enzyme Immunoassay for Plasma Testosterone

Samples were extracted from plasma (100 μl) with ethyl acetate, and testosterone concentrations were determined using a testosterone enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI, USA), according to the manufacturer's instructions. All male samples and randomly selected samples from females (5 for each treatment group) subjected to the socio-sexual and anxiety behavior tests were analyzed.

Immunohistochemistry

The brain samples were coronally sectioned at 30 μm thickness with 90 μm intervals on a freezing microtome. Sections were incubated in PBS-X (0.1 M PBS, pH 7.2 and 0.2% Triton X-100), containing 0.5% hydrogen peroxide for 20 min to

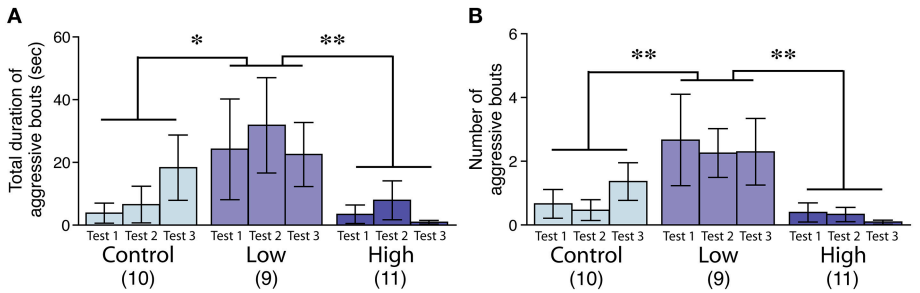

FIGURE 3 | Effects of developmental ACE exposure on male aggressive behavior. (A) The total duration and **(B)** the number of aggressive bouts toward the intruder stimuli. The numbers of animals used are indicated in parentheses. The data are presented as the mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$.

inhibit the endogenous peroxidase activity, and then blocked in an incubation buffer (1% casein in PBS-X) for 2 h at room temperature. Tissue sections were then incubated with a rabbit polyclonal anti-vasopressin antiserum (1:4000; Immunostar Cat. #20069, Hudson, NY, USA) in incubation buffer for 24 h at 4°C. After the completion of the incubation process, the staining was visualized using the DAKO EnVision™ Detection System (Peroxidase/DAB+, K5007). Three anatomically matched sections (30 μm thickness, 90 μm intervals) containing the paraventricular nucleus (PVN, bregma 0.70–0.94 mm) were selected for each mouse. PVN images were photographed at a 10x magnification with a digital camera mounted on a light microscope (Leica DFC290 HD; Leica Microsystems, Wetzlar, Hesse, Germany). The total number of immunoreactive cells was bilaterally counted for each animal. Because of technical issues with the sample preservation and tissue preparation, only the number of samples denoted in **Figure 7** were used for the analysis.

Evaluation of Behavioral Flexibility Using the IntelliCage

Male and female mice were tested separately for their behavioral flexibility using the IntelliCage, a fully automated testing apparatus consisting of a large plastic cage (55 × 37.5 × 20.5 cm) equipped with 4 corner chambers (15 × 15 × 21 cm each). Male mice at the age of 13–20 weeks were introduced to the IntelliCage apparatus and housed for 57 days. The female mice were housed in the apparatus for 56 days, starting at the age of 23–32 weeks. The difference in test timing of test was due to the limited capacity of the IntelliCage. Two to Three days before being introduced to the apparatus, the mice were anesthetized with isoflurane and subcutaneously implanted a glass-covered transponder having a unique ID code for radiofrequency identification (Datamars, Temple, TX, USA).

The behavioral flexibility test paradigm was composed of an acquisition phase and serial reversal phases. Prior to the behavioral flexibility test, the mice were allowed to acclimatize to the IntelliCage for 9 days. In the acquisition phase after acclimatization, mice were allowed to learn the two rewarded corners and shuttle between them. Subsequently, the mice were subjected to serial reversal tasks, in which the diagonal spatial patterns of the rewarded corners was repetitively reversed every

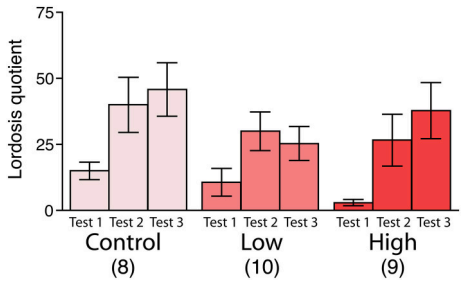

FIGURE 4 | Effects of developmental ACE exposure on female sexual behavior. The comparison of the lordosis quotient [(the number of lordosis responses/15 mounts or intromissions) * 100]. The numbers of animals used are indicated in parentheses. The data are presented as the mean ± SEM.

4–7 sessions. In total, there were 57 sessions for the male mice and 56 sessions for the female mice, including the first 14 sessions of the acquisition phase. Additional sessions were conducted with 10 serial reversals for the male mice and 9 serial reversals for the female mice. The percentage of visits to the non-rewarded corners within the first 100 visits was defined as the discrimination error rate and used to analyze the inter-session comparisons of learning performance. Additionally, the nose-poke frequency per visit within the first 100 visits was calculated for each mouse as an index of compulsive repetitive behavior. The IntelliCage apparatus and behavioral flexibility test paradigm are described in details elsewhere (Endo et al., 2011, 2012). The data from session 38–41 (6th reversal phase) and session 46–53 (8th and 9th reversal phases) in the male mice were excluded from analysis because of a mechanical malfunction of the IntelliCage apparatus. Thus, in the **Figure 8A**, the sessions 42–45, which were the 7th reversal phase, are denoted as Rev 6 and sessions 54–57, which were the 10th reversal phase, are denoted as Rev 7.

Statistical Analyses

All data are presented as mean ± standard error of the mean (SEM). All data, except the comparison of the numbers of ejaculating males, were analyzed using an ANOVA, followed by a Fisher's PLSD *post hoc* test. The incidence of ejaculation during the male sexual behavior test was compared using χ^2 tests. The differences were considered statistically significant when P

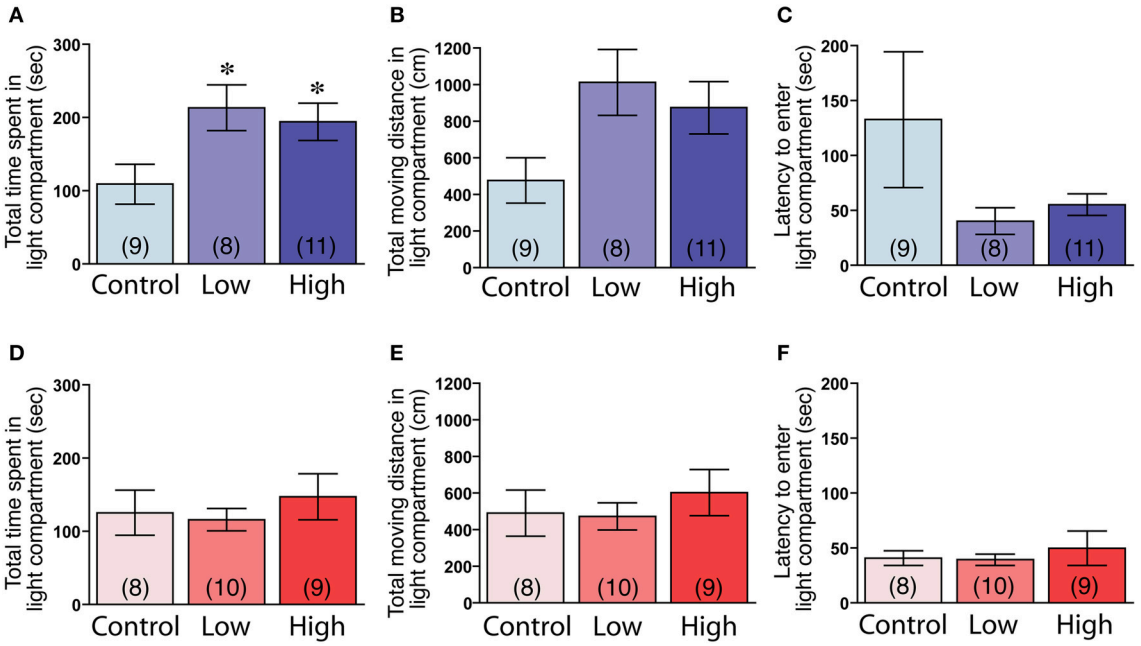

FIGURE 5 | Effects of developmental ACE exposure on anxiety-related behaviors, as measured in the light-dark transition test. (A–C) Male and **(D–F)** female mice of each treatment group. The comparison of **(A,D)** the total time spent in, **(B,E)** the total moving distance in, and **(C,F)** the latency to enter the light compartment of the light-dark transition apparatus. The numbers of animals used are indicated in parentheses. The data are presented as the mean ± SEM. *P < 0.05 vs. control.

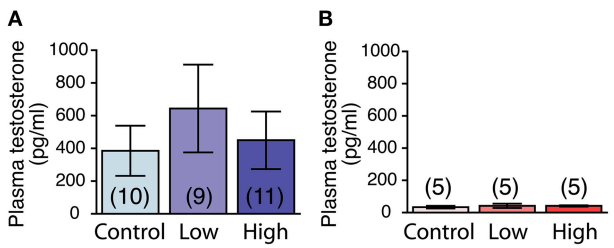

FIGURE 6 | Effects of developmental ACE exposure on plasma testosterone levels. (A) Male and **(B)** female mice of each treatment group. The numbers of animals used are indicated in parentheses. The data are presented as the mean ± SEM.

< 0.05. All data were analyzed using the SPSS 19.0 statistical package (SPSS Inc., Chicago, IL, USA) or R software (The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Body Weight/Litter

In the male and female mice, no differences were found in BW/litter among the groups at birth, PND 21, or 23–26 weeks of age (**Table 1**).

Brain Weight

No differences were found in the brain weights of either male or female mice at PND 21 (**Table 2**). There were no differences in the brain-to-body weight ratio either [data not shown].

Brain Residual Concentration Analysis

The brain residual concentration of ACE was measured in 6 mice (3 male and 3 female) from the control group and 6 mice (3 male and 3 female) from the high-dose group. The concentrations in the high-dose group were 1.29 ± 0.46 and 1.23 ± 0.20 ng/g in males and females, respectively (**Table 3**). In contrast, the concentrations were below the MDL in the control group for both sexes.

Male Sexual Behavior

In the male sexual behavior test (**Figures 2A–D**), the total number of sexual behaviors was significantly increased in the low-dose group [$F_{(2,\ 27)} = 3.72$, $P < 0.05$; Fisher's PLSD, $P < 0.05$, low-dose group vs. control and high-dose groups; **Figure 2A**], particularly for the mean number of mounts [$F_{(2,\ 27)} = 3.77$, $P <$ 0.05; Fisher's PLSD, $P < 0.05$, low-dose group vs. control group; **Figure 2C**]. We found no significant difference in the incidence of ejaculation during the tests (**Table 4**).

Male Aggressive Behavior

The aggression level in the low-dose group was significantly increased compared to that of the control and high-dose groups, as measured by the total duration [$F_{(2,\ 27)} = 4.44$, $P < 0.05$; Fisher's PLSD, $P < 0.05$, low-dose group vs. control; $P <$ 0.01, low-dose group vs. high-dose group; **Figure 3A**] and the number of bouts [$F_{(2,\ 27)} = 6.24$, $P < 0.01$; Fisher's PLSD, $P < 0.01$, low-dose group vs. control and high-dose groups; **Figure 3B**].

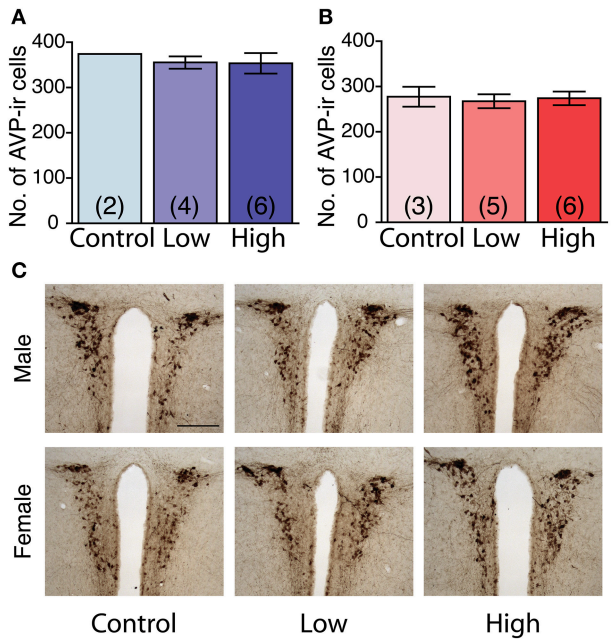

FIGURE 7 | Effects of developmental ACE exposure on the number of vasopressin (AVP) immunoreactive (ir) cells in the paraventricular nucleus (PVN). (A) Male and **(B)** female mice of each treatment group. The data are presented as the mean ± SEM. **(C)** Representative photomicrographs of AVP-ir cells in brain sections from males (top panels) and females (bottom panels) of each treatment group. The numbers of animals used are indicated in parentheses **(A,B)**. The scale bar represents 200 μm.

Female Sexual Behavior

No significant differences were found in the lordosis quotient among the groups, whereas the lordosis quotient increased with repeated testing in all groups [$F_{(2,\ 48)} = 12.83$, $P < 0.001$; **Figure 4**].

The LDT Test

The male mice in both the low-dose and high-dose groups spent significantly more time in the light compartment compared to the control group [$F_{(2,\ 25)} = 3.83$, $P < 0.05$; Fisher's PLSD, $P < 0.05$, low-dose and high-dose groups vs. control group; **Figure 5A**]. The male mice in the low-dose and high-dose groups tended to travel longer distances in the light compartment compared to the control group [$F_{(2,\ 25)} = 3.27$, $P = 0.055$; **Figure 5B**]. We found no significant differences in the latency to enter the light compartment (**Figure 5C**). In contrast to the males, there were no significant group differences in the females in the time spent in the light compartment (**Figure 5D**), the total distance traveled in the light compartment (**Figure 5E**) and the latency to enter the light compartment (**Figure 5F**).

Plasma Testosterone Levels

The plasma testosterone levels were significantly higher in the males compared to those of the females, regardless of treatment [$F_{(1,\ 39)} = 7.48$, $P < 0.01$; **Figures 6A,B**]. No main effect of ACE exposure was found on the plasma testosterone levels in either the males (**Figure 6A**) or females (**Figure 6B**).

AVP Immunoreactivity in the PVN

No main effect of ACE exposure was found on the number of AVP immunoreactive cells in either the males (**Figure 7A**) or females (**Figure 7B**). The number of AVP-immunoreactive cells was significantly higher in the males than in the females, regardless of treatment [$F_{(1,\ 20)} = 26.7$, $P < 0.01$; **Figures 7A,B**] as shown in representative photomicrographs (**Figure 7C**).

Behavioral Flexibility Test

During the acclimatization period, the number of corner visits per week in the IntelliCage did not differ between the groups in both males and females (Supplementary Figure 1), indicating the possibility that the developmental exposure to ACE does not affect home cage activity. In the acquisition phase, the male and female mice in all groups showed a decrease in the discrimination error rate with repeated sessions. No statistical differences were found between the groups, indicating that all groups could acquire the first task (**Figures 8A,B**). In the serial reversal phase, the male and female mice in all groups showed a similar pattern in the discrimination error rate in the reversal stage (Rev), indicating that the developmental ACE exposure had no effect on the spatial learning ability and behavioral flexibility during adulthood. We measured the nose-poke/visit ratio, which is an index of behavioral impulsivity, against rewards in the home cage, but found no significant group differences.

DISCUSSION

We evaluated the general physiological parameters, such as body and brain weights, during the developmental period of mice exposed to ACE *in utero* and via lactation, as well as a battery of socio-sexual and anxiety-related behaviors during their adulthood. ACE was found in the brains of pups of the high-dose group at PND 21, using a residual concentration analysis. These data showed that absorbed ACE was transferred into the developing brain. The ACE exposure at the used doses did not alter the body and brain weights. In addition, no impairments in behavioral flexibility were found in the *in utero* and lactational ACE-exposed adult mice. On the other hand, *in utero* and lactational ACE exposure altered socio-sexual and anxiety-related behaviors in males.

Although, the binding affinity of ACE to nAChRs is approximately 70–80 times lower than that of nicotine (Tomizawa and Casida, 1999; Picciotto et al., 2001), ACE can be bound to nAChRs containing α_4 and β_2 subunits, which are known to mediate the effects of nicotine. Based on this binding affinity difference, we propose that the effect of ACE on certain behaviors may correspond to the effect of nicotine at lower doses. Therefore, in our discussion below, we attempt to compare the present results to previous reports demonstrating the effects of developmental nicotine exposure at relatively low doses, when possible.

We found significant changes in the socio-sexual and anxiety-related behaviors in male mice exposed to ACE *in utero* and via lactation. The most significant change was found by the LDT test. ACE exposure at low or high doses prolonged the time spent in the light compartment, indicating reduction of

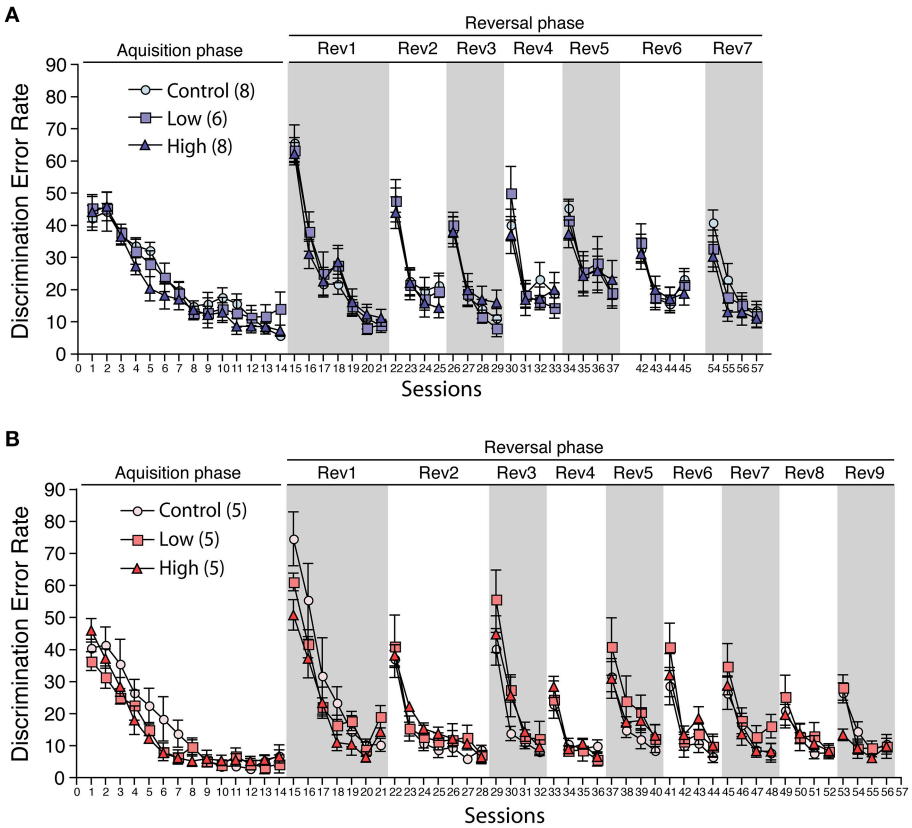

FIGURE 8 | Effects of developmental ACE exposure on behavioral flexibility. The comparison of the discrimination error rates (the number of discrimination errors in the first 100 corner visits in the session) in **(A)** male and **(B)** female mice. The numbers of animals used are indicated in parentheses. The data are presented as the mean ± SEM. The data for session 38–41 (6th reversal phase) and session 46–53 (8th and 9th reversal phases) in male were omitted due to the technical issues with the IntelliCage apparatus. Thus, the session 42 to 45 is denoted as Rev 6 and the session 54–57 is denoted as Rev 7 on **(A)**.

anxiety. This result suggests that there may be altered emotional responses in the ACE-exposed male mice. There have been many reports regarding the effects of nicotine exposure on anxiety-related behaviors during the developmental period. However, the effects of perinatal nicotine exposure reported in these studies are bidirectional, positive or negative, depending on the experimental conditions such as the dose, time of administration, and the behavioral test paradigm. Maternal nicotine exposure at a dose of 0.5 mg/kg BW from GD 10 to delivery in mice is reported to decrease anxiety-related parameters measured in the elevated plus-maze test in male offspring (Ajarem and Ahmad, 1998). In contrast, male mice whose mothers were exposed to nicotine (0.2 mg/ml) in drinking water throughout gestation or from GD 14 to delivery have been reported to exhibit increased anxiety-related responses in both the elevated plus-maze and LDT tests (Alkam et al., 2013). Vaglenova et al. (2004) reported that nicotine exposure in pregnant rats at a daily dose of 6.0 mg/kg BW from GD 3 to delivery also causes increased anxiety-related responses in their offspring as assessed in the elevated plus-maze test. The relationship between developmental nicotine exposure and anxiety shown by many studies allows us to speculate that the neural circuitry responsible for anxiety is vulnerable to cholinergic agents, although reservation is warranted because of inconsistencies among previous studies. In addition, perinatal nicotine exposure is reported to cause hyperactivity in rodents (Ajarem and Ahmad, 1998; Thomas et al., 2000; Pauly et al., 2004; Vaglenova et al., 2004; Paz et al., 2007; Heath et al., 2010; Alkam et al., 2013). We did not find any differences between the groups in corner visits in the IntelliCage, demonstrating no differences in home cage activity between the groups. However, mice exposed to ACE at both the low and high doses tended to travel longer distances in the light compartment, suggesting the possibility that ACE exposure may have induced hyperactivity under specific stressful conditions. Therefore, the longer times spent in the light compartment in the LDT test may reflect not only decreased anxiety, but also hyperactivity under specific stressful conditions. Future experiments measuring locomotor activity with or without stress are necessary to clarify whether ACE exposure decreases low anxiety, induces hyperactivity, or both under stressful conditions.

On socio-sexual behaviors, the effect of ACE appeared to be dose-specific. ACE exposure at the low dose, but not at the high dose, significantly increased male sexual and aggressive behaviors. Socio-sexual behaviors are governed by neural circuits

TABLE 1 | Body weight per litter (BW/litter) at birth, weaning (PND21), and 23–26 weeks of age.

	Groups	Birth (g)	PND21 (g)	23–26 weeks (g)
Male	Control	1.27 ± 0.02 (7)	7.85 ± 0.12 (6)	28.28 ± 0.57 (5)
	Low	1.30 ± 0.02 (6)	8.01 ± 0.37 (6)	30.09 ± 0.68 (5)
	High	1.31 ± 0.04 (9)	7.72 ± 0.39 (9)	28.08 ± 0.66 (6)
Female	Control	1.22 ± 0.02 (7)	7.38 ± 0.14 (6)	25.63 ± 0.72 (4)
	Low	1.29 ± 0.03 (6)	7.76 ± 0.27 (5)	25.82 ± 0.50 (5)
	High	1.31 ± 0.03 (9)	7.43 ± 0.31 (9)	24.85 ± 0.33 (5)

Data presented as mean ± SEM.
Number of dam is given in parentheses.

TABLE 2 | Brain weight at PND 21.

	Groups	Brain weight (g)
Male	Control ($n = 6$)	0.41 ± 0.007
	Low ($n = 2$)	0.44
	High ($n = 4$)	0.41 ± 0.008
Female	Control ($n = 6$)	0.40 ± 0.003
	Low ($n = 4$)	0.41 ± 0.003
	High ($n = 6$)	0.39 ± 0.013

Data presented as mean ± SEM.

TABLE 3 | Residual concentration of ACE in the brains of offspring at PND 21.

	Groups	Brain residual concentration (ng/mg tissue)
Male	Control ($n = 3$)	<MDL
	High dose group ($n = 3$)	1.29 ± 0.46
Female	Control ($n = 3$)	<MDL
	High dose group ($n = 3$)	1.23 ± 0.20

MDL: The method detection limit.
Data presented as mean ± SEM.

TABLE 4 | Number of male mice demonstrated ejaculation in each test.

Groups	Test 1	Test 2	Test 3
Control	2/10 (20%)	2/10 (20%)	2/10 (20%)
Low	3/9 (33.3%)	2/9 (22.2%)	5/9 (55.6%)
High	2/11 (18.2%)	2/11 (18.2%)	4/11 (36.4%)

Number of mice ejaculated/total number of mice tested; the percentage is given in parentheses.

involving various brain regions (Newman, 1999). Peripheral sex steroid hormones play an essential role in the formation of these neural circuits during the critical period. Generally in mammals, the critical period can extend from late gestational period to the early life (Hull and Dominguez, 2007; Nelson and Trainor, 2007). The cholinergic system is also thought to play a role in the formation of the neural bases of these behaviors (Dwyer et al., 2009; Blood-Siegfried and Rende, 2010). Any agents that block or enhance acetylcholine transmission may interfere with this formation.

The idea that low-dose chemical exposure alters specific behaviors has been proposed in previous studies. Dioxin and bisphenol A induce their toxicities in a non-monotonic manner (Endo et al., 2012; Vandenberg, 2014). The actions of nicotine also appear to be complex and non-monotonic, especially when given *in vivo* (Anderson and Brunzell, 2012, 2015; Alkam et al., 2013; Abreu-Villaça et al., 2015). Behaviors as well as specific neural circuit formation may potentially be dysregulated by nicotine in a non-monotonic manner. For example, the effect of developmental nicotine exposure on the development of the vasopressinergic system is complex. Prenatal nicotine exposure at a relatively high dose (6 mg/kg/day throughout the gestational period) greatly reduced vasopressin production and release in the hypothalamus of male rats (Zbuzek and Zbuzek, 1992). Although, the species difference must be taken into consideration, prenatal nicotine exposure at a relatively low dose (total amount of 1.05 mg throughout the gestational period) has been reported to increase the number of vasopressin cell bodies and fibers in the hypothalamus of the golden hamster (Rossi et al., 2003). Since vasopressinergic systems regulate both sexual and aggressive behaviors in a facilitative manner (Ho et al., 2010; Stevenson and Caldwell, 2012), we speculated that ACE exposure specifically at the low dose enhanced the development of neural networks involved in vasopressinergic systems. However, we did not find any differences in the number of AVP-immunoreactive cells in the PVN. Therefore, it is unlikely that *in utero* and lactational ACE exposure at the low dose increased aggression and sexuality in adulthood through alterations of the vasopressinergic system in the PVN. We could not examine the numbers of AVP-immunoreactive cells in areas outside the PVN, such as the bed nucleus of the stria terminalis (BNST), a region known to be critically involved in the facilitation of male socio-sexual behaviors (Newman, 1999; Nelson and Trainor, 2007). Further studies are needed to clarify whether ACE affects AVP immunoreactive cells in brain regions other than the PVN.

There have been several reports regarding the effects of in utero nicotine exposure on plasma testosterone levels in animal models, but these reports are also inconsistent with each other, presumably due to differing exposure conditions. On one hand, the plasma testosterone levels decreased in 10-week-old male rat offspring born to dams that had been administered nicotine at a dose of 0.5 mg/kg throughout gestation (Segarra and Strand, 1989). In contrast, the plasma testosterone levels increased in 13-week-old male rat offspring born to dams that had been given nicotine at a dose of 2 mg/kg throughout gestation increased (Paccola et al., 2014). In our study, we did not find any differences in plasma testosterone levels measured at 23–26 weeks of age, which excludes the possibility that increased testosterone levels in adult males caused the abnormalities in the socio-sexual and emotional behaviors. However, we did not determine plasma testosterone levels during the perinatal period, which is a critical

period for the testosterone-induced masculinization of socio-sexual behaviors. Therefore, we cannot exclude the possibility that developmental ACE exposure altered the testosterone levels during the perinatal periods, resulting in the impaired sexual differentiation of socio-sexual behaviors.

Behavioral flexibility was unaffected by developmental ACE exposure under our exposure conditions. Behavioral flexibility is considered a part of executive function, which is dependent on hippocampal and prefrontal cortical function (Kosaki and Watanabe, 2012; Malá et al., 2015). Various studies have reported the negative effects of developmental nicotine exposure at relatively high doses on the cognitive performance of offspring (Yanai et al., 1992; Wickström, 2007; Parameshwaran et al., 2012). However, the effects of developmental nicotine exposure to neonicotinoids or lower doses of nicotine have not been clarified for cognitive functions. Our study demonstrated that the developmental ACE exposure did not affect the cognitive functions, at least at the ACE doses and animal ages tested in this study. However, we cannot exclude the possibility that late-onset impairment of cognitive function occurs in mice exposed to ACE. Since malfunction of the cholinergic system is associated with cognitive deficits such as those in Alzheimer's disease (Schliebs and Arendt, 2011), examining behavioral flexibility in aged mice exposed to ACE perinatally would be an interesting avenue of our research.

Sex differences in the effects of ACE exposure on socio-sexual and emotional behaviors should also be addressed since *in utero* and lactational ACE exposure at either dose had no effect on female sexual behavior and anxiety. The present study was unable to elucidate the neuropathologies underlying the sex-specific effect of ACE. Several lines of evidence suggest that the male is more susceptible to developmental nicotine exposure than the female (Fung and Lau, 1989; Ribary and Lichtensteiger, 1989; von Ziegler et al., 1991; Pauly et al., 2004). The sex-specific effect of ACE observed in this study is consistent with the reported male–biased effects of nicotine, possibly suggesting that nicotine and ACE elicit toxic effects through a common sex-specific pathway.

It is also important to mention that ACE was administered to mice throughout the gestational and lactational periods in this study. The effect of developmental nicotine exposure has been reported to vary depending on the timing of exposure (Alkam et al., 2013). Therefore, if ACE shares a common toxic pathway with nicotine, it is highly likely that the effect of ACE also varies depending on the timing of exposure. The developmental action of ACE in different time-windows should be further examined in future studies.

CONCLUSION

Our results suggest the possibility that ACE affects socio-sexual and anxiety-related behaviors in a male-specific manner. Further experiments are needed to understand the behavioral alterations and examine the mechanisms underlying the ACE-induced impairments in brain function. The action of ACE appears to be non-monotonic for the socio-sexual behaviors, as the effects were only found in mice exposed to ACE at low doses. Further empirical studies using mouse models are required to evaluate whether ACE doses equivalent to the human exposure level have detrimental effects.

AUTHOR CONTRIBUTIONS

KS, TI, TW, SN, TK, GS, SH, and FM designed the experiment. KS, TI, JY, TW, MY, TK, and FM performed the experiment. KS, TI, JY, MY, and FM analyzed the data. KS wrote the manuscript. KN, CT, and FM critically revised the manuscript.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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Prenatal Exposure to Arsenic Impairs Behavioral Flexibility and Cortical Structure in Mice

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Exposure to arsenic from well water in developing countries is suspected to cause developmental neurotoxicity. Although, it has been demonstrated that exposure to sodium arsenite ($NaAsO_2$) suppresses neurite outgrowth of cortical neurons *in vitro*, it is largely unknown how developmental exposure to $NaAsO_2$ impairs higher brain function and affects cortical histology. Here, we investigated the effect of prenatal $NaAsO_2$ exposure on the behavior of mice in adulthood, and evaluated histological changes in the prelimbic cortex (PrL), which is a part of the medial prefrontal cortex that is critically involved in cognition. Drinking water with or without $NaAsO_2$ (85 ppm) was provided to pregnant C3H mice from gestational days 8 to 18, and offspring of both sexes were subjected to cognitive behavioral analyses at 60 weeks of age. The brains of female offspring were subsequently harvested and used for morphometrical analyses. We found that both male and female mice prenatally exposed to $NaAsO_2$ displayed an impaired adaptation to repetitive reversal tasks. In morphometrical analyses of Nissl- or Golgi-stained tissue sections, we found that $NaAsO_2$ exposure was associated with a significant increase in the number of pyramidal neurons in layers V and VI of the PrL, but not other layers of the PrL. More strikingly, prenatal $NaAsO_2$ exposure was associated with a significant decrease in neurite length but not dendrite spine density in all layers of the PrL. Taken together, our results indicate that prenatal exposure to $NaAsO_2$ leads to behavioral inflexibility in adulthood and cortical disarrangement in the PrL might contribute to this behavioral impairment.

Keywords: sodium arsenite, developmental neurotoxicity, behavioral impairment, neurite outgrowth, prelimbic cortex

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INTRODUCTION

The developing brain is vulnerable to disruption by environmental factors including toxic chemical exposure. Environmental exposures may therefore account for an increase in the prevalence of neurodevelopmental and neuropsychiatric disorders including autism spectrum disorders, attention deficit hyperactivity disorders (ADHD), and learning disabilities (Grandjean and Landrigan, 2006, 2014). Recent studies suggest that *in utero* and lactational exposure to

toxic chemicals affects the development of the brain. For example, exposure to inorganic lead, methylmercury, and polychlorinated biphenyls during gestation and early childhood are associated with the prevalence of mental retardation, cerebral palsy, and ADHD in children (Grandjean and Landrigan, 2006; Bisen-Hersh et al., 2014). These studies indicate that early life environmental exposures play a role in the etiology of neurodevelopmental disorders.

It has been long suspected that arsenic exposure can lead to developmental neurotoxicity. More than 200 million people worldwide have been estimated to be chronically exposed to arsenic in drinking water at concentrations above the World Health Organization (WHO) recommended safety limit of 10 μg/L (WHO, 2008). A large number of epidemiological studies have demonstrated that chronic exposure to arsenic produces peripheral neuropathies and decreases cognitive performance in children such as lowered memory and intelligence quotient scores on standardized tests (Rocha-Amador et al., 2007; Rosado et al., 2007; Wasserman et al., 2007), which are indicative of higher brain function deficits. Additionally, follow-up studies on victims of arsenic poisoning from the Morinaga formula incident in Japan revealed an association between oral exposure to arsenic during infancy and various brain disorders, including mental retardation and epilepsy (Dakeishi et al., 2006). These studies suggest that early life arsenic exposure can affect higher brain function later in life. This notion is supported by some studies in animal models. For example, exposure to low level arsenic in maternal drinking water throughout gestational and lactational period increased indices of anxiety in mouse offspring during a novel object exploration task (Martinez-Finley et al., 2009). Moreover, few behavioral deficits such as an increase in pivoting, a type of abnormal gait behavior, was observed in younger mouse offspring following a short period of gestational exposure to arsenic (Colomina et al., 1996).

Arsenic exposure could produce behavioral changes through effects on the developing brain directly since arsenic freely crosses the fetus-placenta and blood-brain barrier in human (Willhite and Ferm, 1984; Hirner and Rettenmeier, 2010). *In vitro* and *in vivo* experimental models have been used to elucidate how arsenic exposure impairs higher brain function. A previous study showed that sodium arsenite ($NaAsO_2$) exposure produces both apoptotic and necrotic cell death in developing brain cells in rat (Chattopadhyay et al., 2002). Our *in vitro* studies have shown that $NaAsO_2$ exposure induces apoptotic cell death and inhibits neuritogenesis (Koike-Kuroda et al., 2010; Aung et al., 2013). The inhibitory effect of $NaAsO_2$ on neuritogenesis is in part result from alterations in cytoskeletal components (Aung et al., 2013) and the downregulation of AMPA receptors, which are known to regulate the expression of cytoskeletal proteins (Maekawa et al., 2013). In animal studies, embryonic arsenic exposure produces neural tube defects, increase neuronal apoptosis, disrupt neural outgrowth, and reduce overall head size in both mouse and zebrafish models (Chaineau et al., 1990; Li et al., 2009). Further, it has been reported that arsenic exposure in rats from gestation throughout lactation and development until the age of 4 month alters morphology of nerve fibers and axon in the corpus striatum (Rios et al., 2009). These studies indicate that structural changes of brain such as neural network formation might contribute to the impairment of higher brain function following early life exposure to $NaAsO_2$. However, the precise mechanism by which developmental arsenic exposure produces impairments in higher brain function remains largely unknown.

Executive function such as planning, goal-directed action, and behavioral flexibility are core units of higher brain function, and impairment of these functions has been observed in a variety of neurodevelopmental disorders (Valencia et al., 1992; Kipp, 2005; Hill and Bird, 2006). To evaluate these executive processes in mice, a model of behavioral flexibility was recently established using the IntelliCage system, which is a fully automated behavioral testing apparatus for mice under group-housing conditions (Endo et al., 2011, 2012). This testing apparatus allows for the comprehensive and reproducible evaluation of behavioral flexibility. In humans, the brain areas responsible for executive function of goal-directed actions and behavioral flexibility are located in the medial prefrontal cortex (Yan et al., 2015). Several studies have shown that the prelimbic cortex (PrL), a part of the medial prefrontal cortex, is critically involved in a variety of cognitive and executive processes (Dalley et al., 2004; Marquis et al., 2007; Ragozzino, 2007). It has been also reported that the PrL is affected by exposure to chemicals, such as methylmercury, lead, and dioxin, and that exposure-associated impairments in the PrL are associated with decreased executive function in rodents (Ferraro et al., 2009; Tomasini et al., 2012). Accordingly, we decided to investigate the structure of the PrL as a possible target of arsenic-induced brain impairment.

In the present study, we used a fully automated behavioral analysis system to investigate the effects of prenatal $NaAsO_2$ exposure on murine behavioral flexibility in adulthood, and then analyzed the morphology of neuronal cells in these animals in order to determine how early life $NaAsO_2$ exposure produces neurotoxicity.

MATERIALS AND METHODS

Animals and $NaAsO_2$ Exposure

Pregnant C3H mice were purchased from JAPAN SLC (Shizuoka, Japan) and housed on a 12-h light/dark cycle at a temperature of 24 ± 1°C with free access to water and food. From gestational days 8–18, pregnant females were given *ad libitum* access to regular water or water containing 85 parts per million (ppm) $NaAsO_2$ (equivalent to 85 mg/L). To examine the water consumption of pregnant dams, the weight of water bottle for each dam was measured before and after providing *ad libitum* access to water. Pregnant dams tolerated the dose of $NaAsO_2$ at 85 ppm, and no obvious effects on maternal toxicity or teratogenicity were observed.

The pups were weaned at post-natal day 21 and housed under the same conditions as the dams. The number and sex of pups born from dams were then measured. At 60 weeks of age, control and $NaAsO_2$-exposed offspring were prepared for behavioral flexibility testing using the IntelliCage system (TSE Systems GmbH, Bad Homburg, Germany). Mice were randomly selected per dams (number of dams: control = 6 and $NaAsO_2$ = 9) to minimize the litter effects. After selecting mice, they were lightly

anesthetized with diethyl ether and subcutaneously implanted with a glass-covered transponder. Each transponder had a unique ID code for radiation frequency identification (RFID) for use with the IntelliCage system. Males and females were separately tested using different IntelliCage apparatuses. The control and $NaAsO_2$-exposed groups includes 9 mice per group for females and 6 or 10 mice per group for males. All procedures were approved by the Institutional Animal Care and Use Committee of the National Institute for Environmental Studies (NIES) and conducted strictly in accordance with NIES guidelines.

Intellicage Apparatus

The IntelliCage is a computer-based, fully-automated testing apparatus that can be used to monitor the spontaneous and cognitive behaviors of group-housed RFID-tagged mice in a large home cage (**Figure 1A**). Briefly, a large standard plastic cage (55 × 37.5 × 20.5 cm^3) was equipped with four triangular operant learning chambers (hereafter referred to as corners) (15 × 15 × 21 cm^3) that fit into each corner of the cage. RFID readers and other sensors allowed the simultaneous monitoring of up to 16 transponder-tagged mice living in the same cage. Mice were allowed to enter each corner (hereafter referred to as a "corner visit") through a short, narrow tunnel that functioned as an RFID antenna. Only one mouse was able to enter a given corner at any one time due to the limited size of the tunnel. In the inner space of each corner was equipped with two nose poke holes that were monitored via an infrared beam-break response detector. Nose poke behavior triggered to open a motorized gate access to a water bottle nipple. For each behavioral event (corner visit, nose poke, and licking), mouse ID and corner ID were automatically recorded through the RFID readers, infrared sensors, and lickometers.

Intellicage Test Procedures

Acclimation

The control and $NaAsO_2$-exposed male or female mice at 67 weeks of age were separately introduced to IntelliCage apparatuses on the same day. The number of mice in each IntelliCage apparatus was counterbalanced within groups. Acclimation and behavioral tests were then conducted according to test procedures. In acclimation phase 1 (3 days), the motorized gates controlling access to water bottle nipples were kept open in all 4 corners; thus, mice were allowed to drink water in each corner *ad libitum*. In acclimation phase 2 (1 day), the mice were trained to perform the nose poke task. Initially, all motorized gates were closed and mice were only given access to water bottle nipples after a nose poke event. The gate remained open for 4 sec following each nose poke, and water was available through the nose poke task for 24 h. In acclimation phase 3 (5 days), mice were only given the opportunity to gain access to water through the nose poke task for a 3-h period (19:00–22:00) per day. During acclimation phase, four female mice (one from control group and three from $NaAsO_2$-exposed group) were not able to learn how to access water drinkable corners, and such mice were not used in the following behavioral tasks.

Behavioral Sequencing Task

The behavioral flexibility test, also referred to as the behavioral sequencing task, was composed of an acquisition phase and a serial reversal task phase. The acquisition phase consisted of 11 or 21 sessions of the behavioral sequencing task (**Figure 1B**) and the serial reversal task phase consisted of repetitions of a reversal task (Rev. 1−11, **Figure 1C**). Water-deprived mice had 4 sec of access to water as a reward when they visited designated corners during a daily 3-h test session (19:00−22:00; **Figure 1D**). A total of 67 sessions for male mice and 83 sessions for female mice were conducted. In each session, mice were rewarded continuously if they alternated visits between two particular diagonally opposed corners (**Figure 1C**). The diagonal pair of corners was either active or inactive in a mutually exclusive manner, meaning that there was always one active reward corner, one inactive reward corner, and two never-rewarded corners. Mice were able to open the gate in an active corner by nose poke, and the gate remained open for 4 s to permit drinking. After the reward period, the corner instantly became inactive, and this signal was synchronized with the activation of the diagonally opposed corner. The alteration of corner assignments was controlled for each mouse independently by the IntelliCage software. Thus, the mice had to alternate between two diagonally opposed reward corners in order to acquire rewards continuously. A visit to either of the two never-rewarded corners was regarded as a discrimination error. The number of discrimination errors within the first 100 visits in each session provided a discrimination error rate that was used to analyze inter-session learning performance.

Histological Staining

After the last session of behavioral experiments, the same female mice were immediately sacrificed for morphometrical analysis of neuronal cells, while male mice were used for gene expression analyses (not described in this study). Mice were deeply anesthetized with sodium pentobarbital (60 mg/kg) and brains were harvested for analysis. Brains were histologically processed using the FD Rapid GolgiStain Kit (FD NeuroTechnologies, Ellicot City, MD, USA). Briefly, brains were rinsed with distilled water, immersed in 5 mL of equal parts Solution A and Solution B at room temperature, and stored for 2 weeks in the dark. Storage solution was replaced with fresh solution on the second day. Tissues were next immersed in Solution C at 4°C for at least 48 h. Solution C was replaced with fresh solution on the second day. Samples were then quickly frozen at −70°C and stored at −20°C until use. Coronal brain sections (60 μm) were cut using a cryostat (Leica CM1900; Leica Microsystems, Wetzlar, Germany), where the temperature of chamber and specimen head were set to −22°C and −23°C, respectively. Brain sections were mounted on gelatin-coated glass slides and allowed to dry at room temperature. Sections were then stained with a solution of 1 part Solution D, 1 part Solution E, and 2 parts distilled water for 10 min at room temperature. Golgi-stained brain sections were rinsed in distilled water twice for 4 min each and counterstained with 0.1% Cresyl Fast Violet solution. The Golgi- and Cresyl Fast Violet-stained sections were used for stereological analysis. The group average number of neurons and glial cells, length of

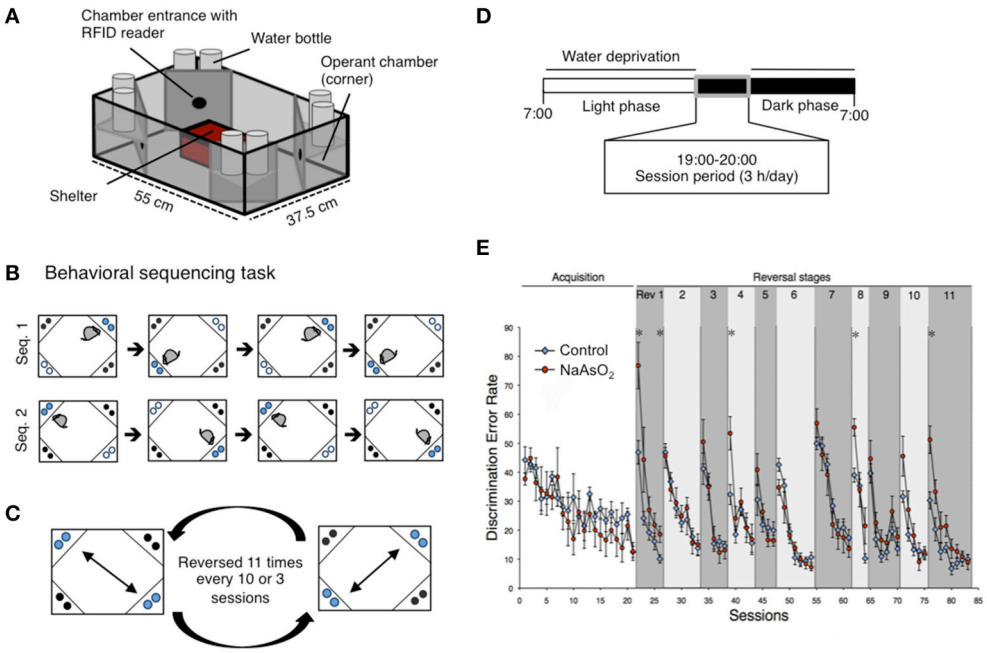

FIGURE 1 | Impaired behavioral flexibility in adult mice born to dams that were administered $NaAsO_2$ during gestation. (A) Overview of an IntelliCage apparatus. **(B)** Behavioral sequencing task. Mice were allowed to make an access to water as a reward for 4 s during their visits at an active reward corner (blue circle). The location of the active reward corner was automatically alternated to a diagonally opposed corner each time the mouse received a reward. Therefore, mice obtained rewards continuously by acquiring a behavioral sequence (alternating between the two reward corners). A visit to a never-rewarded corner (black circle) was counted as a discrimination error. **(C)** Serial reversal task. For each mouse, the assigned spatial patterns of the rewarded corners (sequence 1 or sequence 2) were reversed 11 times every 3 or 8 sessions. **(D)** Timeline of the experiment for each day. **(E)** Learning performance of control or $NaAsO_2$-exposed female mice in the behavioral flexibility test. Discrimination error rates (the number of discrimination errors in the first 100 corner visits of a session) are expressed as mean ± SEM (control group $n = 8$, $NaAsO_2$-exposed group $n = 6$). An asterisk indicates a statistical difference ($p < 0.05$) from the control group.

neurites, and density of dendritic spines were calculated from five or seven brains of mice, which was randomly selected and blind from behavioral data, for control and $NaAsO_2$-exposed group, respectively.

Stereological Analysis of the Length of Neurites and the Number of Neuronal Cells

The length of neurites and the number of neurons and glial cells in the PrL were measured using StereoInvestigator software (MicroBrightField (MBF) Bioscience, Williston, VT., USA) and a light microscope (DM5000B; Leica Microsystems) connected to a CCD camera. The boundaries between the PrL and the infralimbic cortex (IL) and between the PrL and the dorsal anterior cingulate (ACd) were determined by observing differences in cell size and density in the cortical layers of Cresyl Fast Violet-stained brain sections (Van De Werd et al., 2010). The rostrocaudal level of the PrL was determined by referring to an atlas of the mouse brain (Franklin and Paxinos, 2008; **Figure 2A**).

The length of neurites on Golgi-stained neurons in the left PrL was measured using the Space Ball probe utility of the Stereo Investigator software. The contours of the PrL in each brain section were drawn using a 5X objective lens magnification according to the criteria mentioned above. We set grid sizes of 150 × 200 μm, and used a sphere with a 40-μm radius and a highest top guard zone of 2.5 μm for the quantification of neurite length. The intersectional points between Golgi-stained neurites and the spherical line (**Figure 3B**) were counted in three consecutive sections of the PrL using a 100X oil immersion objective lens magnification, and the number of intersection points was used to compute the estimated length of Golgi-stained neurite in the selected region of the PrL for each mice (Mouton et al., 2002). The estimated length of Golgi-stained neurite was then normalized by dividing it by the estimated volume of the selected region in each animal. The coefficient of error (Gundersen, $m = 1$) for the estimation of neurite length was 0.05–0.09 for each animal.

The optical fractionator method was used to measure the number of Cresyl Fast Violet-stained neurons (pyramidal and non-pyramidal neurons) and glial cells in three different cortical layers (layer II−III, V, and VI) of the left PrL in accordance with the system work flow of the Stereo Investigator software. Since cortical layer IV is completely disappeared in the mice PrL (Van De Werd et al., 2010), it was not included in this analysis. The cortical layer boundaries were distinguished based on differences in cell size and density in Cresyl Fast Violet-stained brain sections (**Figure 2B**). The contours for layers II–III, V, and VI were drawn in each section using a 5X objective lens magnification and a frame size of 30 × 30 μm within the grid size of 150 × 150 μm. The height of the optical dissector was 40 μm and the top guard zone was 2.5 μm. Cell numbers were counted manually

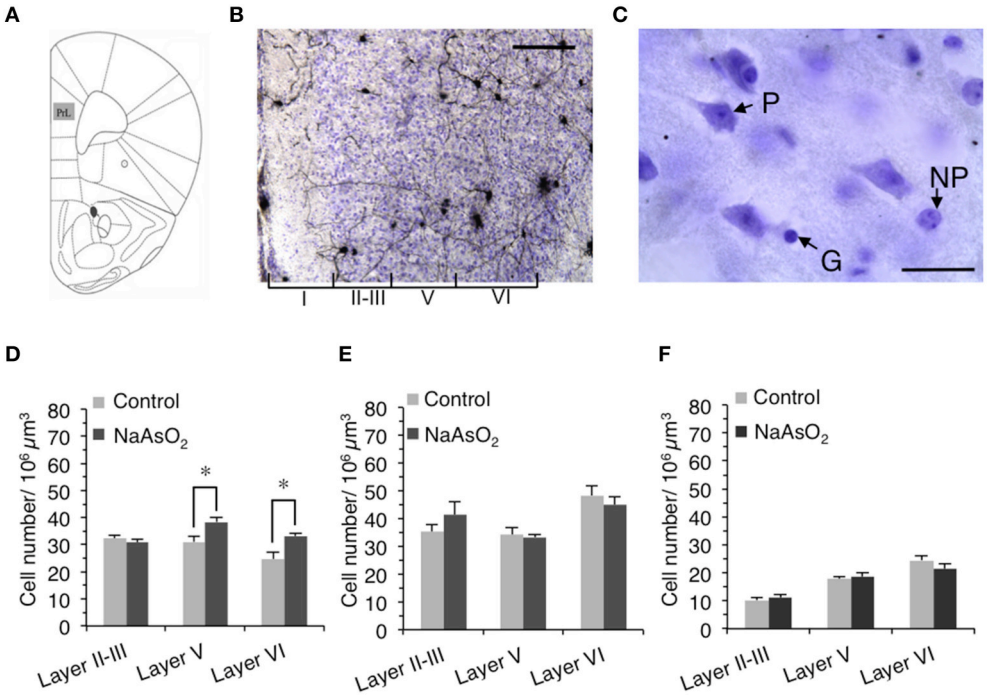

FIGURE 2 | Alteration in the number of neuronal cells in the PrL of adult female mice born to dams that were administered $NaAsO_2$. (A) Illustration of the region of interest, the PrL, adapted from a mouse brain atlas (Franklin and Paxinos, 2008). **(B)** Representative photomicrograph of the PrL in Golgi- and Cresyl Fast Violet-stained coronal brain sections. The Greek numbered (I–VI) regions indicate the cortical layers of the PrL. Scale bar = 20 μm. **(C)** Photomicrographs of the PrL at high magnification showing a Cresyl Fast Violet-stained pyramidal neuron (P), a non-pyramidal neuron (NP), and a glial cell (G). The length of scale bar indicates 5 μm. The number of pyramidal neurons **(D)**, non-pyramidal neurons **(E)**, and glial cells **(F)** in the PrL. Values are expressed as mean ± SEM (control group $n = 7$, $NaAsO_2$-exposed groups $n = 5$). An asterisk indicates $p < 0.05$ vs. the control group.

using a 100X oil immersion objective lens magnification. The setting for cell counting was sufficient to generate a coefficient of error (Gundersen, $m = 1$) of 0.05–0.06. The estimated number of pyramidal neurons, non-pyramidal neurons, and glial cells in each layer of the PrL was normalized by dividing each number by the estimated volume of its respective layer. The morphological criteria used to identify neuronal and glial cells observed in Cresyl Fast Violet-stained brain sections have been previously reported (Tsukahara et al., 2011). To distinguish pyramidal neurons from non-pyramidal neurons, the following criteria were used: (1) the cell bodies of pyramidal neurons exhibited a characteristic triangular shape with a single large apical dendrite extending vertically toward the pial surface, (2) non-pyramidal neurons were identified by the absence of the preceding criteria and exhibited a relatively smaller cell body size than that of pyramidal neurons (**Figure 2C**).

Imaging and Analysis of Dendritic Spine Morphology

The dendritic segments of Golgi-stained pyramidal neurons were used in morphometrical analyses. Sequential z-series images of dendritic segments were taken every 0.4 μm with an oil immersion lens (Plan Apo VC 100X, Numerical Aperture 1.40, Oil; Nikon, Tokyo, Japan) and a BioRevo 9000 microscope (Keyence Co., Osaka, Japan). The applied zoom factor (1.5X) provided images with 0.14 μm/pixel resolution. Images were then deconvoluted using Keyence BZ II Analyzer software (Keyence) and constructed into three-dimensional images using ImageJ software (National Institutes of Health, Bethesda, MD, USA) for analyses of dendritic spine morphology. The density and head diameter of dendritic spines were analyzed using Spiso-3D automated dendritic spine analysis software, which has an equivalent capacity to Neurolucida (MBF Bioscience, USA) (Mukai et al., 2011). The primary basilar dendritic segments of Golgi-stained pyramidal neurons, lying between 10 and 100 μm from the soma, were used to analyze the morphology of dendritic spines. For each cortical layer (layer II—III, V, or VI), 30—45 dendritic segments were analyzed per experimental group. Spine density was calculated from the number of spines existing on the total length of 40—100 μm dendritic segments. To examine spine morphological changes in response to prenatal $NaAsO_2$ exposure, the diameter of the spine head was classified into three categories: (1) small-head spines with a diameter of 0.2—0.4 μm, (2) middle-head spines with a diameter of 0.4—0.5 μm, and (3) large-head spines with a diameter of 0.5—1 μm.

Statistical Analysis

Changes in mouse behavioral flexibility were analyzed using the non-parametric Mann Whitney *U*-test with R software (The R Foundation for Statistical Computing, Vienna, Austria) because

the sample size of each group for each session was relatively small and it didn't follow a normal distribution. Morphometrical and other general assessments (including body weight, number of pups, and water intake) were analyzed with the parametric Student's unpaired *t*-test with Welch's correction with Prism software (GraphPad Software, La Jolla, CA, USA). Statistical differences were evaluated between the control and $NaAsO_2$-exposed groups. $P \leq 0.05$ were considered to be statistically significant.

RESULTS

Maternal and Embryonic Toxicity

No dams were found to develop significant abnormalities in general health parameters including the body weight gain of the dams during pregnancy (Figure S1) and maternal death. In addition, there were no differences in the number of live pups between the control and $NaAsO_2$-exposed groups (Figure S2).

Basal Activity Levels of Offspring

No toxic effects of prenatal $NaAsO_2$ exposure on body weight gain and blood glucose level of offspring were observed (Figure S3). In addition, there were no apparent differences in most of basal activity indices of the offspring in the acclimation phase of the behavioral flexibility test were observed between the two groups, except significant increase in duration of nose poke in $NaAsO_2$-exposed female mice (Tables S1, S2).

Impaired Behavioral Flexibility in $NaAsO_2$-Exposed Mice

Behavioral flexibility was examined by evaluating the number of incorrectly visiting the two never-rewarding corner within the first 100 visits of a given test session (discrimination error rate). In acquisition phase of the behavioral sequencing task, mice were imposed to discriminate rewarded corners from never-rewarded corners with acquirement of shuttling behavior between the two distantly positioned rewarded corners to obtain water continuously (**Figure 1B**). No apparent differences in the acquisition of the behavioral sequencing tasks were observed between the control and $NaAsO_2$-exposed groups of both sexes (**Figure 1E**, Figure S4). However, a delay in acquiring the behavioral sequencing tasks was observed in both the control and $NaAsO_2$-exposed females. While the discrimination error rate of both the control and $NaAsO_2$-exposed males was significantly decreased to approximately 10% by session 11 (Figure S4), the discrimination error rate of both groups of females was decreased to approximately 15% by session 21 (**Figure 1E**). It indicates that female mice took longer time to be able to adapt the behavioral sequencing task than that taken by male mice regardless of prenatal exposure to $NaAsO_2$.

In the subsequent serial reversal task, the discrimination error rate for the control and $NaAsO_2$-exposed groups of both female and male mice was elevated in the first session of each reversal phase (Rev 1−11; **Figure 1E**, Figure S4), indicating that each group of both male and female mice properly acquired the behavioral sequence assigned in the previous phase. However, in $NaAsO_2$-exposed female mice, the discrimination error rate was significantly higher than that of the control mice in the first session of reversals 1, 4, 8, and 11, and in the fifth session of reversal 1 (**Figure 1E**). These results suggest that $NaAsO_2$-exposed female mice are impaired in the initial adaptation process of reversal learning. Nevertheless, the increased discrimination error rate in the first session of reversals was significantly reduced in subsequent reversal phase sessions of both groups of female mice (**Figure 1E**), demonstrating a day-to-day improvement in the adaptive behavior in female mice. In male mice, tendency of overall increases in discrimination error rate between the control and and $NaAO_2$-exposed groups was observed. Significant increases were observed in the second session of reversals 5 and 11 (Figure S4), whereas significant decrease in discrimination error rate was found in first session of Rev 6. These results suggest that $NaAsO_2$-exposed male mice showed impairment in adaptation to reversals, but the degree of impairment in males might not be severe compared to that in females.

Alteration in the Number of Neurons and Glial Cells in the PrL of $NaAsO_2$-Exposed Mice

To determine whether $NaAsO_2$ exposure-related behavioral alterations in mice are associated with changes in brain histology, the number of Cresyl Fast Violet-stained pyramidal neurons, non-pyramidal neurons, and glial cells were measured in three different layers (layer II−III, V, and VI) of the PrL (**Figures 2B,C**). Stereological analysis revealed that the number of pyramidal neurons in layers V and VI but not in layer II−III of the PrL was significantly ($p < 0.05$) increased in the $NaAsO_2$-exposed group as compared to the control group (**Figure 2D**). No significant differences in the number of non-pyramidal neurons and glial cells were observed between the control and *$NaAsO_2$-exposed* groups in any observed layer of the PrL (**Figures 2E,F**).

Alteration of the Morphology of Neurites in the PrL of $NaAsO_2$-Exposed Mice

We next evaluated the morphology of neurites on Golgi-stained neurons in the PrL. A reduction in the length of neurites on Golgi-stained neurons in the PrL was observed in $NaAsO_2$-exposed mice as compared to neurites in the control group (**Figure 3A**). Space Ball probe analysis indicated that $NaAsO_2$ exposure was associated with a significant ($p < 0.05$) decrease in the length of neurites in Golgi-stained neurons of the PrL as compared to the control group (**Figure 3C**).

The Density and Morphology of Dendritic Spines of Pyramidal Neurons in the PrL of $NaAsO_2$-Exposed Mice

The density and head diameter of dendritic spines from Golgi-stained pyramidal neurons were measured in three different cortical layers (layer II−III, V, and VI) of the PrL (**Figure 4A**). The total density of dendritic spines in pyramidal neurons was not significantly different between control mice and $NaAsO_2$-exposed mice in any observed layer of the PrL (**Figure 4B**). Spine head diameters were also not significantly different between the

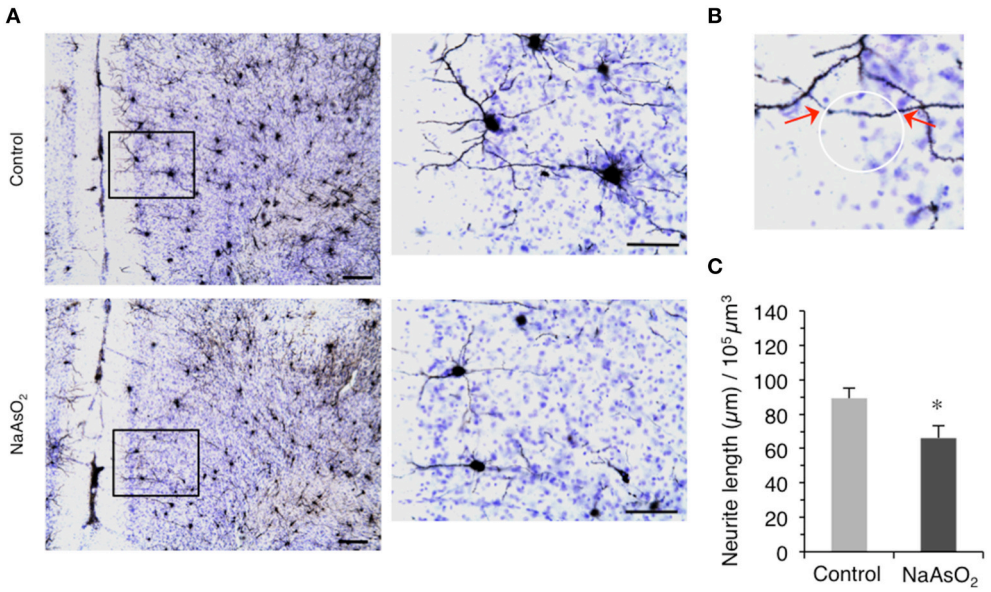

FIGURE 3 | Altered morphology of neurites in the PrL of adult female mice born to dams that were administered $NaAsO_2$ during gestation. (A) Representative photomicrographs of the PrL in Golgi- and Cresyl Fast Violet-stained coronal brain sections from female mice with or without prenatal exposure to $NaAsO_2$. The rectangular area was magnified and shown in the corresponding right panel. Bars = 20 μm (low magnification) and 10 μm (high magnification). **(B)** Demonstrative photomicrograph for the intersection points (indicated by red arrows) between a virtual sphere "Space Ball" (white circle) and Gogli-stained neurite. **(C)** Estimated neurite lengths in Golgi-stained PrL neurons as measured by the Space Ball method. Values (mean ± SEM) were normalized to the estimated volume of the selected PrL region (control group $n = 7$, $NaAsO_2$-exposed group $n = 5$). An asterisk indicates $p < 0.05$ vs. the control group.

control and $NaAsO_2$-exposed groups in any observed layer of the PrL (**Figures 4C–E**).

DISCUSSION

In the present study, we investigated the effect of prenatal $NaAsO_2$ exposure on the behavioral flexibility/reversal learning of adult mice using the IntelliCage system, which is an efficient tool for monitoring multiple aspects of cognitive behavior in a social environment (Endo et al., 2012; Benner et al., 2015). The core finding of our work is that exposure of dams to $NaAsO_2$ produces behavioral inflexibility to reversal learning and abnormal formation of the PrL in adult offspring. These findings suggest that behavioral impairments caused by $NaAsO_2$ exposure are associated with structural changes of brain, particularly in the PrL cortical region.

Here, we provided pregnant female mice with drinking water that contained 85 ppm $NaAsO_2$ during a critical period of embryonic brain development (gestational day 8–18). A series of studies by Colomina et al. evaluated the effect of $NaAsO_2$ exposure on development of nervous system. Exposure to $NaAsO_2$ at 10 mg/kg/day throughout gestational day 15–18 delayed neurodevelopmental indices such as eye opening in female offspring (Colomina et al., 1997). Furthermore, they have shown that single $NaAsO_2$ exposure at 30 mg/kg induces deficit in neuromotor development (Colomina et al., 1996). In accordance with these studies, we considered that providing $NaAsO_2$ about 10 mg/kg to pregnant mice during gestation might induce higher brain function deficits in offspring. It has been reported that mice drink around 5 ml of water daily (Bachmanov et al., 2002). However, our own measurement showed that a 25–35 g pregnant mouse drink about 3.5 ml of water daily when the mouse is provided with $NaAsO_2$-containing water (Figure S5). Based on these estimates, a 25–35 g dam consumes 0.39 mg $NaAsO_2$/day (which is equivalent to 8.5–12 mg/kg/day) when dams are provided with drinking water containing 85 ppm $NaAsO_2$. The selected $NaAsO_2$ dose used in the current study did not produce any obvious maternal toxicity or embryonic toxicity (Figures S1, S2), which is consistent with the previous studies (Rodriguez et al., 2002; Waalkes et al., 2003; Markowski et al., 2012).

Behavioral flexibility describes the ability of an organism to adapt to a changing environment. Behavioral flexibility occurs in many kinds of animal, such as mice, rat, and monkey, and is often assessed using rule-shift learning task paradigms that include a sequencing reversal task. In this study, we assessed the behavioral flexibility of control or $NaAsO_2$-exposed female mice using an altered action-outcome contingency paradigm during inter-sessions, and inter-reversal stages that included a serial reversal task (Endo et al., 2012). In the inter-session analysis, day-to-day improvements in adaptive behavior, as observed in a decreasing trend of discrimination error rate in each reversal phase, were clearly observed not only in control but also arsenite-exposed groups. However, $NaAsO_2$-exposed mice demonstrated a lower degree of achievement in reversal learning than the control group. It may be inferred that the repetition of reversal learning lead to difficultly in re-acquiring reversal learning for the $NaAsO_2$-exposed group. Such effects of arsenic have

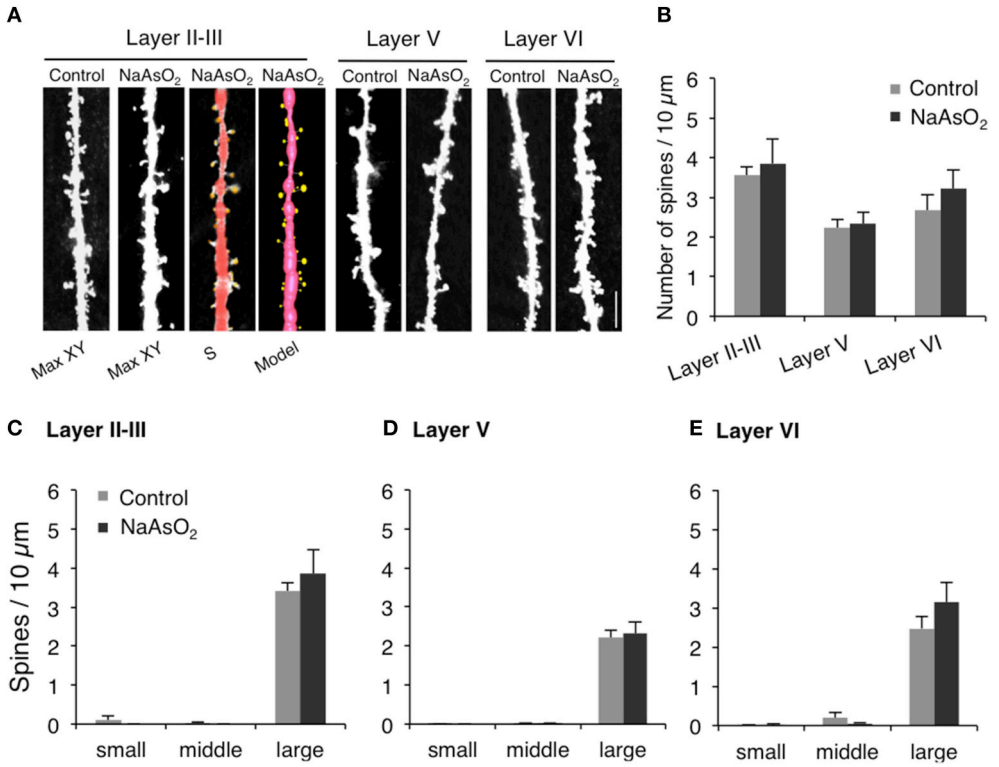

FIGURE 4 | The density and the morphology of dendritic spines in the PrL of adult female mice born from dams exposed to $NaAsO_2$ during gestation. Dendritic spines were analyzed along the primary basilar dendrites of Golgi-stained pyramidal neurons in three different cortical layers (layer II–III, V, and VI) of PrL. **(A)** Representative photomicrographs of dendritic spines with maximal intensity projections onto the XY plane from Z-series images (Max-XY) for the control group (Control) and $NaAsO_2$-exposed group. Photomicrographs analyzed by Spiso-3D (S) and three-dimensional models (Model) are also shown for the analysis of dendritic spines in the layer II–III of the $NaAsO_2$-exposed group. **(B)** Effect of $NaAsO_2$ exposure on the total dendritic spine density of pyramidal neurons in three different cortical layers. Effect of $NaAsO_2$ exposure on the density of three subtypes of dendritic spines, small-head spines (small), middle-head spines (middle), and large-head spines (large), in three cortical layers of the PrL: Layer II–III **(C)**, Layer V **(D)**, and Layer VI **(E)**. The density of dendritic spines is expressed as the number of spines per 10 μm of dendrite. A total 500–1200 spines from 30 to 45 dendritic segments of 30–45 neurons were analyzed for each cortical layer. Values are expressed as mean ± SEM (control group $n = 7$, $NaAsO_2$-exposedgroup $n = 5$).

been reported in adulthood exposure. A series of studies have demonstrated that arsenic exposure in adult mice produces an increase in the number of errors in an egocentric task (Rodriguez et al., 2001, 2002). Our present findings suggest that $NaAsO_2$ exposure in early life also produces behavioral impairments in learning function in mice. The first session of each reversal stage in the serial reversal-learning task tests the ability of mice to adapt to a changing task, because in this first session, mice must alter their behavioral sequence in order to receive a reward. Mice in the control group adapted to new behavioral sequences after a series of reversals, whereas mice in the $NaAsO_2$-exposed group did not adequately adapt to changing tasks (**Figure 1E**) that was likely indicative of behavioral inflexibility.

We also revealed a possible link between behavioral alterations and structural changes in the PrL cortical region. The PrL is known to be involved in the regulation of cognitive and executive processes (Dalley et al., 2004; Marquis et al., 2007; Ragozzino, 2007). It has been demonstrated that the PrL plays a fundamental role in behavioral flexibility. For example, patients who have the frontal lobe (including PrL) damage show impaired adaptation to changes in reinforcement contingencies in spite of the fact that these patients can acquire novel skills or adopt new rules with relative ease (Owen et al., 1993). It has also demonstrated that either lesion or inactivation of the PrL impairs behavioral flexibility in rodents (Ragozzino et al., 1999). Accordingly, structural changes in the PrL can contribute to the impairment of behavioral flexibility. $NaAsO_2$ is known to produce neurotoxicity by inducing apoptotic cell death (Wong et al., 2005; Keim et al., 2012) and/or cellular necrosis (Chattopadhyay et al., 2002; Yang et al., 2003). Therefore, in the present study, we measured the number of neurons and glial cells in the PrL in order to determine whether cell viability was affected by prenatal $NaAsO_2$ exposure. Contrary to our expectations, these morphometrical analyses revealed that $NaAsO_2$ exposure increased the number of pyramidal neurons in layers V and VI of the PrL (**Figure 2D**). Our previous *in vitro* work showed that a high concentration of $NaAsO_2$ (2 μM) reduced the viability of mouse primary cortical neurons, but that a low concentration of $NaAsO_2$ (0.5 μM) conversely increased cell viability and promoted cellular proliferation (Maekawa et al., 2013). It suggests

that the concentration of $NaAsO_2$ (85 ppm) used in the present study reflect the low concentration of $NaAsO_2$ exposure resulting in the increase in the number of pyramidal neurons in the present. On the other hand, the number of non-pyramidal neurons and glial cells was not affected by $NaAsO_2$ exposure in the present study. The difference between the effect of $NaAsO_2$ exposure on pyramidal and non-pyramidal cells (**Figures 2D,E**) could be due to differences in the timing of neurogenesis and neuronal migration. The majority of cerebral cortical neurons are generated during embryonic day 11–17 in mouse (Price and Lotto, 1996; Price et al., 1997), whereas neurogenesis for each cortical layer is not simultaneous and occurs with variable timing (Finlay and Darlington, 1995). Additionally, pyramidal neurons are generated from the ventricular zone and migrate through the cortical layers radially, while interneurons including non-pyramidal neurons are generated from the ganglionic eminence and migrate tangentially (Nadarajah et al., 2003). Therefore, the timing of generation and migration of pyramidal neurons is different from that of non-pyramidal neurons, and these differences may reflect the layer-specific and cell type-specific effects of $NaAsO_2$ on the number of neurons in the PrL observed in the present study. Several studies have already shown that chemical exposures affect neuronal migration by disrupting the inside-out pattern of migration (Kakita et al., 2002; Schreiber et al., 2010). Taken together, the generation and migration of neurons may be at least partially affected by prenatal $NaAsO_2$ exposure, although the mechanisms by which $NaAsO_2$ exposure specifically increases the number of pyramidal neurons in a layer-dependent manner has not yet been identified. Regarding glial cells, morphological or functional changes have been shown to occur at higher $NaAsO_2$ concentrations than those, which affect the morphology of neurons (Wang et al., 2012). Therefore, the observed lack of effect of $NaAsO_2$ exposure on the number of glial cells in this study was expected, and may be due to an insufficient level of prenatal $NaAsO_2$ exposure.

Another critical finding of our study is that behavioral inflexibility is clearly associated with structural changes in PrL neurons. We previously demonstrated that $NaAsO_2$ disrupts neuritogenesis in primary cultured neurons (Maekawa et al., 2013) and neuronal cell lines (Aung et al., 2013), and that inhibition of neuritogenesis by $NaAsO_2$ is caused by alterations in the expression of cytoskeletal genes, tau, tubulin, and neurofilament (Aung et al., 2013), and suppression of glutamate AMPA receptor expression (Maekawa et al., 2013). Thus, inorganic arsenic adversely affects the fate and maturation processes of young neurons, which may lead to abnormal formation of neural circuits. In the present study, we found that the length of neurites in the PrL was significantly lower in the $NaAsO_2$-exposed group, suggesting that prenatal exposure to $NaAsO_2$ has an adverse effect on neuritogenesis. Elongation of the axon and dendrites is an essential event for the formation of basic neuronal circuitry. Impairments in the length and morphology of dendrites in the frontal cortex are involved in the pathogenesis of cognitive deficits and mental retardation (Armstrong et al., 1998). It indicates that the alteration in the morphology of neuron, particularly the PrL neuron, is strongly associated with the pathophysiological states of cognitive and learning dysfunction and that prenatal exposure to $NaAsO_2$ may contribute to the pathogenesis. Additionally, it has been reported that the degree of learning disability is positively correlated with the severity and extent of dendritic abnormalities (Kaufmann and Moser, 2000). Therefore, we subsequently examined the density and morphology of pyramidal neuron dendritic spines in different layers of the PrL. In contrast to the impairment in neurite length, the morphology and the density of dendritic spines in PrL pyramidal neurons were not affected by $NaAsO_2$ exposure. We recently demonstrated in cultured neurons that $NaAsO_2$ specifically alters the gene expression of cytoskeletal proteins including tau, tubulin, and neurofilaments, but does not affect the expression of actin protein (Aung et al., 2013). Since dendritic spines are actin-rich protrusions from dendrites that form the post-synaptic component of a synapse (Hotulainen and Hoogenraad, 2010), our current finding that $NaAsO_2$ exposure did not have an effect on synapse number agrees with our previous study regarding the expression of actin protein. $NaAsO_2$ exposure has however been reported to impair the expression of AMPA and NMDA glutamate receptors (Maekawa et al., 2013; Ramos-Chavez et al., 2015), suggesting that $NaAsO_2$ exposure can affect glutamate transmission. Because glutamate transmission is critically involved in the regulation of synapse formation (Rasse et al., 2005), we cannot exclude the possibility that the exposure to $NaAsO_2$ alters synapse formation in areas other than the PrL. Taken together, the present study highlights the possible association between behavioral impairment in mice caused by prenatal $NaAsO_2$ exposure and morphological alteration of brain, particularly cortical disarrangement in the prelimbic cortex.

On the other hand, the suggested association between the behavioral inflexibility and morphological alteration of the PrL was come from the morphometrical analysis, which was however carried out following the behavioral flexibility test. It has been demonstrated in human subjects that goal-directed learning is strongly associated with increase neural activity in prefrontal cortex (Valentin et al., 2007) and higher neurite density in medial orbitofrontal cortex (Morris et al., 2016). Since the control mice performed better than $NaAsO_2$-exposed mice in this study of behavioral flexibility tasks, we could not deny the possibility of increase neurite length in the PrL of the control mice, which might be outcome of better goal-directed learning in behavioral flexibility tasks. In addition, it is important to note that the reduced maternal water consumption was observed in the group of dams provided with water containing $NaAsO_2$, and the difference between the two groups was about 2 ml per day (Figure S5). It might be due to unpalatability of dams to water containing $NaAsO_2$. Although we did not observe the obvious signs of maternal or embryonic toxicity such as maternal weight (Figure S1) and the number of pups (Figure S2) between the two groups of this study, several studies reported the possibility that maternal dehydration due to reduced water intake during pregnancy was associated with long-term physiologic effects on offspring such as development of brain function and plasma composition (Desai et al., 2005; Ross et al., 2005; Zhang et al., 2011). Therefore, we had to assume that behavioral inflexibility observed in mice prenatally

exposed $NaAsO_2$ could be induced by the combinatorial effect of the toxicity of prenatal $NaAsO_2$ exposure and maternal dehydration.

In this study, we used male and female mice at 67 week (15.5 month) of their age in this study of behavioral flexibility tests, which additionally lasted for 10–12 weeks (about 2–3 month). Therefore, the age of mice in the last day of behavioral tests was being 17.5–18.5 month, which could be generally considered as old aged mice. It has been demonstrated that arsenic-induced increase in oxidative stress (such as glutathione level in the blood) was more prominent in young and old rats compared to adults (Jain et al., 2011, 2012). Motor impairments caused by prenatal arsenic exposure were observed in young juvenile mice, but such effects observed in young mice were subsided with advancing age (Markowski et al., 2012). These studies indicate age-dependent effects of arsenic-induced toxicity. Therefore, although we observed $NaAsO_2$-induced behavioral inflexibility in old aged mice in the present study, we need further studies to test the age-dependent effects of prenatal $NaAsO_2$ exposure on behavioral flexibility.

In conclusion, we demonstrate the possibility that *in utero* $NaAsO_2$ exposure leads to behavioral inflexibility to changing tasks in adulthood, and cortical disarrangement in the PrL might contribute to this behavioral impairment. Further studies are required to elucidate how $NaAsO_2$ disrupts neuronal development including axonal and dendritic elongation particularly in prefrontal cortex. Since behavioral inflexibility is observed in children with neurodevelopmental disorders such as autism spectrum disorders, our findings put forth a new perspective on how environmental exposures affect the pathogenesis of neurodevelopmental disorders.

AUTHOR CONTRIBUTIONS

KA designed and performed experiments, analyzed data and wrote the paper; CT, KS performed experiments and analyzed data; KaN, AT, KeN, MK, and CT edited the paper; ST designed experiments and edited the paper; and FM designed experiments, analyzed data and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2016.00137

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Arsenic Exposure Induces Unscheduled Mitotic S Phase Entry Coupled with Cell Death in Mouse Cortical Astrocytes

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There is serious concern about arsenic in the natural environment, which exhibits neurotoxicity and increases the risk of neurodevelopmental disorders. Adverse effects of arsenic have been demonstrated in neurons, but it is not fully understood how arsenic affects other cell types in the brain. In the current study, we examined whether sodium arsenite ($NaAsO_2$) affects the cell cycle, viability, and apoptosis of *in vitro*-cultured astrocytes isolated from the cerebral cortex of mice. Cultured astrocytes from transgenic mice expressing fluorescent ubiquitination-based cell cycle indicator (Fucci) were subjected to live imaging analysis to assess the effects of $NaAsO_2$ (0, 1, 2, and 4 μM) on the cell cycle and number of cells. Fucci was designed to express monomeric Kusabira Orange2 (mKO2) fused with the ubiquitylation domain of hCdt1, a marker of G1 phase, and monomeric Azami Green (mAG) fused with the ubiquitylation domain of hGem, a marker of S, G2, and M phases. $NaAsO_2$ concentration-dependently decreased the peak levels of the mAG/mKO2 emission ratio when the ratio had reached a peak in astrocytes without $NaAsO_2$ exposure, which was due to attenuating the increase in the mAG-expressing cell number. In contrast, the mAG/mKO2 emission ratio and number of mAG-expressing cells were concentration-dependently increased by $NaAsO_2$ before their peak levels, indicating unscheduled S phase entry. We further examined the fate of cells forced to enter S phase by $NaAsO_2$. We found that most of these cells died up to the end of live imaging. In addition, quantification of the copy number of the glial fibrillary acidic protein gene expressed specifically in astrocytes revealed a concentration-dependent decrease caused by $NaAsO_2$. However, $NaAsO_2$ did not increase the amount of nucleosomes generated from DNA fragmentation and failed to alter the gene expression of molecules relevant to unscheduled S phase entry-coupled apoptosis (p21, p53, E2F1, E2F4, and Gm36566). These findings suggest that $NaAsO_2$ adversely affects the cell cycle and viability of astrocytes by inducing unscheduled S phase entry coupled with cell death that may be caused by mechanisms other than apoptosis.

Keywords: astrocytes, sodium arsenite, cell cycle, cell death, live imaging

INTRODUCTION

Several environmental chemicals are suspected to exert deleterious effects on development of the brain, which may result in an increased risk of neurodevelopmental disorders such as autism, attention-deficit hyperactivity disorder, and cerebral palsy (Grandjean and Landrigan, 2006, 2014). Arsenic is one of these environmental toxicants that disrupt brain development. According to a long-term prospective study of survivors of arsenic poisoning from the Morinaga milk incident in Japan, arsenic poisoning during infancy leads to a risk of mortality from neurological diseases in adulthood (Tanaka et al., 2010). Epidemiological studies suggest that chronic consumption of arsenic-contaminated water causes a reduction in the cognitive performance of school-age children (Calderon et al., 2001; Tsai et al., 2003; Wasserman et al., 2007). Thus, arsenic exposure via drinking water may be a risk factor for neurodevelopmental disorders.

Animal studies support the notion that developmental exposure to arsenic increases the risk of neurodevelopmental disorders. Exposure to sodium arsenite ($NaAsO_2$), an inorganic arsenical compound, via drinking water during gestational and/or postnatal periods causes impairment of spatial learning and memory, neuromotor reflex alteration, and spontaneous locomotor deficits in adult rats (Rodriguez et al., 2002; Xi et al., 2009). Offspring of female mice, which had been chronically exposed to $NaAsO_2$ via drinking water, display depression-like behavior (Martinez et al., 2008), and exhibit deficits in a hippocampus-dependent learning tasks (Martinez-Finley et al., 2009) during adulthood. Thus, $NaAsO_2$ exposure during developmental periods and the associated adverse effects on brain development induce behavioral abnormalities, although the toxic mechanisms of arsenic remain to be elucidated.

In vitro studies of neurons have revealed that $NaAsO_2$ induces apoptotic cell death in primary cultured neurons (Namgung and Xia, 2001; Wong et al., 2005) and neuronal cell lines (Koike-Kuroda et al., 2010; Keim et al., 2012). In addition, $NaAsO_2$ disrupts neuritogenesis in primary cultured neurons (Maekawa et al., 2013) and neuronal cell lines (Frankel et al., 2009; Aung et al., 2013). We previously reported that $NaAsO_2$-induced apoptosis is mediated by activation of caspase-3 (Koike-Kuroda et al., 2010), and that inhibition of neuritogenesis by $NaAsO_2$ is caused by alterations in the expression of cytoskeletal genes tau, tubulin, and neurofilament (Aung et al., 2013), and suppression of glutamate AMPA receptor expression (Maekawa et al., 2013). The toxic mechanisms by which developmental exposure to $NaAsO_2$ impairs the aforementioned brain functions and behaviors remain to be uncovered. However, based on *in vitro* studies of neurons, inorganic arsenic adversely affects the fate and maturation processes of young neurons, which may lead to abnormal formation of the neural circuits responsible for the brain functions and behaviors.

In addition to neurons, there may be other target cells of arsenic in the developing brain. Astrocytes are the largest population of glial cells, which are more abundant in the brain compared with neurons, and contribute to the formation and maintenance of the blood–brain barrier (BBB). The BBB is composed of endothelial cells, which line capillary blood vessels and connect to each other via tight junctions, and astrocytes surrounding blood capillaries via their end feet (Abbott, 2002). The BBB is not considered as a perfect barrier, although it contributes to protection of the brain against circulating xenobiotics that disrupt brain functions. The developing brain is considered to be vulnerable to toxic chemicals compared with the adult brain. One of the reasons is that the immature BBB during early development provides only partial protection against entry of chemicals into the brain (Zheng et al., 2003). Arsenite and arsenate are transferred to offspring through the placenta of pregnant mice that are exposed via drinking water, and arsenic species easily crossing the immature BBB accumulate in the brains of newborn offspring (Jin et al., 2006). Astrocytes are therefore the first brain cells that appear to be targeted by inorganic arsenic when it is transferred from the blood to the brain. Arsenite inhibits glutamate metabolism in astrocytes by reducing the activity and expression of glutamine synthase and glutamate transporters (Zhao et al., 2012). Synapse formation of primary cultured neurons is impaired by culture in conditioned medium from arsenite-exposed astrocytes (Wang et al., 2013). Taken together, the neurotoxicity of inorganic arsenic may be, at least in part, caused by its effects on astrocytes.

During brain development, neuron generation occurs first, followed by the generation of glial cells. In the cerebral cortex of rodents, astrocyte generation begins on embryonic day 18 following neurogenesis during embryonic days 12–18, and the number of astrocytes peaks in the neonatal period (Miller and Gauthier, 2007). It is assumed that neurotoxicant exposure during the developmental period affects not only neurogenesis but also the generation and proliferation of astrocytes, followed by altering the cell numbers. A reduced number of cortical glial cells is related to the pathological changes of schizophrenia and depression, indicating a causal link between glial cell abnormalities and psychiatric disorders (Cotter et al., 2001). In primary cultured rat astrocytes, inorganic arsenic decreases cell viability and increases DNA damage (Catanzaro et al., 2010). Such toxic effects of arsenite are stronger than those of arsenate (Jin et al., 2004). However, the mechanisms by which inorganic arsenic reduces the viability of astrocytes are largely unknown. Fluorescent ubiquitination-based cell cycle indicator (Fucci), which consists of monomeric Kusabira Orange2 (mKO2) fused with the ubiquitylation domain of human Cdt1 to monitor G1 phase and monomeric Azami Green (mAG) fused with the ubiquitylation domain of human Geminin to monitor S/G2/M phases, is useful to visualize the dynamics of cell cycle progression (Niwa et al., 1991; Sakaue-Sawano et al., 2008). In this study, we carried out live imaging analysis of primary cultured astrocytes originating from the cerebral cortex of Fucci transgenic (tg) mice to determine whether $NaAsO_2$ exposure decreases cell viability by affecting the cell cycle. Additionally, we examined the effects of $NaAsO_2$ exposure on the viability, apoptotic cell death, and expression of genes related to the cell cycle and apoptosis in cultured cortical astrocytes.

MATERIALS AND METHODS

Animals

Fucci tg mice were bred and maintained at the National Institute for Environmental Studies (Tsukuba, Japan). Wild-type C57BL/6J mice (Sankyo Labo Service, Tokyo, Japan) were bred at Saitama University (Saitama, Japan). They were housed under a controlled temperature (23 ± 2°C) and photoperiod (12:12, light:dark) with free access of tap water and standard chow. Animal procedures were conducted according to the approval and guidelines of Animal Care and Use Committee at the National Institute of Environmental Studies and Saitama University.

In vitro Culture of Astrocytes

Fucci tg and C57BL/6J mice were sacrificed on postnatal days 1 or 2 (postnatal day 1 = day of birth). After isolation of the cerebral cortex from the brain, the meninges were removed and the cortex tissue were placed in ice-cold Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 3.7 mg/L sodium bicarbonate (Sigma-Aldrich), 10 mL/L antibiotic antifungal solution (Sigma-Aldrich), which contained 10,000 units penicillin, 10 mg streptomycin, and 25 μg amphotericin B per mL, and 10% fetal bovine serum (FBS; Gibco-Invitrogen, Carlsbad, CA, USA), which is hereafter referred to as culture medium (pH 7.2). Cells were isolated from the cerebral cortex in fresh culture medium by gentle mechanical trituration, seeded on poly-L-ornithine (15 μg/L)-precoated culture dishes or plates, and maintained in culture medium at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was changed at 3 days after seeding, followed by medium changes at intervals of 3–4 days.

Cells obtained from Fucci tg mice were seeded on a four-compartment glass bottom dish (Greiner Bio-One, Kremsmünster, Austria) at 1×10^5 cells per compartment (1.9 cm^2), cultured for more than 1 week, and then applied to fluorescence microscopy (see Section Fluorescence Microscopy of Fucci-Expressing Astrocytes) and live imaging analyses (see Section Time-Lapse Analysis of Fucci-Expressing Astrocytes). Cells obtained from C57BL/6J mice were seeded on a 6-well culture plate (Asahi Glass, Tokyo, Japan) at 3×10^5 cells per well (9.4 cm^2) and cultured until 90–100% confluence. The cells were re-seeded onto new culture plates at 1×10^5 cells per well after trypsinization and then used to analyze the protein expression of glial fibrillary acidic protein (GFAP), a marker of astrocytes (see Section Analysis of GFAP Immunoreactivity), and the GFAP gene copy number (see Section Analysis of GFAP Gene Copy Numbers). For gene expression analysis (see Section Analysis of mRNA Levels), the cells were re-seeded at 4×10^5 cells per well after trypsinization. For apoptosis analysis, the cells were re-seeded on a 96-well culture plate (Asahi glass) at 5×10^3 cells per well (see Section Apoptosis Assay). All culture dishes and plates were precoated with poly-L-ornithine (15 μg/L) before use.

Analysis of GFAP Immunoreactivity

To check the purity of cells in primary culture, we performed immunocytochemistry of GFAP. Cultured cells fixed with 4% paraformaldehyde were reacted with a polyclonal rabbit anti-GFAP antibody (1:500; Dako, Glostrup, Denmark) at 4°C overnight and then Alexa Fluor 647 goat anti-rabbit IgG (1:400; Life Technology, Carlsbad, CA, USA) for 30 min at room temperature. 4,6-Diamidino-2-phenylindole (DAPI) staining was performed to count the total cell number.

Fluorescence Microscopy of Fucci-Expressing Astrocytes

To determine the cell cycle duration of astrocytes, we observed astrocytes from Fucci tg mice under a fluorescence microscope (BioZero 8100; Keyence, Osaka, Japan) equipped with an mKO2 filter (excitation filter: 542AF15; emission filter: 585QM30; dichroic mirror: 560 DRLP; Opto science, Tokyo, Japan) and a mAG filter (excitation filter: 475QM20; emission filter: 518QM32; dichroic mirror: 500DRLP; Opto science). First, the cell cycle of each astrocyte was synchronized by serum starvation. Culturing in medium supplemented with a low concentration of FBS (0.5%) for 72 h is effective to increase the population of cells at G1 phase (Khammanit et al., 2008). Therefore, 30–40% confluent astrocytes were incubated in culture medium supplemented with 0.1% FBS for 3 days. The astrocytes were again incubated in culture medium containing 10% FBS, and fluorescence images of mKO2 and mAG were captured every 6 h for 150 h using an objective lens (Plan Fluor ELWD DM 20 × C, NA 0.45; Nikon, Tokyo, Japan) and a CCD camera in the BioZero 8100 fluorescence microscope. In each culture, digital images were obtained in three regions (0.58 mm^2/region, 1.74 mm^2 in total) that were randomly selected in the culture dish. Fluorescence microscopy was performed in four primary cultures derived from different animals.

Image analysis of mKO2 and mAG expression was performed with BZ-II analyzer software (Keyence). The digital images were modified to remove the background signal, and the red-green-blue (RGB) digital images of mKO2 and mAG were converted to monochromatic color images (red, mKO2; green, mAG). The monochromatic color images of mKO2 and mAG were merged at each time point to determine the intensities of mKO2 and mAG expression in the same region, which were obtained by measuring the brightness of the red and green signals, respectively. After measuring the brightness values of red and green signals in the same region of each merged image, the mAG/mKO2 emission ratio was calculated by dividing the brightness value of the green signal by that of the red signal. The mAG/mKO2 emission ratio at each time point was calibrated using the ratio of the same area at 0 h after the end of serum starvation, which was set at 100.

Time-Lapse Analysis of Fucci-Expressing Astrocytes

Exposure to $NaAsO_2$ and Live Imaging

$NaAsO_2$ (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in sterile-filtered water (Sigma-Aldrich) at a concentration of 100 mM. The $NaAsO_2$ solution (100 mM) was further diluted with culture medium to obtain the indicated concentrations. Primary cultured Fucci-expressing astrocytes were subjected to serum starvation (see Section Fluorescence Microscopy of Fucci-Expressing Astrocytes) to synchronize the

cell cycle. After synchronization, the cells were exposed to $NaAsO_2$ in culture medium at concentrations of 0, 1, 2, or 4 μM. Immediately after starting $NaAsO_2$ exposure, astrocytes were placed in an incubation chamber (Tokai Hit, Shizuoka, Japan) equipped to the BioZero 8100 fluorescence microscope. In the incubation chamber, the temperature was controlled at 37°C and the CO_2 concentration was maintained at 5%. Time-lapse fluorescence imaging began at 1 h and ended at 73 h after initiation of $NaAsO_2$ exposure. Fluorescence images of mKO2 and mAG expression, and bright field images were captured every 2 h. The digital image data were obtained from three regions (0.58 mm^2/region, 1.74 mm^2 in total) that were randomly selected in each culture dish compartment. Live imaging of Fucci-expressing astrocytes was performed in six primary cultures derived from different animals.

Image Analysis

We analyzed the intensity of mKO2 and mAG signal emissions, the number of cells expressing mKO2 and mAG, and the cell fate after S phase entry using the digital image data from live imaging. Image analyses were performed using the BZ-II analyzer software. The images were modified to remove the background signal and change the RGB colors of mKO2 and mAG to monochromatic colors. The modified images were used for analysis as described below.

The monochromatic color images of mKO2 (red) and mAG (green) were merged at each time point to measure the intensities of mKO2 and mAG in the same region, which were obtained by measuring the brightness of red and green signals, respectively. The mAG/mKO2 emission ratio was then calculated by dividing the brightness value of the green signal by that of the red signal. The mAG/mKO2 emission ratio at each time point was calibrated using the ratio of the same area at 5 h after $NaAsO_2$ exposure, which was set at 100.

To count cells expressing mKO2 and mAG, we used the monochromatic color images for each fluorescent protein, which were obtained every 8 h from 1 h after initiation of $NaAsO_2$ exposure. After counting the number of mKO2- and mAG-expressing cells, the value at each time point was then calibrated using the number of cells in the same area at 1 h after $NaAsO_2$ exposure, which was set at 100.

To analyze cell fate after S phase entry, the digital images of mKO2, mAG, and bright field, which were obtained from astrocytes with or without exposure to 4 μM $NaAsO_2$, were merged at each time point. The merged images at all-time points were then saved as a movie file to observe temporal changes in the expression of mKO2 and mAG, and the morphology of target cells. Target cells were astrocytes that expressed mAG at 41 h in the control group and at 9 h in the $NaAsO_2$-exposed group after initiation of $NaAsO_2$ exposure, because the population of mAG-expressing cells at these time points was the largest during live imaging for each group. These cells were traced until the end of live imaging to determine their viability. Live cells were defined as cells that expressed mAG followed by expression of mKO2 with normal morphology. Dead cells were defined as cells that expressed mAG followed by loss of fluorescent signals with abnormal morphology. A total of 434 cells in the control group and 426 cells in the $NaAsO_2$-exposed group were followed up to determine their cell fate. For each group, the total cell number was defined as 100%, and the percentages of live and dead cell populations were calculated.

Analysis of GFAP Gene Copy Numbers

Exposure to $NaAsO_2$ and DNA Extraction

Astrocytes originating from the cerebral cortex of C57BL/6J mice were incubated in culture medium containing $NaAsO_2$ at concentrations of 0, 1, 2, or 4 μM for 73 h. The astrocytes were then rinsed in Dulbecco's phosphate-buffered saline without calcium and magnesium, and collected in microcentrifuge tubes by scraping the culture plates filled with the buffer. After centrifugation (10,000 rpm, 10 min), the resulting cell pellet was subjected to extraction of total DNA with a DNeasy blood and tissue kit (Qiagen, Hilden, Germany). DNA samples were obtained from eight independent primary cultures derived from different animals.

Real-Time PCR

Real-time PCR was performed using a LightCycler 96 (Roche Diagnostics, Mannheim, Germany). To prepare standard samples for the GFAP gene, a partial fragment of the mouse GFAP gene (430 bp from 13,801 to 14,230 nt) was cloned by insertion of the DNA fragment into the pCR2.1-TOPO vector supplied in a TOPO TA cloning kit (Invitrogen). The plasmid vector containing a copy of the GFAP gene was serially diluted with nuclease-free water at 3.2×10^4, 8.0×10^3, 2.0×10^3, 5.0×10^2, and 1.25×10^2 copies per 2 μl. Two microliters of the standards and unknown samples containing total DNA extracted from the cultured cells were amplified in a 20 μl reaction mixture containing 200 nM of each primer (forward: 5′-TCCTTTCCACCTCCGCTAAC-3′; reverse: 5′-GTTGGGTCTTGCCTGTCTTC-3′) and 10 μl of 2× SYBR Premix Ex Taq (Takara Bio, Otsu, Japan). Real-time PCR conditions were initial activation of Taq polymerase for 30 s at 95°C, followed by 40 cycles of 5 s at 95°C for denaturation, and then 30 s at 60°C for annealing and extension with a temperature transition rate of 20°C/s. After real-time PCR, melting curve analysis was carried out to demonstrate the specificity of the PCR product (estimated amplicon size: 138 bp), resulting in a melting curve with a single peak (data not shown). After measurement of the GFAP gene copy number in each sample, the values were expressed as a percentage of the value obtained from cells without $NaAsO_2$ exposure, whose viability was set at 100%.

Apoptosis Assay

Primary cultured astrocytes originating from the cerebral cortex of C57BL/6J mice were subjected to serum starvation (see Section Fluorescence Microscopy of Fucci-Expressing Astrocytes) to synchronize the cell cycle and then incubated in culture medium containing $NaAsO_2$ at concentrations of 0, 2, or 4 μM for 72 h. The effects of $NaAsO_2$ on DNA fragmentation was examined using a Cell Death Detection ELISA Plus Assay kit (Roche Diagnosis) in accordance with the manufacturer's protocol. This assay was performed using six independent primary cultures derived from different animals. The amount of nucleosomes

generated from DNA fragmentation is expressed as relative to that in the controls, which was set at 100% for each culture derived from the same animal.

Analysis of mRNA Levels

Exposure to $NaAsO_2$, RNA Extraction, and Reverse Transcription

Primarily cultured astrocytes were subjected to serum starvation (see Section Fluorescence Microscopy of Fucci-Expressing Astrocytes) to synchronize the cell cycle and then exposed to 0 or 4 μM $NaAsO_2$ in culture medium for 9 and 41 h. After exposure, total RNA was extracted and purified with an RNeasy Mini kit (Qiagen) in accordance with the manufacturer's protocol. For each sample, total RNA (650 ng) was reverse transcribed into cDNA in a final reaction volume of 20 μl using a PrimeScript RT Reagent kit (Takara Bio) in accordance with the manufacturer's protocol.

Real-Time PCR

To measure the gene expression levels of molecules involved in unscheduled S phase entry and apoptosis, p21, p53, E2F1, E2F4, and Gm36566, real-time PCR was performed using a LightCycler 96 (Roche Diagnostics). Equal amounts of cDNA from each sample were combined together and serially diluted with EASY dilution (Takara Bio) to prepare standard samples for each gene. One microliter of standards and diluted unknown samples were amplified in a 10 μl reaction mixture containing 5 μl of 2× SYBR Premix Ex Taq (Takara Bio) and 100 nM of each primer for specific genes (see **Table 1**). The real-time PCR settings were the same as those described in Section Real-Time PCR. The amounts of the mRNAs for target genes in each sample were normalized to the mRNA level of the housekeeping gene cyclophilin B (CPB) in the same sample. The normalized mRNA level of each target gene was expressed relative to that in the controls, which was set at 100%. The percentage values were averaged from five different primary cultures.

Statistical Analysis

Two-way factorial analysis of variance (ANOVA) for repeated measures was used to examine the effects of $NaAsO_2$ and time on the mAG/mKO2 emission ratio. One-way ANOVA was performed to determine the difference among groups with respect to the number of cells expressing mAG and mKO2, the copy number of the GFAP gene, and the amount of nucleosomes generated from DNA fragmentation. When significant overall effects were detected by one-way ANOVA, the Tukey–Kramer test was used for *post-hoc* analysis. Two-way ANOVA was performed to determine the effects of concentrations and exposure time of $NaAsO_2$ on the mRNA levels of target genes. Differences in the populations of live and dead cells between groups with or without exposure to $NaAsO_2$ were analyzed by the nonparametric Mann–Whitney *U*-test.

TABLE 1 | Primer sequences used for real-time PCR.

	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	NCBI reference sequence
p21	TAGCTCCTTCCCTGGGATTC	ATAGCAAAGGGGCAGAAAAG	AF035683
p53	GCTTCTCCGAAGACTGGATG	GTCCATGCAGTGAGGTGATG	AB020317
Gm36566	TCCCATTCCCCTATCTGTGT	GCTATTCTCTGCTCCGATCC	XM_011247417
E2F1	ACTGTGACTTTGGGGACCTG	CAGAGGGTATGGATCGTGCT	L21973
E2F4	CTGGCACTTGTGACTGTGCT	AGCACCACCCTCTCTCTGAA	NM_148952
CPB	AGACTGTTCCAAAAACAGTGGA	GATGCTCTTTCCTCCTGTGC	M60456

RESULTS

Immunoreactivity of GFAP in Cultured Cells

Microscopy and counting DAPI-stained cells with or without GFAP immunoreactivity showed that most DAPI-stained cells exhibited GFAP-immunoreactive signals (98 ± 0.4% in four primary cultures derived from different animals; **Figure 1**). Thus, cells that were isolated from the mouse brains and cultured *in vitro* were astrocytes expressing GFAP.

Cell Cycle Distribution of Fucci-Expressing Astrocytes

Most primary cultured astrocytes from the cerebral cortex of Fucci tg mice expressed mKO2 after their cell cycles were synchronized by serum starvation (**Figure 2A**). mAG-expressing astrocytes emerged at 12 h after synchronization of the cell cycle. Forty-two hours after synchronization was the first time that expression of mAG reached a peak. mAG expression then decreased and the second peak of mAG expression occurred at 66 h after synchronization. This cycle of mAG expression was repeated during fluorescence microscopic observation.

The emission ratio of mAG to mKO2 in Fucci-expressing astrocytes changed over time (**Figure 2B**). The mAG/mKO2 emission ratio was low until 12–18 h after synchronization of the

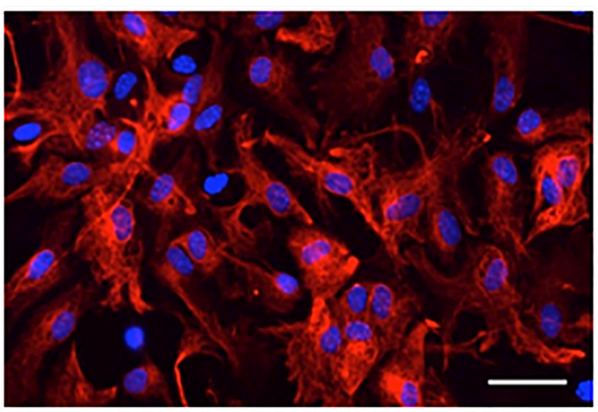

FIGURE 1 | Digital photomicrograph of primary cultured astrocytes. Astrocytes were immunostained for GFAP (red) and counterstained with DAPI (blue). Scale bar: 50 μm.

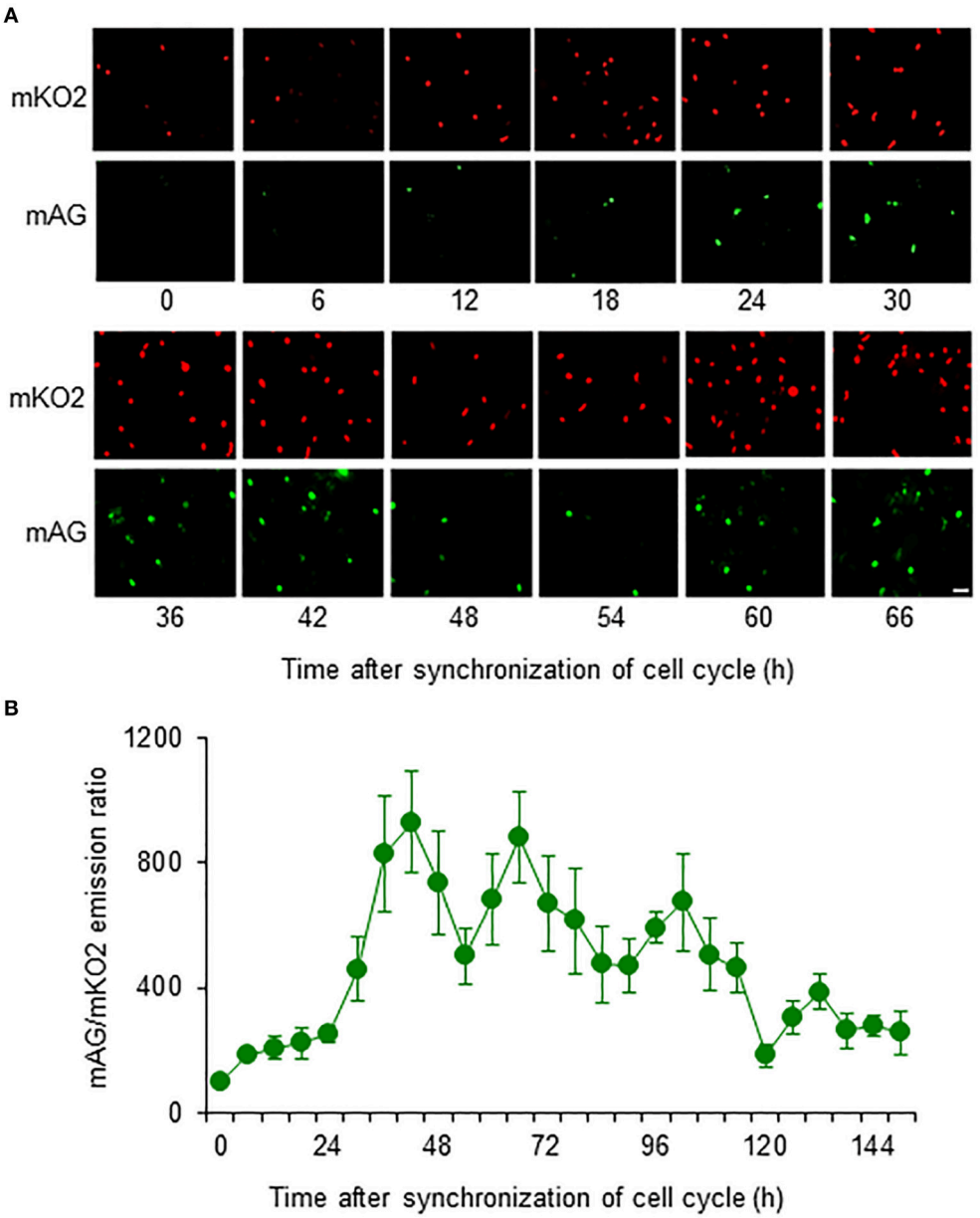

FIGURE 2 | Temporal changes in mAG and mKO2 emission signals of Fucci-expressing astrocytes. After astrocytes were serum starved for 72 h to synchronize the cell cycle, they were cultured in medium containing 10% FBS and applied to cell cycle monitoring. **(A)** Time-lapse images of mAG and mKO2 emission signals after the original fluorescent colors were converted to pseudocolors (mAG, green; mKO2, red). Scale bar: 50 μm. **(B)** Temporal changes in the mAG/mKO2 emission ratio of Fucci-expressing astrocytes. All data points are the means ± standard error of the mean (SEM) of four primary cultures derived from different animals. The mAG/mKO2 emission ratio at each time point was calibrated using the ratio at 0 h after the cells were cultured in 10% FBS-containing medium, which was set at 100.

cell cycle. The ratio dramatically increased from 24 to 42 h after synchronization of the cell cycle and then decreased until 54 h after synchronization. The ratio increased again and reached a peak at 66 h after synchronization of the cell cycle in four primary cultures derived from different animals. The cyclic changes in the mAG/mKO2 emission ratio were observed four times during the analysis period. The mean interpeak interval of the mAG/mKO2 emission ratio for each culture was 30, 28, 32, and 30 h. When these values were represented as the cell cycle duration in each culture, the mean of four different experiments was 30 ± 0.82 h, indicating that the cell cycle duration of Fucci-expressing astrocytes is ∼30 h.

Effects of $NaAsO_2$ on the Cell Cycle of Astrocytes

Fucci-expressing astrocytes mostly emitted mKO2 fluorescence signals at the beginning of live imaging with or without $NaAsO_2$ exposure, while little emission of mAG signals was

observed (**Figure 3**; Supplementary Movies 1–4). In control and $NaAsO_2$ (1 and 2 μM)-exposed groups, astrocytes emitting mAG fluorescence signals were frequently observed between 33 and 49 h after initiation of $NaAsO_2$ exposure, which then decreased over time. On the other hand, in astrocytes exposed to 4 μM $NaAsO_2$, mAG fluorescence signals were found at 5–9 h after $NaAsO_2$ exposure. In some astrocytes exposed to 4 μM $NaAsO_2$, the fluorescent emissions of mAG and mKO2 had disappeared after 31 h of exposure to $NaAsO_2$.

Two-way ANOVA for repeated measures indicated that the temporal changes in the mAG/mKO2 emission ratio differed significantly among groups [$F_{(3, 175)} = 49.3$, $p < 0.05 \times 10^{-26}$], over time [$F_{(34, 175)} = 2.91$, $p < 0.000005$], and by interactions between the main factors [$F_{(102, 525)} = 1.84$, $p < 0.00001$]. In control and $NaAsO_2$ (1 and 2 μM)-exposed groups, the mAG/mKO2 emission ratio was low and maintained a stable level until 23 h after $NaAsO_2$ exposure, which then increased over time and reached a peak at 39–45 h after $NaAsO_2$ exposure, followed by a gradual decrease until the end of live imaging (**Figure 4**). The peak levels of the mAG/mKO2 emission ratio in $NaAsO_2$ (1 and 2 μM)-exposed groups were lower than those in the control group. The temporal change in the mAG/mKO2 emission ratio of the $NaAsO_2$ (4 μM)-exposed group was different from that of other groups. In the $NaAsO_2$ (4 μM)-exposed group, a peak of the mAG/mKO2 emission ratio was found at 7 h after initiation of $NaAsO_2$ exposure, whereas no obvious peak in the ratio was observed when the ratio showed a peak in the control and $NaAsO_2$ (1 and 2 μM)-exposed groups.

Effects of $NaAsO_2$ on the Number of Fucci-Expressing Astrocytes

In control and $NaAsO_2$ (1 μM)-exposed groups, the numbers of mAG-expressing cells at 33 and 41 h after $NaAsO_2$ exposure were larger than those at other time points for each group (**Figure 5A**). Compared with the control group, the number of mAG-expressing cells was significantly ($p < 0.05$) smaller at 41 h after $NaAsO_2$ exposure in the $NaAsO_2$ (2 μM)-exposed group

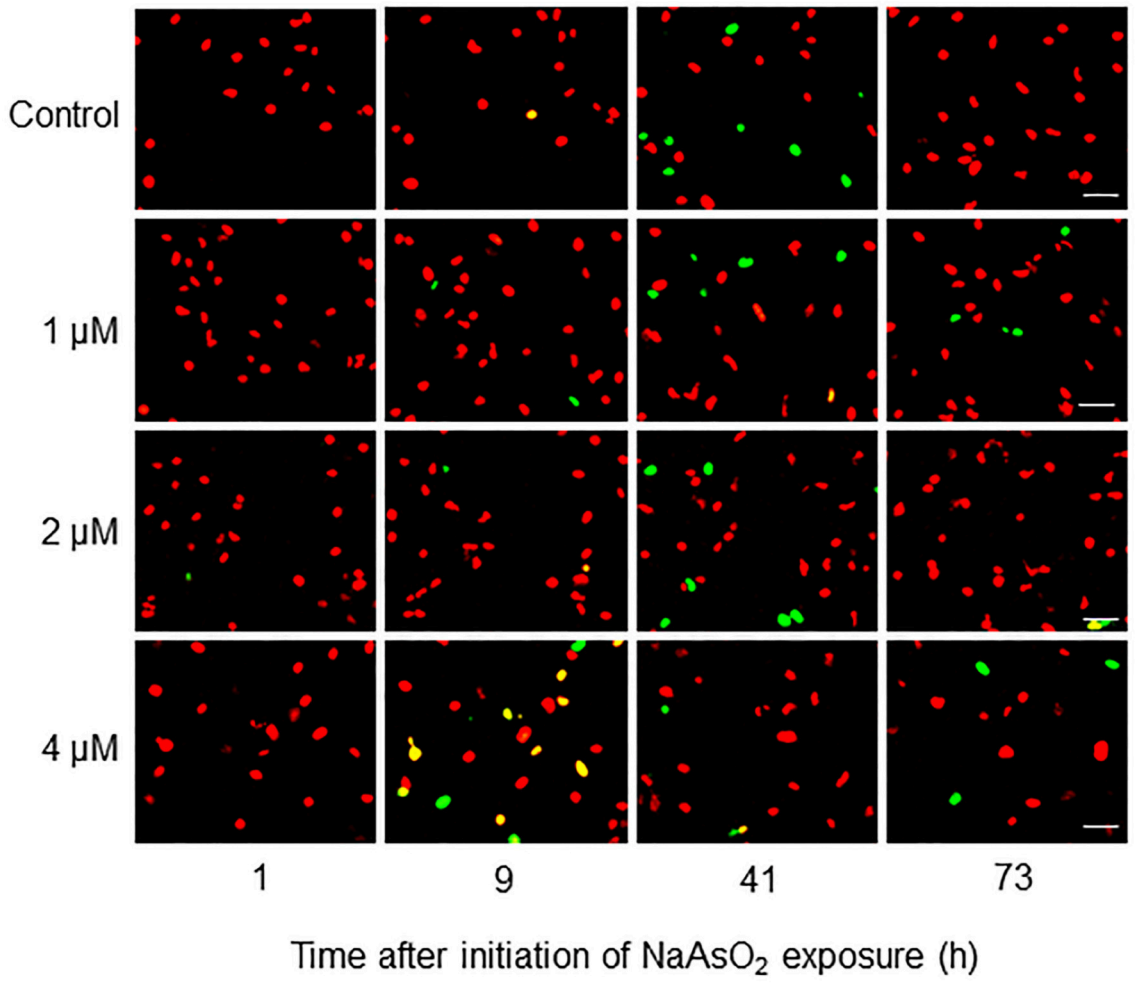

FIGURE 3 | Time-lapse images of Fucci-expressing astrocytes after initiation of $NaAsO_2$ exposure. Images of mAG and mKO2 signals were merged after the original colors were converted to pseudocolors (mAG, green; mKO2, red). Images were obtained from Fucci-expressing astrocytes exposed to $NaAsO_2$ at concentrations of 0 (control), 1, 2, or 4 μM for 1–73 h. Scale bar: 50 μm.

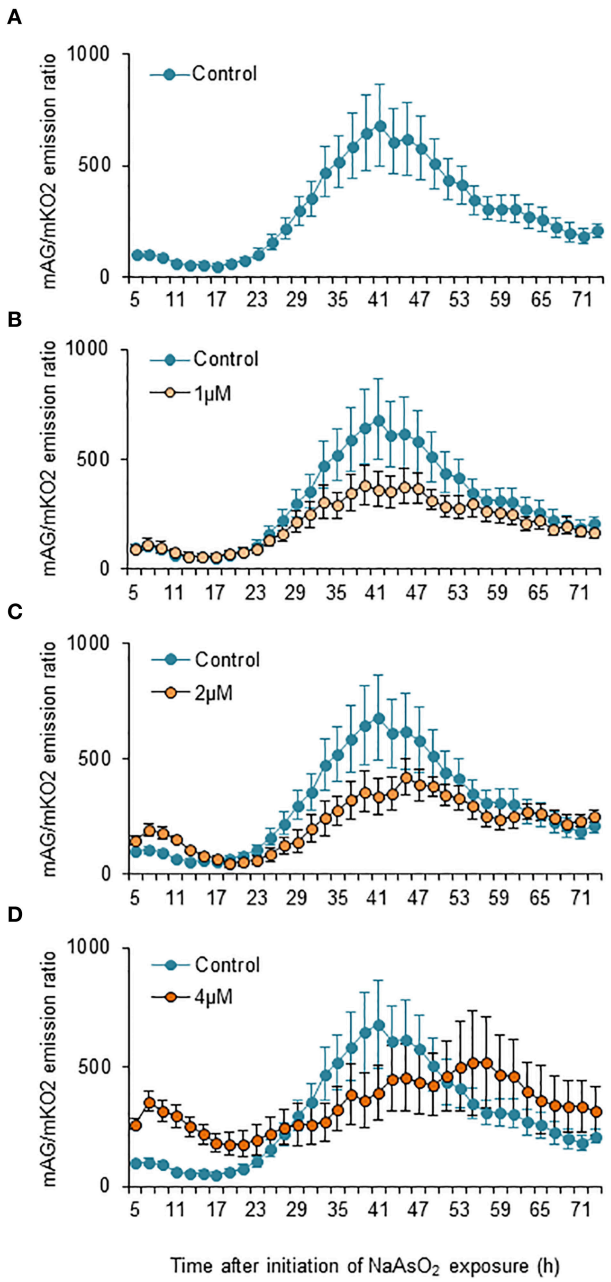

FIGURE 4 | Effects of $NaAsO_2$ exposure on the mAG/mKO2 emission ratio of Fucci-expressing astrocytes. Temporal changes in the mAG/mKO2 emission ratio of the control group **(A)** and groups exposed to $NaAsO_2$ at 1 μM **(B)**, 2 μM **(C)**, or 4 μM **(D)** from 5 to 72 h after initiation of $NaAsO_2$ exposure. Significant effects of the concentration and time of $NaAsO_2$ exposure and the interaction between the concentration and time on the ratio were found in two-way ANOVA for repeated measures. The ratio at each time point was calibrated using the ratio at 5 h after $NaAsO_2$ exposure, which was set at 100. All data are the means ± SEM of six primary cultures derived from different animals.

and at 25, 33, and 41 h after $NaAsO_2$ exposure in the $NaAsO_2$ (4 μM)-exposed group. In the $NaAsO_2$ (4 μM)-exposed group, the number of mAG-expressing cells was highest at 9 h after $NaAsO_2$ exposure, which was significantly ($p < 0.05$) larger than that in the control group.

There was tendency toward an increase in the number of cells expressing mKO2 from the beginning to the end of live imaging in control and $NaAsO_2$ (1 μM)-exposed groups (**Figure 5B**). At 57, 65, and 73 h after $NaAsO_2$ exposure, the total number of mKO2-expressing cells showed a concentration-dependent decrease, although there was no significant difference in the number of mKO2-expressing cells among the groups at each time point.

Effects of $NaAsO_2$ on Cell Death after S Phase Entry

To analyze cell fate after S phase entry, we traced Fucci-expressing astrocytes that emitted mAG fluorescence signals at 41 h in the control group and at 9 h after initiation of $NaAsO_2$ exposure in the $NaAsO_2$ (4 μM)-exposed group. As a result, most cells in the control group were alive at the end of live imaging, because the fluorescent emission signals changed from mAG to mKO2, and the morphology was normal (**Figure 6A**). Conversely, in most astrocytes of the $NaAsO_2$ (4 μM)-exposed group, the emission signal of mAG, which was observed at 9 h after $NaAsO_2$ exposure, had disappeared by the end of live imaging (**Figure 6B**). In addition, the cells did not express mKO2 and exhibited abnormal morphology with a debris-like structure. The population of live cells after S phase entry was significantly ($p < 0.01$) larger in the control group than in the $NaAsO_2$ (4 μM)-exposed group (**Figure 6C**). In contrast, the population of dead cells after S phase entry was significantly ($p < 0.01$) larger in the $NaAsO_2$-exposed group than in the control group.

Effects of $NaAsO_2$ on the Copy Number of the GFAP Gene and DNA Fragmentation

Exposure to $NaAsO_2$ for 73 h significantly [$F_{(3, 28)} = 4.30$, $p < 0.05$] reduced the GFAP gene copy number in a concentration-dependent manner (**Figure 7**). The copy number of the GFAP gene in the $NaAsO_2$ (4 μM)-exposed group was significantly ($p < 0.05$) smaller than that in the control group. We found no significant effect of lower $NaAsO_2$ concentrations.

The amount of nucleosomes generated from DNA fragmentation in astrocytes did not significantly [$F_{(1, 15)} = 2.21$, $p = 0.14$] change after exposure to $NaAsO_2$ (2 and 4 μM) for 72 h (**Figure 8**).

Effect of $NaAsO_2$ on the mRNA Expression of Apoptotic Markers and Cell Cycle Regulators

We did not find any significant effects of $NaAsO_2$ exposure on the mRNA levels of E2F1, E2F4, Gm36566, p21, or p53 in astrocytes (**Figure 9**). The mRNA levels of E2F1, E2F4, and p21, but not Gm36566 and p53, changed over time after synchronization of the cell cycle of astrocytes. The mRNA levels of E2F1 [$F_{(1, 16)} = 300.69$, $p < 0.01 \times 10^{-9}$] and p21 [$F_{(1, 16)} = 37.74$, $p < 0.00005$] were significantly higher at 41 h than 9 h after synchronization of the cell cycle, while the mRNA levels of E2F4 [$F_{(1, 16)} = 16.28$, $p < 0.001$] were higher at 9 h than 41 h after synchronization.

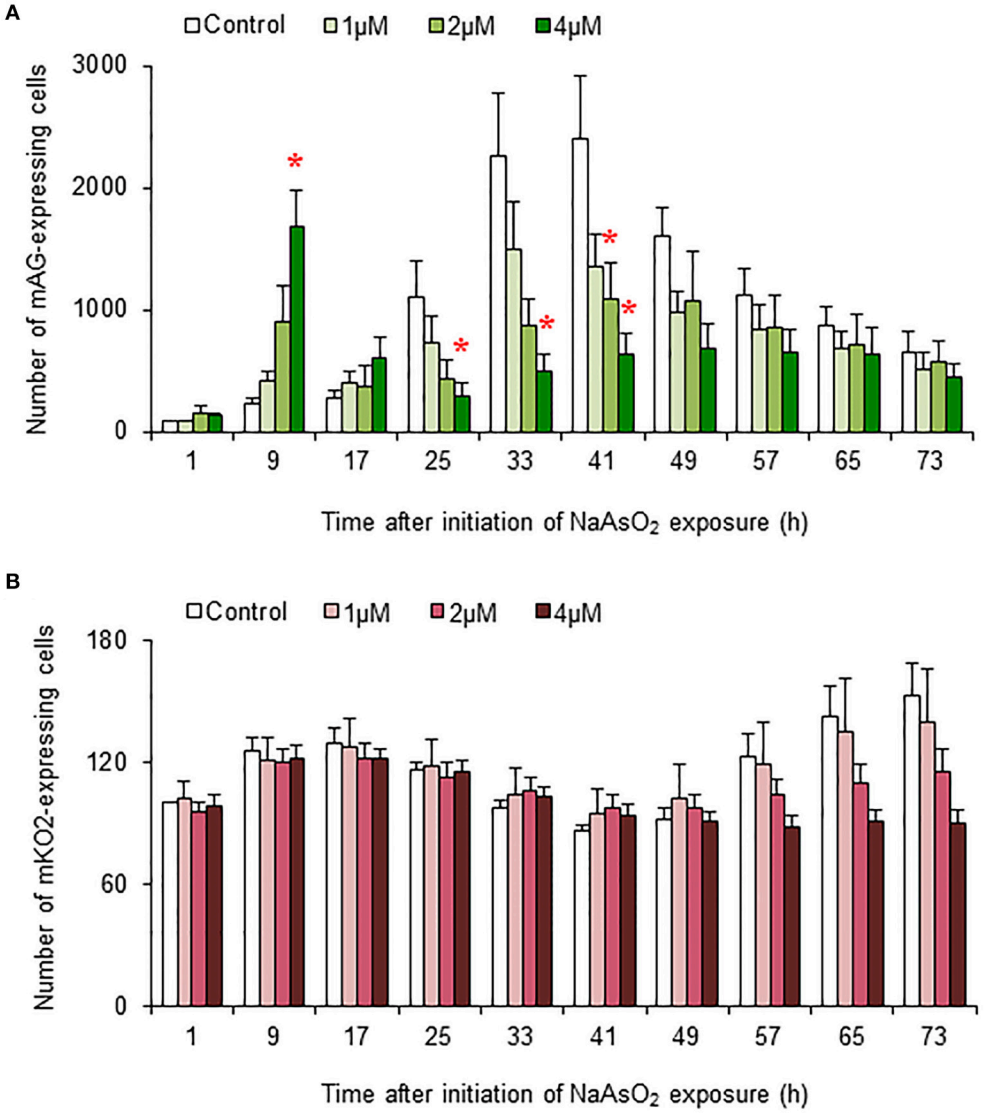

FIGURE 5 | Effects of $NaAsO_2$ exposure on the number of Fucci-expressing astrocytes. The number of cells expressing mAG **(A)** and mKO2 **(B)** at the indicated time point after initiation of $NaAsO_2$ exposure. The value for each time point was calibrated with the number of cells in the control group at 1 h after $NaAsO_2$ exposure, which was set at 100. All data are the means ± SEM of six primary cultures derived from different animals. *$p < 0.05$ vs. control at the same time point.

DISCUSSION

There is serious concern about contamination of ground water with arsenic, because chronic consumption of arsenic-contaminated water causes an impairment of cognitive functions (Calderon et al., 2001; Tsai et al., 2003; Wasserman et al., 2007). The World Health Organization recommends a limit of 0.01 mg/L (0.01 ppm) arsenic in water (World Health Organization, 2011). However, arsenic contamination in tube well water was found in Bangladesh at a concentration of more than 0.3 mg/L (0.3 ppm; Smith et al., 2000). In a native Andean population living in a part of Argentina, where drinking water contains arsenic at about 0.2 mg/L (0.2 ppm), the concentrations of arsenic were 9 μg/L in cord blood, 11 μg/L in maternal blood, 34 μg/kg in placenta, and 2.3 μg/kg in breast milk (0.009, 0.011, 0.034, and 0.0023 ppm, respectively; Concha et al., 1998a,b). According to a study measuring the accumulated levels of arsenic in newborn mice of mothers that were chronically exposed to $NaAsO_2$ (10–80 ppm) during the gestational period via drinking water, the accumulated level of arsenic in the brain was higher than that in the liver and blood, and ranged from ∼100 to 700 ng/g (0.1–0.7 ppm; Markowski et al., 2011). We previously reported that $NaAsO_2$ at 0.5–10 μM induced cell death and suppressed neuritogenesis of cultured neurons (Koike-Kuroda et al., 2010; Aung et al., 2013; Maekawa et al., 2013). In this study, to examine whether $NaAsO_2$ at the micromolar concentrations affect cultured astrocytes, we set the exposure concentrations

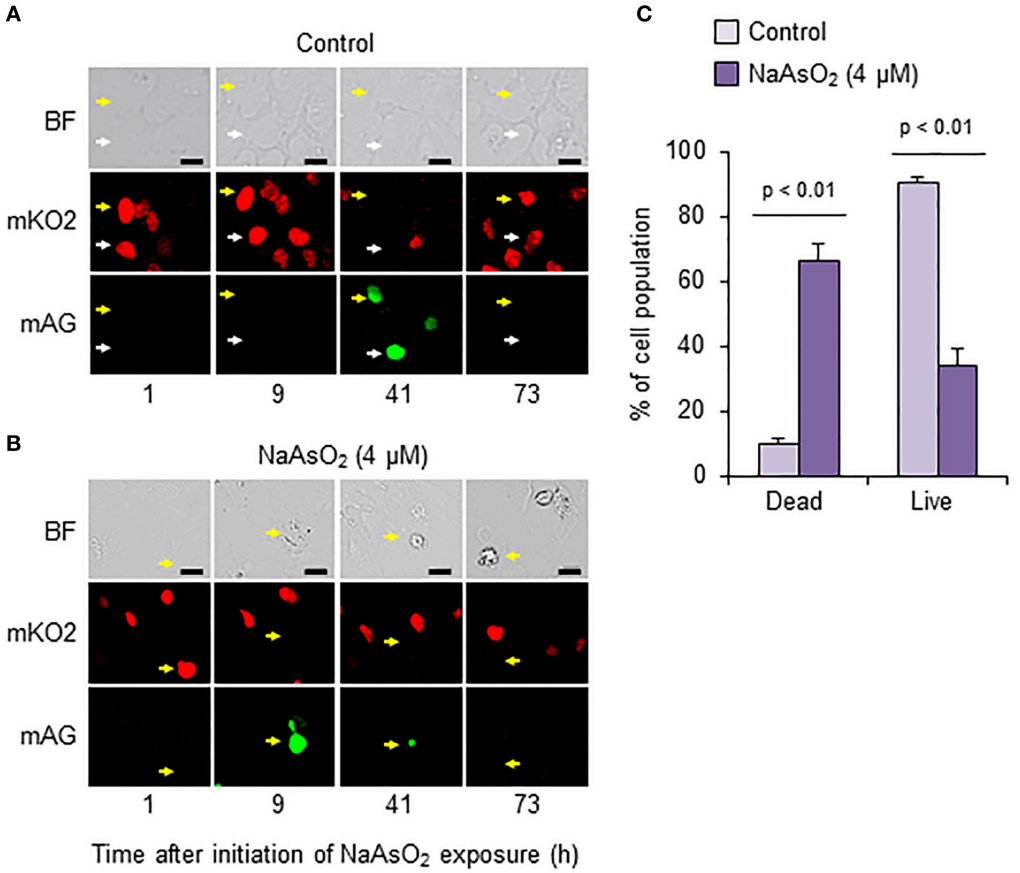

FIGURE 6 | Effects of $NaAsO_2$ exposure on cell fate after S phase entry. Digital photomicrograph of Fucci-expressing astrocytes captured with bright field (BF) and emission of mKO2 and mAG after pseudocolor conversion from the original fluorescent colors (mKO2, red; mAG, green) in control and $NaAsO_2$ (4 μM)-exposed groups **(A,B)**. Yellow and white arrows indicate the same cell or area. Scale bar: 20 μm. Cell population after Fucci-expressing cells entered S phase **(C)**. The percentage values of dead and live cells after S phase entry were calculated with the total number of cells that entered S phase, which was set at 100%. Values are the means ± SEM of six primary cultures derived from different animals.

of $NaAsO_2$ at 1, 2, and 4 μM (equivalent to 0.129, 0.258, and 0.516 ppm, respectively), which were similar to environmental pollution levels and higher than human exposure levels by ∼200-times or less.

It is known that cultured cells undergo phototoxic damage induced by frequent illumination with excitation light under a fluorescence microscope (Cervinka et al., 2008). Our previous study revealed that serum supplementation (the combination of 1% horse serum and 0.5% FBS) is vital to protect PC12 cells from phototoxic damage and enables live cell imaging without phototoxic damage (Koike-Kuroda et al., 2010). On the other hand, the doubling time of primary cultured astrocytes from the rat cerebral cortex is 6 days under the culture condition of 2% fetal calf serum (Geisert et al., 1996). This finding indicates that culturing astrocytes with serum supplementation at low concentrations is not beneficial for live imaging to monitor the cell cycle of astrocytes, because it requires a longer time (more than 6 days), which would increase the risk of phototoxic damage. Therefore, to promote cell proliferation and shorten the doubling time of astrocytes, we performed live imaging analysis of primary cultured Fucci-expressing astrocytes under the culture condition of 10% FBS. The duration of the cell cycle in Fucci-expressing astrocytes subjected to live imaging corresponded to that determined by fluorescence microscopy, suggesting that the live imaging technique in our current study could monitor the cell cycle without severe phototoxic damage.

Live imaging analysis of Fucci-expressing astrocytes showed that $NaAsO_2$ exposure significantly altered the cell cycle. The cell population entering S phase, when the cell population had reached to a peak level in the control group without $NaAsO_2$ exposure, was decreased by $NaAsO_2$ in a concentration-dependent manner. In contrast, the cell population entering unscheduled S phase at 9 h after initiation of $NaAsO_2$ exposure was increased by $NaAsO_2$ in a concentration-dependent manner. The findings in our current study suggest that $NaAsO_2$ exposure disrupts the cell cycle and forces astrocytes to enter S phase at an unscheduled timing. In particular, 4 μM $NaAsO_2$ had significant effects to disrupt cell cycle regulation and induce unscheduled S phase entry. Moreover, we traced the morphology

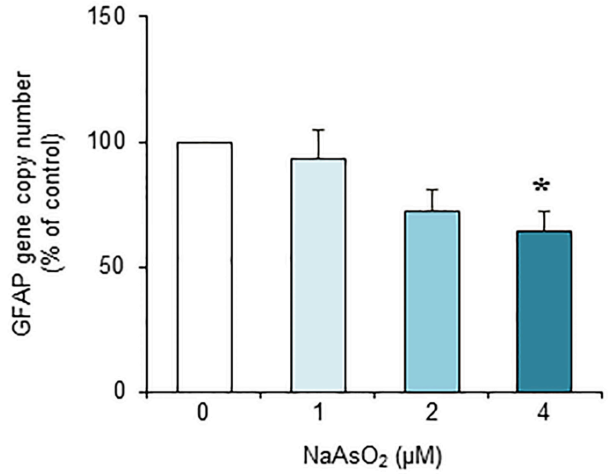

FIGURE 7 | Effects of $NaAsO_2$ exposure on the copy number of the GFAP gene. The GFAP gene copy number is presented as a percentage of the control. Values are the means ± SEM of eight primary cultures derived from different animals. *$p < 0.05$ vs. control.

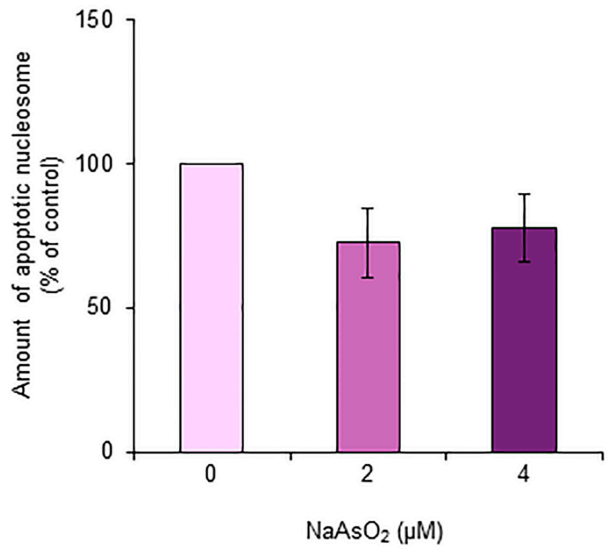

FIGURE 8 | Amount of apoptotic nucleosomes in astrocytes. The amount of apoptotic nucleosomes was assayed in astrocytes exposed to $NaAsO_2$ (0, 2, and 4 μM) and is represented as a percentage of the control. Values are the means ± SEM of six primary cultures derived from different animals.

as well as mKO2 and mAG expression of Fucci-expressing astrocytes to determine the fate of astrocytes that underwent unscheduled S phase entry induced by 4 μM $NaAsO_2$. As a result, more than 60% of astrocytes that entered S phase at 9 h after $NaAsO_2$ exposure had died, with disappearance of fluorescent mKO2 and mAG signals, and transforming debris-like structures until the end of live imaging. We also measured the copy number of the GFAP gene at 73 h after $NaAsO_2$ exposure, and found a significant decrease in the gene copy number induced by 4 μM $NaAsO_2$. Taken together, it appears likely that $NaAsO_2$ exposure at 4 μM decreases the number of astrocytes by inducing unscheduled S phase entry-coupled cell death. There is a report showing a slight increase in the viability of cultured rat cerebellar astrocytes by $NaAsO_2$ exposure at 1 μM for 24 h, but higher concentrations (5–50 μM) decrease the cell viability (An et al., 2016). In contrast, there is a report indicating that the cell viability is unaffected even when cultured rat astrocytes are exposed to 0.3 mM arsenite for 8 h, but it is decreased at 24 h after exposure (Koehler et al., 2014). Although the detailed mechanisms are largely unknown, arsenic may have dual effects to increase or decrease cell viability, which are dependent on the concentration and exposure time. Our current study supports the notion that arsenic decreases the viability of cultured astrocytes, and suggests that decreased cell viability is due to the reduction of cell number induced by unscheduled S phase entry-coupled cell death.

It is well-known that unscheduled S phase entry is linked to apoptotic cell death after DNA damage (Dimova and Dyson, 2005; Cho and Liang, 2011). The live imaging analysis in our current study showed that the mAG/mKO2 emission ratio and the number of mAG-expressing cells increased faster with $NaAsO_2$ exposure, indicating that up-regulation of geminin expression at an unscheduled timing is induced by $NaAsO_2$ exposure. It has been reported that overexpression of geminin induces apoptotic cell death (Shreeram et al., 2002). In this context, we speculated that apoptosis is responsible for the unscheduled S phase entry-coupled cell death induced by $NaAsO_2$ exposure. To test this hypothesis, we examined the effects of $NaAsO_2$ on the amount of nucleosomes generated from DNA fragmentation in astrocytes. However, in contrast to our expectations, we did not find any significant effect of $NaAsO_2$ on the amount of nucleosomes generated from DNA fragmentation. In addition, we examined whether $NaAsO_2$ exposure affected the expression levels of certain molecules involved in unscheduled S phase entry and apoptosis. We measured the mRNA levels of p53, a principal regulator of apoptosis (Fridman and Lowe, 2003; Cho and Liang, 2011), E2F1, an S phase gene transcriptional activator and programed cell death inducer (Hou et al., 2000; Pardee et al., 2004; Dimova and Dyson, 2005; Lazzerini Denchi and Helin, 2005; Cho and Liang, 2011), E2F4, an S phase gene transcriptional suppressor (Dimova and Dyson, 2005), p21, a cyclin dependent kinase inhibitor (Cho and Liang, 2011) and unscheduled S phase inducer (Bedelbaeva et al., 2010), and Gm36566, the mouse ortholog of Killin, which acts as a S-phase-coupled apoptosis regulator (Cho and Liang, 2011). Considering the results of the apoptosis assay with reference to the amount of nucleosomes, the gene expression of these molecules was not significantly affected by $NaAsO_2$ exposure, although the mRNA levels of E2F1, E2F4, and p21 had temporally changed with or without $NaAsO_2$ exposure. Taken together, the unscheduled S phase entry that occurred in astrocytes after $NaAsO_2$ exposure may be induced without alteration of the expression of these molecules. In addition, the subsequent cell death may be

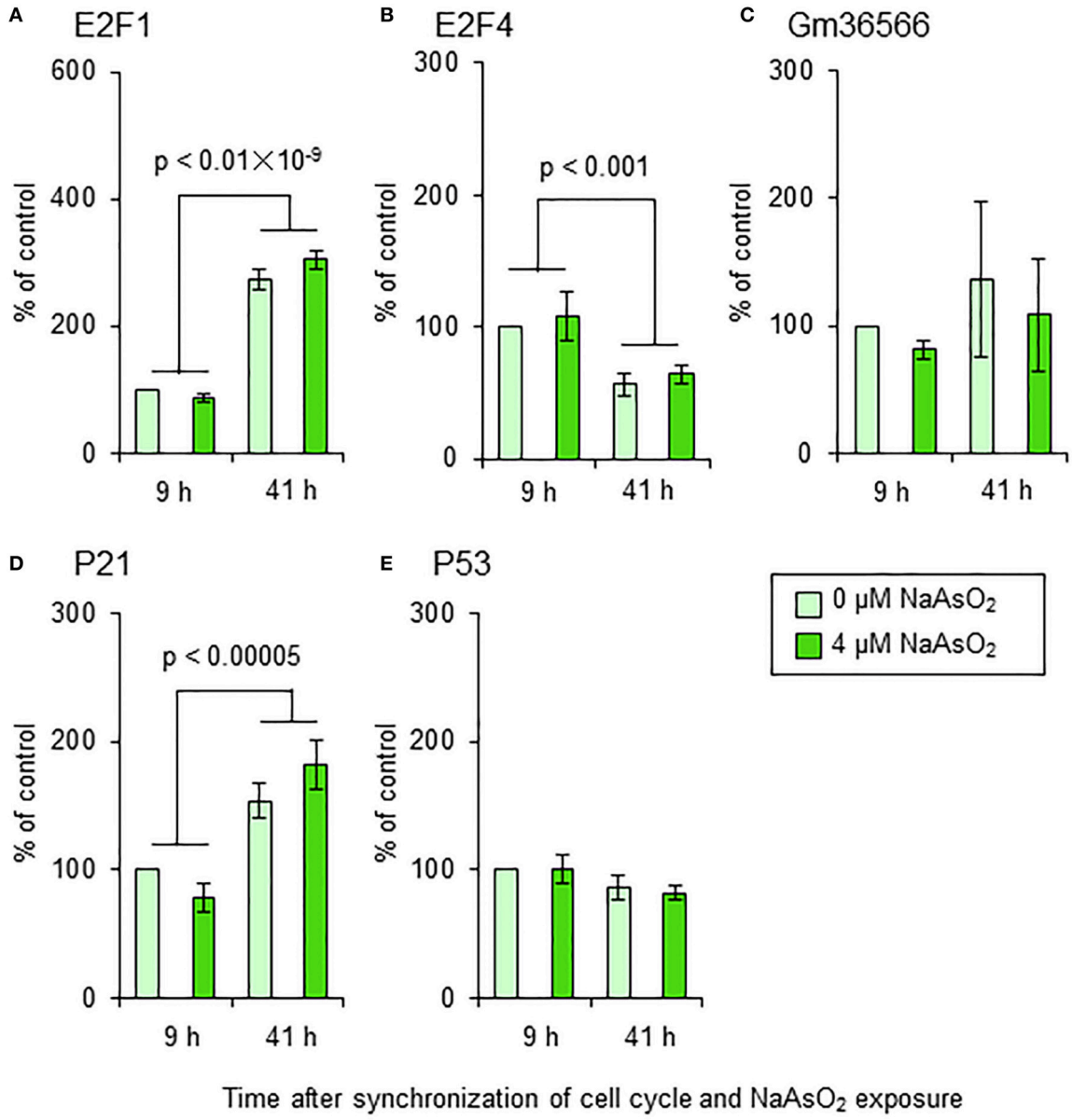

FIGURE 9 | mRNA levels of E2F1 (A), E2F4 (B), Gm36566 (C), p21 (D), and p53 (E) in astrocytes. Astrocytes were exposed to $NaAsO_2$ (0 and 4 μM) for 9 and 41 h. The mRNA level of each gene was normalized to that of CPB and are expressed relative to that of the control (0 μM for 9 h), which was set at 100%. Values are the means ± SEM of five primary cultures derived from different animals.

caused by mechanisms other than apoptosis. However, the mechanisms were not determined in this study. Further studies are needed to clarify the molecular mechanisms of arsenic toxicity, which induce unscheduled S phase entry-coupled cell death in astrocytes.

In summary, we examined the effects of $NaAsO_2$ exposure on the cell cycle, viability, and apoptotic cell death of cultured mouse cerebral astrocytes. The results of these analyses suggest that 4 μM $NaAsO_2$ significantly induces unscheduled S phase entry that is coupled with cell death by mechanisms other than apoptosis.

AUTHOR CONTRIBUTIONS

ST and FM designed the study; NH, HS, KS, and KA performed live imaging analyses; NH, HS, and SM performed molecular analyses; NH and ST wrote the manuscript; FM and MT critically revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2016.00297

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MINI REVIEW
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Metal Ion Toxins and Brain Aquaporin-4 Expression: An Overview

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Metal ions such as iron, zinc, and manganese are essential to metabolic functions, protein synthesis, neurotransmission, and antioxidant neuroprotective mechanisms. Conversely, non-essential metals such as mercury and lead are sources of human intoxication due to occupational activities or environmental contamination. Essential or non-essential metal accumulation in the central nervous system (CNS) results in changes in blood-brain barrier (BBB) permeability, as well as triggering microglia activation and astrocyte reactivity and changing water transport through the cells, which could result in brain swelling. Aquaporin-4 is the main water channel in the CNS, is expressed in astrocyte foot processes in brain capillaries and along the circumventricular epithelium in the ventricles, and has important physiological functions in maintaining brain osmotic homeostasis and supporting brain excitability through regulation of the extracellular space. Some evidence has pointed to a role of AQP4 during metal intoxication in the brain, where it may act in a dual form as a neuroprotector or a mediator of the development of oxidative stress in neurons and astrocytes, resulting in brain swelling and neuronal damage. This mini-review presents the way some metal ions affect changes in AQP4 expression in the CNS and discuss the ways in which water transport in brain cells can be involved in brain damage.

Keywords: aquaporin-4, neurotoxicity, astrocytes, metal ions, oxidative stress, brain edema

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INTRODUCTION

Several metals, including zinc, iron, and manganese are important as major or trace elements in cellular biological functions, acting as catalytic cofactors of enzymes (Kress et al., 2002), cellular antioxidants, and neuromodulators (Paoletti et al., 1997). In contrast, metals such as mercury, lead, cadmium, and nickel have no known biological functions. Both essential and non-essential metal ions may lead to brain damage when they accumulate in the central nervous system (CNS).

Non-essential metals are an important cause of human intoxication due to occupational exposure or air, soil, and water contamination, resulting in serious health problems (Valko et al., 2005; Park and Zheng, 2012), including severe hematopoietic, renal, and neurological conditions (Tchounwou et al., 2012). More recently, the role of glial cells in protecting neuronal damage caused by metal ion accumulation in the brain has been studied, showing that astrocytes have a central role in reducing neural excitotoxicity by taking up metals that cross the blood-brain barrier (BBB) (Ni et al., 2011; Noguchi et al., 2013), while microglia release mediators of inflammatory and immune responses when activated by metal ions. As a consequence, oxidative stress is generated in brain cells, and reactive nitrogen and oxygen species (RNOS) contribute to the apoptotic process, leading to neurodegenerative diseases (Yuste et al., 2015).

Metal intoxication often leads to increased water transport through the BBB and astrocytes, which could have important consequences on the expression of aquaporins in the brain. Aquaporins are integral membrane proteins that mediate the bi-directional transport of water through the cells, regulating the osmolarity of the intra- and extracellular medium. Aquaporins possess six membrane-spanning domains and five connecting loops. To date, thirteen main isoforms of aquaporins have been described (AQP0 to AQP12). The isoforms AQP3, 7, 9, and 10 are known as aquaglyceroproteins, which mediate the transport of glycerol, urea, and carbon dioxide in addition to water.

In the CNS, AQP1 is mainly found in the apical membrane of the epithelium of the choroid plexus and in the ependyma and pia (Nielsen et al., 1993), while AQP4 is the main water channel expressed in glial cells (Jung et al., 1994). In astrocytes, AQP4 is localized in the foot processes apposed to brain capillaries and along the circumventricular epithelium in the ventricles (Nielsen et al., 1997). The distribution of AQP4 in astrocyte processes is polarized, and the channels are assembled as orthogonal arrays of particles (OAPs; Yang et al., 1996), which share the same distribution as the inwardly rectifying K^+ channel Kir4.1 (Nagelhus et al., 1999), showing an expressive role for AQP4 in regulating homeostasis of brain osmolarity and excitability via the extracellular clearance of K^+.

Therefore, oxidative stress due to metal intoxication seems to have an important role in brain excitotoxicity and damage, with cells swelling as a consequence or cause of neuronal damage. This mini-review aims to bring an overview of the role of AQP4 during metal intoxication and cellular mechanisms involved in neuroprotection and toxicity of the brain.

EXPOSURE TO NON-ESSENTIAL METALS AND THE ROLE OF ASTROCYTE WATER TRANSPORT IN BRAIN INJURY (TABLE 1, FIGURE 1)

Mercury

Human intoxication due to mercury exposure is associated with occupational activities, including mining and smelting of cinnabar ore, environmental pollution, and consumption of seafood contaminated with mercury. Central nervous system symptoms related to mercury exposure include paresthesia, cerebellar ataxia, and decrease of cognition (Ye et al., 2016).

Elemental mercury is lipid-soluble and crosses the BBB by diffusion. In brain cells, it is oxidized through catalase and peroxidase to inorganic mercury (Hg^+ and Hg^{2+}). Methylmercury (MeHg) is an important source of human intoxication through seafood intake. MeHg is carried through the endothelial cells of the BBB by a neutral L-cysteine amino acid carrier, and is preferentially taken up by astrocytes and microglia. In astrocytes, MeHg promotes RNOS production through decreased availability of cysteine, reducing the antioxidant responses. Moreover, MeHg stimulates arachidonic acid synthesis, which in turn inhibits glutamate uptake by astrocytes, leading to neurotoxicity through ROS production (Ni et al., 2012).

Aquaporins are mercury-sensitive water channels. Most AQPs are inhibited by Hg^{2+}, therefore decreasing cell water permeability (Agre et al., 1998), except for AQP6, which is activated by Hg^{2+} exposure (Yasui et al., 1999). The effects of mercury on cell's water transport were described in an early study in ghost erythrocytes treated with p-chloromercuribenzene sulphonate. In erythrocytes, mercury inhibited ∼36% of water diffusion (Benga et al., 1985).

AQP4 has two isoforms (M1 and M23) that have been described as mercurial-insensitive water channels (MIWC), since their osmotic water permeability was not inhibited by mercury compounds (Shi and Verkman, 1996). In cells, the AQP4 monomer is normally oriented with the cysteine residue in loop D facing the intracellular side, preventing mercury binding to the channel and rendering AQP4 mercury-insensitive. However, mutagenic assays directed to the cysteine residues in the rat AQP4M23 isoform in proteoliposomes, in which the cysteine residue in loop D was randomly positioned inside or outside the proteoliposome membrane, demonstrated that when the AQP4M23 Cys178 in loop D faced the outside of proteoliposomes, mercury was efficient in reducing water permeability through AQP4. Covalent binding of mercury to the Cys178 residue might induce conformational changes in the AQP4 monomer, reducing water permeability (Yukutake et al., 2008). One *in vivo* study demonstrated the effects of 14 days of MeHg exposure (1.5 mg Hg/kg/day p.o.) on AQP4 expression in the brain of marmosets. MeHg intoxication increased AQP4 mRNA in the frontal lobe, occipital lobe, and cerebellum, while the AQP4 protein was increased in the occipital lobe and cerebellum (Yamamoto et al., 2012).

Lead

Lead (Pb) intoxication effects in the CNS include lethargy, memory deficits, encephalopathy, and coma. Lead poisoning in humans occurs by breathing dust or swallowing paint, water, or food containing lead. When bound to sulfhydryl groups of hemoglobin, lead reaches brain vessels and crosses the BBB by diffusion or competition with the same carrier system for other metal ions, as iron. Mechanisms of lead intoxication are mainly related to ROS production and disturbed ionic mechanisms, as lead can substitute for bivalent cations as Ca^{+2}, Mg^{+2}, and Fe^{+2}, leading to neurotransmission deficits, impaired subcellular signaling, and oxidative stress (for review, see Needleman, 2004).

Lead intoxication changes BBB permeability as a result of its accumulation in endothelial cells, leading to capillary weakness and brain swelling. Cultured rat astrocytes exposed to lead showed a 40% increase in osmotic water permeability (*Pf*) in AQP4-expressing astrocytes. The lead chelator DMSA (meso-2, 3-dimercaptosuccinic acid) abolished the effects of lead on water permeability, showing that AQP4 in astrocytes is central to cell swelling after lead intoxication (Gunnarson et al., 2005).

Changes in astrocyte water permeability would be caused by the calcium/calmodulin-dependent protein kinase II (CaMKII) pathway; astrocyte exposure to a CaMKII inhibitor abolished the lead effects on water permeability. The AQP4 phosphorylation site for CaMKII is located at the Ser111 residue; when mutated to Ser111Ala, the effect of lead on water permeability was

TABLE 1 | Effects of metal-ions on aquaporin-4 (AQP4) expression in the brain.

Metal Ion	Experimental approach	AQP4 expression	Water permeability	References
Mercury	*in vivo*			
	Rat brain	↑mRNA ↑protein	–	Yukutake et al., 2008
	in vitro			
	Xenopus oocytes	–	↓	Shi and Verkman, 1996
	AQP4 cystein-residue mutants			Yukutake et al., 2008
	AQP4 reconstituted in proteoliposomes	–	↓	
Lead	*in vivo*			
	Rat brain	no changes mRNA	–	Gunnarson et al., 2005
	in vitro			
	Cultured astrocytes	–	↑	Gunnarson et al., 2005
Manganese	*in vitro*			
	cultured astrocytes	↑protein in plasma membrane no changes mRNA	↑	Rao et al., 2010
Zinc	*in vitro*			
	AQP4.M23 proteolipossomes	–	↓	Yukutake et al., 2009
Iron	*in vivo*			
	Rat brain	↑protein	↑	Qing et al., 2009
	in vitro			
	cultured astrocytes	↑protein	–	Wang et al., 2015

↑, *Increased;* ↓, *Decreased.*

prevented. Assentoft et al. (2013, 2014) have questioned the effect of Ser111 residue phosphorylation on AQP4's regulation of water permeability. Mutation of the Ser111Ala residue to abolish the potential site of AQP4 phosphorylation and mutation to aspartate (S111D) to mimic serine phosphorylation did not change water permeability in *Xenopus* oocytes. Similarly, primary culture of astrocytes exposed to a cGMP-dependent protein kinase (PKG) activator did not change water permeability, indicating that phosphorylation of AQP4 could not be implicated in cell swelling. Another *in vivo* study in Sprague-Dawley rats did not indicate lead-related changes in AQP4 expression. Ten and fourty day old rats that received lead acetate intraperitoneally or by gavage showed no difference in AQP4 mRNA in the cerebellum and cerebrum at either age, although significantly increased brain lead levels could be detected (Gunnarson et al., 2005).

BIOLOGICALLY-NECESSARY METALS AND THE ROLE OF ASTROCYTE WATER TRANSPORT IN BRAIN INJURY (TABLE 1, FIGURE 1)

Manganese

Manganese is an essential metal and a constituent of metalloproteins and mitochondrial enzymes in oxidative metabolism (Aschner, 2000). Manganese poisoning occurs mainly through occupational exposure of miners, industrial steel workers, or welders to heavy metals. High exposure to manganese results in neurological symptoms, including bradykinesia, dystonia, and gait disturbance. At a cellular level, manganese poisoning will disturb antioxidant defense and water transport in cells, leading to swelling (Erikson et al., 2004). Primary cultures of rat astrocytes treated with manganese showed increased AQP4 proteins in the plasma membrane. This effect was time-dependent, and there was no corresponding increase in mRNA. Conversely, astrocyte cultures transfected with siRNA targeted to AQP4 showed a significant reduction (∼86%) of astrocyte swelling mediated by the AQP4 protein when exposed to manganese. The effects of manganese on cell swelling seem to involve mitogen-activated protein kinases (MAPKs) in astrocytes, since inhibition of ERK1/2/3 and p38-MAPK prevented AQP4 protein increases in the plasma membrane (Rao et al., 2010).

Zinc

Zinc is an essential trace element for all cells, involved in various metabolic and signaling pathways as component of regulatory and catalytic proteins (Mizuno and Kawahara, 2013). In the brain, zinc is mostly bound to proteins and has important modulatory functions in glutamatergic synapses (Tamano and Takeda, 2011). Zinc intoxication is a consequence of inhalation, ingestion, or manipulation of metal. Free intracellular Zn^{2+}, which is present during intoxication, generates oxidative stress in neurons and astrocytes and modulates neuronal activity.

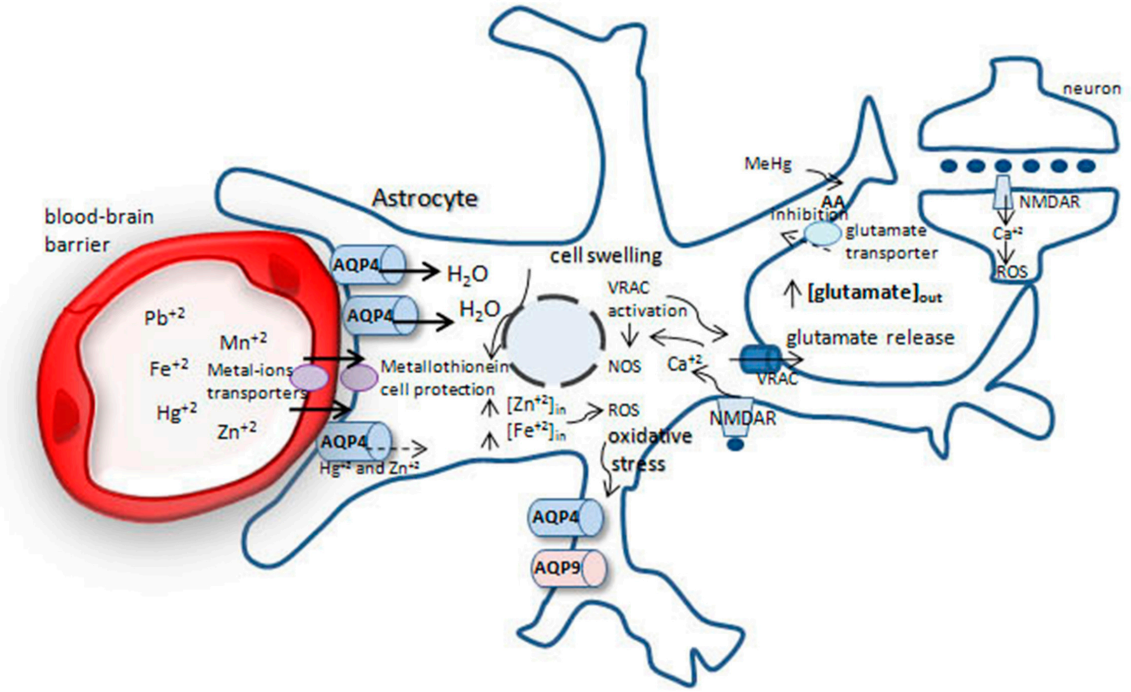

FIGURE 1 | Possible mechanisms involved in brain protection or swelling after metal-ion intoxication. After crossing the blood-brain barrier metal-ions are taken up by astrocytes. Fe^{+2}, Mn^{+2}, Pb^{+2} increase water permeability throughout aquaporin-4 (AQP4), leading to cell swelling and glutamate release from astrocytes by volume-regulated anion channels (VRACs), increasing extracellular glutamate pool. Glutamate acts via NMDA receptor in neurons and astrocytes, increasing $[Ca_i^{+2}]$ and reactive oxygen species (ROS) production, generating oxidative stress. Methylmercury (MeHg) reduces cystein uptake, increasing ROS production. Oxidative stress leads to increase in AQP4 and AQP9 in astrocytes, which leads to cell swelling.

In the brain, Zn^{2+} is mainly distributed in membrane-bound metalloproteins and presynaptic vesicles in glutamatergic neurons. When the amount of free Zn^{2+} increases in the brain, oxidative stress is triggered and activates nitric oxide synthetase (NOS), which in turn releases Zn^{2+} from intracellular stores and activates apoptosis. Increases in free Zn^{2+} promote cellular swelling (Kruczek et al., 2009). Cultured rat astrocytes exposed to a hypo-osmotic milieu (205 mosm/L) increased Zn^{2+} concentrations in the cytoplasm, mitochondria, and nucleus. Hypo-osmotic-dependent zinc increase in astrocytes seems to be trigged by Ca^{2+} and ROS intracellular signaling, as antagonists of the NMDA receptor prevent hypo-osmotic Zn^{2+} increase (Kruczek et al., 2009). Hypo-osmotic effects in astrocytes can be mediated in part by the recently identified AQP4e isoform (Moe et al., 2008). Rat astrocytes transfected with AQP4e and maintained in hypo-osmolar solution (200 mosm/L) showed a transitory increase of AQP4e membrane insertion, concomitant with diminished mobility of the AQPe-carrying vesicles. Depolymerization of vimentin filaments in the cytoskeleton under hypo-osmotic conditions would contribute to the AQPe mobility and membrane insertion (Potokar et al., 2013).

Zinc seems to have an inhibitory effect on water permeability, as demonstrated in AQP4.M23 expressed in proteoliposomes. The Cys178 residue in AQP4 is a potential site for the inhibitory effect of zinc on water permeability, since mutation of this residue resulted in no change in water permeability after zinc exposure (Yukutake et al., 2009).

Iron

Iron is an essential metal in multiple metabolic reactions, including DNA synthesis, enzymatic reactions, and electron transport. Iron accumulation is very deleterious for brain functions due to its wide participation in metabolic reactions (Schipper, 2012; Rouault, 2013). Iron intoxication is not common; however, iron deposition in cells occurs frequently after intracerebral hemorrhage (ICH), a subtype of stroke with high morbidity and mortality in humans.

As early as 24 h after ICH, iron content increases in the perihematomal zone and peaks at day 7. AQP4 expression peaks at day 3 and is maintained until day 7. Brain water content follows the initial increase of AQP4 and then declines slowly until day 14 post ICH onset. AQP4 expression is increased in astrocytes near the perihematomal area. The iron chelator deferoxamine (DFO) reduced iron deposition, brain water content, and AQP4 level in the perihematomal area, demonstrating a correlation between free iron content and brain swelling mediated by AQP4. Additionally, increased iron deposition and brain water permeability are likely to initiate apoptosis in perihematomal areas (Qing et al., 2009).

Cell damage as a result of increased Fe^{2+} is mediated by the NF-κB p65 protein, which activates ROS production and

release of proinflammatory cytokines in astrocytes and microglia, respectively, and consequently increases AQP4 and AQP9 in astrocytes (Wang et al., 2015). These studies evidenced increased iron-dependent water permeability in astrocytes mediated by AQP4, showing a role for free iron brain deposition and increased risk of brain damage.

CONCLUSIONS

Metal ions including iron, zinc, and manganese are essential to metabolic functions, protein synthesis, neurotransmission, and antioxidant neuroprotective mechanisms. However, in the CNS, unbalanced essential metal ion amounts, as well as non-essential metal accumulations, are detrimental to brain function.

Toxic amounts of non-essential metals and breakdown of metal ion homeostasis result in changes in brain metabolism and water permeability. These changes are particularly related to increased AQP4 expression in the astrocytes surrounding the BBB, the development of oxidative stress in neurons and astrocytes, and brain swelling, leading to neurodegeneration.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Gliotransmitter Release from Astrocytes: Functional, Developmental, and Pathological Implications in the Brain

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Astrocytes comprise a large population of cells in the brain and are important partners to neighboring neurons, vascular cells, and other glial cells. Astrocytes not only form a scaffold for other cells, but also extend foot processes around the capillaries to maintain the blood–brain barrier. Thus, environmental chemicals that exist in the blood stream could have potentially harmful effects on the physiological function of astrocytes. Although astrocytes are not electrically excitable, they have been shown to function as active participants in the development of neural circuits and synaptic activity. Astrocytes respond to neurotransmitters and contribute to synaptic information processing by releasing chemical transmitters called "gliotransmitters." State-of-the-art optical imaging techniques enable us to clarify how neurotransmitters elicit the release of various gliotransmitters, including glutamate, D-serine, and ATP. Moreover, recent studies have demonstrated that the disruption of gliotransmission results in neuronal dysfunction and abnormal behaviors in animal models. In this review, we focus on the latest technical approaches to clarify the molecular mechanisms of gliotransmitter exocytosis, and discuss the possibility that exposure to environmental chemicals could alter gliotransmission and cause neurodevelopmental disorders.

Keywords: astrocytes, exocytosis, glial cell, gliotransmitter, neurodevelopmental disorders, optical imaging, synaptic activity

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INTRODUCTION

Astrocytes are the most abundant glial cells in the central nervous system (CNS) of mammals (Ventura and Harris, 1999). Based on electron microscopic analyses, astrocytes are located near to neurons and blood vessels (**Figure 1A**). Regarding vasculature, capillary endothelial cells are surrounded by pericytes and basal lamina, and astrocytes tightly wrap these microvascular structures (Abbott et al., 2006). Together with pericytes, astrocytes are an essential component of the blood–brain barrier (BBB), which selects and transports molecules from the bloodstream, and allows for the transfer of nutrients to neurons (**Figure 1B**). Regarding their relationship with neurons, astrocytic foot processes make close contact with pre- and post-synaptic areas, forming structures called "tripartite synapses" (Araque et al., 1999; Halassa et al., 2007). Indeed, in the hippocampus, 57% of synapses are associated with astrocytes (Ventura and Harris, 1999), suggesting that astrocytes might contribute to neural information processing in the CNS.

Despite their morphological characteristics described above, astrocytes have long been considered as mere metabolic supporters that nurture adjacent neurons (Halassa et al., 2007; Wang and Bordey, 2008; Calì et al., 2009). However, recent electrophysiology and optical imaging analyses have provided strong evidence that astrocytes respond to neurotransmitters and release chemical transmitters called "gliotransmitters" (Li et al., 2013). Gliotransmitters, including glutamate, D-serine, and ATP, bind to their respective receptors on neurons to modulate their firing frequency and/or synaptic transmission (**Figure 1C**; Halassa et al., 2007; Koizumi, 2010). In fact, the dysfunction of gliotransmitter release-related proteins (e.g., vesicular transporters and vesicle-associated membrane proteins) in astrocytes can cause serious brain disorders and abnormal behaviors (Rossi et al., 2011; Verkhratsky et al., 2014). At the same time, traumatic injury, stroke, or infection-induced astrogliosis (also known as reactive astrocytes). These reactive astrocytes produce and release neurotoxic levels of glutamate (Rossi et al., 2011; Verkhratsky et al., 2014). Astrocytes also contribute to proper development of the BBB by aligning endothelial cells and pericytes, transporting molecules selected from the bloodstream to neurons (Abbott et al., 2006), and providing a protective barrier against toxic substances (Pentreath and Slamon, 2000; Calabrese, 2008). Thus, chronic exposure to environmental chemicals, or inflammatory molecules from vasculature, may potentially affect the function of astrocytes and gliotransmitter release (Kim et al., 2014; Orellana et al., 2014; Avendano et al., 2015).

In this review, we present the latest methods that enable scientists to decipher the molecular mechanisms of gliotransmitter secretion. In particular, we focus on the vesicular exocytosis of gliotransmitters from astrocytes using optical microscopic imaging. We further discuss how genetic alterations, acute injuries, and chronically toxic conditions (including exposure to stress *in utero*) could impair gliotransmission and consequently lead to neuronal and behavioral disorders.

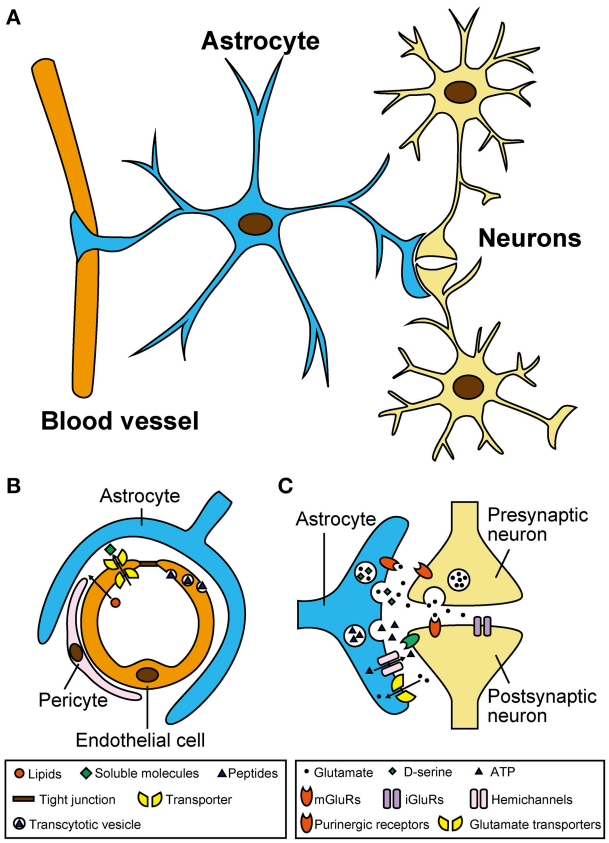

FIGURE 1 | Astrocytes have close morphological and functional associations with microvasculature and neurons. (A) Location of astrocytes around blood vessels and neurons in the central nervous system. Note that single astrocytes make contact with a large number of blood vessels and neurons through their numerous processes. **(B)** Schematic diagram showing the blood–brain barrier and its functions in selecting and transporting various molecules from the blood stream. Although, vascular endothelial cells form robust tight junctions that prevent infiltration of most soluble molecules, hydrophobic lipids can penetrate across the plasma membrane. In addition, certain soluble molecules such as glucose are actively transported across the endothelial cells via their specific transporters, and some peptides are taken up by selective vesicular transcytosis. **(C)** Schematic diagram showing the tripartite synapse and complex signaling interactions mediated by neurotransmitters and gliotransmitters. Neurotransmitters released from presynaptic terminals such as glutamate act not only on postsynapses but also on astrocytes. Activated astrocytes release gliotransmitters including glutamate, D-serine, and ATP, via vesicular exocytosis (and also possibly via hemichannels for ATP). Released gliotransmitters bind to presynaptic and postsynaptic receptors to regulate synaptic transmission. Astrocytes also take part in clearance of extracellular glutamate via glutamate transporters.

MOLECULAR MECHANISMS UNDERLYING THE RELEASE OF GLIOTRANSMITTERS

There have been two major methodological breakthroughs that have allowed for profound understanding of astrocytic activities including gliotransmission: calcium imaging and advanced optical microscopy (Li et al., 2013). The initial discovery made by using chemical calcium indicators was that astrocytes exhibit increased intracellular calcium concentration ($[Ca^{2+}]_i$), which spreads to adjacent astrocytes. This phenomenon is called Ca^{2+} waves (Cornell-Bell et al., 1990; Charles et al., 1991; Rusakov et al., 2014). Genetically encoded calcium indicators have enabled more detailed analysis of astrocyte functions (Shigetomi et al., 2013).

Two-photon microscopy enabled scientists to observe fluorescence with superior penetration depth. Thus, studies on astrocytes have been expanded to experiments using brain slices and *in vivo* models (Nimmerjahn et al., 2004; Nishida and Okabe, 2007). Moreover, thanks to total internal reflection fluorescence microscopy, which can visualize fluorescent molecule behaviors beneath the plasma membrane, the interaction between $[Ca^{2+}]_i$ elevation and subsequent vesicular trafficking became precisely clarified (Bezzi et al., 2004; Shigetomi et al., 2012; Oya et al., 2013).

Because of these experimental advancements, accumulating evidence suggests the paradigm that: (1) inositol 1,4,5-trisphosphate-mediated Ca^{2+} release from endoplasmic

reticulum causes $[Ca^{2+}]_i$ increases in astrocytes in response to the activity of adjacent astrocytes and neurons; (2) elicited $[Ca^{2+}]_i$ elevation induces release of gliotransmitters (Halassa et al., 2007; Oya et al., 2013; Khakh and McCarthy, 2015). Although the exact mechanisms of gliotransmission are unclear, recent studies have partially revealed the release mechanisms of glutamate, D-serine, and ATP in astrocytes (**Figure 2**; Gucek et al., 2012; Li et al., 2013).

GLUTAMATE

Although, glutamate is well-known as a neurotransmitter, it also acts as a gliotransmitter. Application of bradykinin to cultured astrocytes induces glutamate release and influences adjacent neurons through N-methyl-D-aspartate (NMDA) receptors (Parpura et al., 1994). In contrast, application of clostridium, tetanus, and botulinum neurotoxins, which differentially cleave the exocytosis-regulating soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, reduces Ca^{2+}-dependent glutamate release. These findings suggest that the SNARE proteins, including vesicle-associated membrane protein-2 (VAMP2), syntaxin-1, and synaptosome-associated protein-23, mediate Ca^{2+}-dependent glutamate release (Montana et al., 2006; Parpura and Zorec, 2010).

The uptake of cytoplasmic glutamate into exocytotic vesicles is mediated by vesicular glutamate transporters (VGLUTs), which are driven by a proton gradient produced by vacuolar-type H^+ ATPases (V-ATPases; Takamori et al., 2000; Gucek et al., 2012). Inhibition of V-ATPases blocks Ca^{2+}-dependent glutamate release (Parpura and Zorec, 2010). Furthermore,

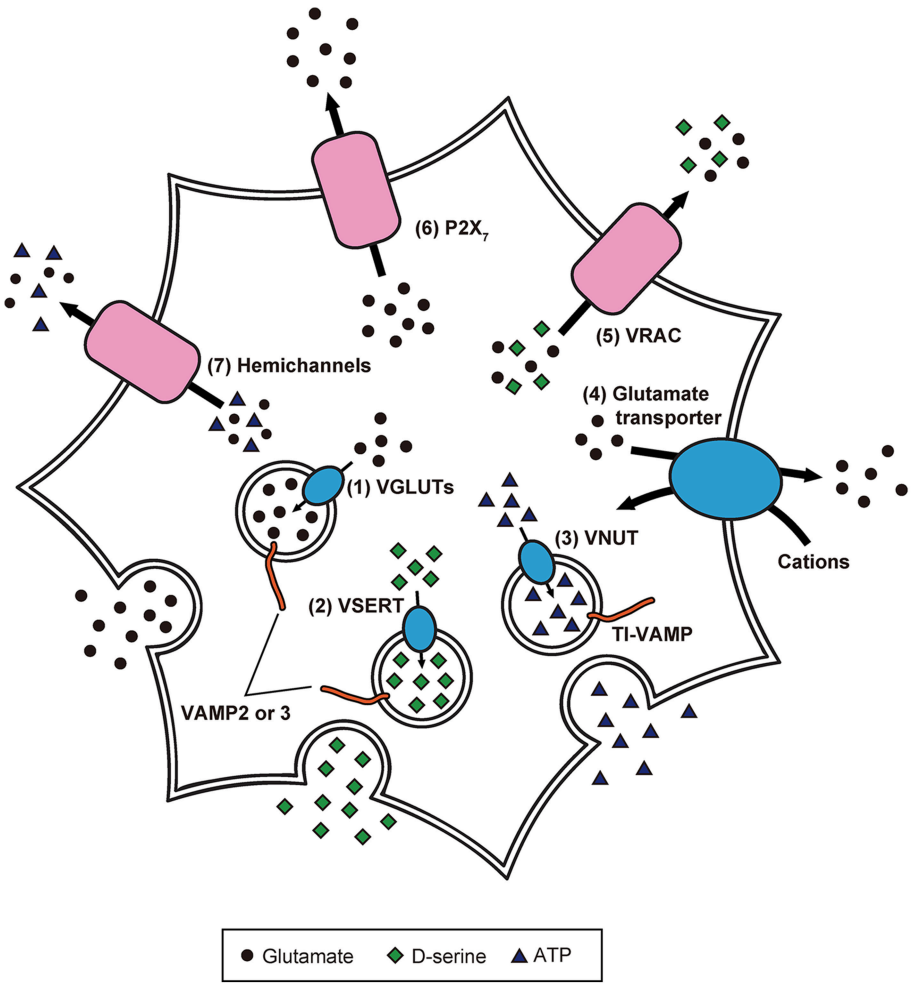

FIGURE 2 | Precise intracellular machinery involved in the release of glutamate, D-serine, and ATP from astrocytes. Glutamate and D-serine are taken up into synaptic-like vesicles through (1) VGLUT and (2) vesicular D-serine transporters (VSERT), respectively. These synaptic-like vesicles fuse to the plasma membrane, mediated by SNARE proteins including VAMP2 or VAMP3, in response to $[Ca^{2+}]_i$ increase. In contrast, ATP is released through secretory lysosomes. Storage of ATP into secretory lysosomes is achieved by (3) VNUT. Through the interaction of SNARE proteins including TI-VAMP, ATP-containing secretory lysosomes are Ca^{2+}-dependently exocytosed. Moreover, the existence of other release mechanisms has been discovered: (4) reverse operation of plasma membrane glutamate transporters, (5) cell swelling-induced anion transporter (VRAC) opening, (6) release via $P2X_7$ receptors, and (7) gap junction channels (hemichannels) on the cell surface of astrocytes.

VGLUT1 and 2 are colocalized with synaptic-like vesicles (Bezzi et al., 2004), suggesting that glutamate is packaged into synaptic-like vesicles and released from astrocytes in a Ca^{2+}-dependent manner.

Meanwhile, other release mechanisms have been identified: (1) reverse operation of plasma membrane glutamate transporters (Longuemare and Swanson, 1995); (2) cell swelling-induced anion transporter opening (Kimelberg et al., 1990); (3) release via $P2X_7$ receptors (Duan et al., 2003); (4) gap junction channels (i.e., hemichannels) on the cell surface of astrocytes (Ye et al., 2003). However, it is not clear how often and to what extent astrocytes employ these different mechanisms. Further studies will be needed to clarify whether there are specific release mechanisms that operate under particular conditions.

D-SERINE

The discovery of D-serine as a gliotransmitter was remarkable because it was long thought that mammalian tissues only produced L-isomers of amino acids (Oliet and Mothet, 2006; Henneberger et al., 2012). D-serine is thought to be produced from L-serine by serine racemase (de Miranda et al., 2002). In cultured astrocytes, application of glutamate enhanced Ca^{2+}-dependent secretion of D-serine via the activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors (AMPA/KARs) and metabotropic glutamate receptors (Mothet et al., 2005). Correspondingly, agonists for AMPA/KARs and metabotropic glutamate receptors were found to increase $[Ca^{2+}]_i$ as well as subsequent secretion of D-serine, which is reduced by inhibition of these receptors. Furthermore, tetanus neurotoxins and V-ATPase inhibitors suppress agonist-evoked secretion of D-serine, and VAMP2/3 and VGLUT2-containing vesicles that are colocalized with D-serine. These results suggest that D-serine is stored in the synaptic-like vesicles and released from the vesicles in a Ca^{2+}-dependent manner (Martineau et al., 2013).

ATP

Although ATP is the primary energy currency of the cells, ATP can also act as a signaling molecule through purinergic receptors. A recent study showed that culture medium from cultured astrocytes exhibiting Ca^{2+} waves contained more ATP than control culture medium. Interestingly, the addition of collected culture medium to astrocytes induced Ca^{2+} waves that were inhibited by purinergic receptor antagonists (Guthrie et al., 1999). Thus, the ATP released from astrocytes induces Ca^{2+} waves, which astrocytes use to communicate with each other. However, the ATP release mechanisms still remain controversial; several lines of investigation have suggested various putative models for ATP release from astrocytes (Koizumi, 2010).

Connexin 43 (Cx43) assembles into a hemichannel which constitutes gap junctions in astrocytes, and exchanges signaling molecules, including Ca^{2+} and inositol 1,4,5-trisphosphate,between adjacent astrocytes (Orellana and Stehberg, 2014). Bioluminescence imaging of ATP combined with single channel recording showed that Cx43 hemichannels in rat glioma C6 cells and CA1 hippocampal astrocytes are permeable to ATP (Kang et al., 2008). Consistent with this finding, glutamate evoked $[Ca^{2+}]_i$ increase and ATP release in astrocytes of hippocampal slices, which were inhibited by application of a hemichannel blocker and in Cx43/Cx30 knockout mice (Torres et al., 2012), suggesting that ATP is released extracellularly through Cx43 hemichannels.

However, some studies have shown the involvement of secretory lysosomes in ATP release from astrocytes. In fact, primary cultured astrocytes express a secretory lysosome marker called vesicle-associated membrane protein-7 (also called TI-VAMP), and TI-VAMP-positive secretory lysosomes contain ATP which is Ca^{2+}-dependently released (Verderio et al., 2012). In an experiment using primary cultured astrocytes and C6 cells, vesicular nucleotide transporter (VNUT)-positive lysosomes were labeled with fluorescent ATP, and application of VNUT inhibitor reduced the number of fluorescent ATP-containing vesicles. Observation by total internal reflection fluorescence microscopy revealed exocytotic events of secretory lysosomes in the cells following the application of a calcium ionophore, ATP, and glutamate. Thus, ATP is stored in lysosomes and released from lysosomes in a Ca^{2+}-dependent manner (Oya et al., 2013).

CONTRIBUTION OF GLIOTRANSMITTER RELEASE TO DEVELOPMENT AND DISEASE

Release of gliotransmitters regulates synaptic transmission between neurons and the extracellular environment in the brain. It is known that glutamate and D-serine excite synaptic transmission. However, whether ATP potentiates or inhibits synaptic transmission is still under debate because adenosine, a metabolite synthesized from ATP, usually inhibits synaptic activity via adenosine A_1 receptors (Koizumi, 2010; Nam et al., 2012; Delekate et al., 2014). It is therefore reasonable to speculate that imbalance in the release of these gliotransmitters could result in altered neuronal activity. Various pathological conditions, including CNS diseases, traumatic brain injuries, developmental disorders, and prenatal exposure to deleterious molecules have been reported to be closely associated with impairment of gliotransmission.

CNS DISEASES

Many CNS diseases are attributed to hyperactivity of neurons or unregulated neuronal cell death. Although such conditions have long been the focus of "neurocentric" studies, recent progress in the study of astrocytic gliotransmission has provided accumulating evidence for the contribution of astrocytes (Rossi et al., 2011; Verkhratsky et al., 2014).

Epilepsy is one of the most common CNS diseases, and is characterized by sudden and frequent seizures resulting from excessive firings by neurons (Wetherington et al., 2008). In slices from epilepsy model mice, astrocytic glutamate release was found to cause abnormal and prolonged depolarization in

neurons (Tian et al., 2005). Furthermore, tumor necrosis factor-α (TNFα) and prostaglandins (PGs), released from astrocytes under traumatic events, can reactivate their calcium signaling, and can cause increased glutamate release (Bezzi et al., 1998, 2001; Domercq et al., 2006).

Reactive astrocytes are also involved in the pathogenesis of other neuronal disorders. In a mouse model of Huntington's disease, cultured astrocytes exhibited hyperactivated Ca^{2+}-dependent glutamate release. This activity was owing to increased expression of pyruvate carboxylase (Lee et al., 2013), or reduced expression of glutamate transporter-1 and $K_{ir}4.1$ K^+ channels, which are key regulators for the clearance of extracellular glutamate and maintenance of membrane potentials, respectively (Behrens et al., 2002; Tong et al., 2014). In addition to overpotentiating neuronal activity, excessive accumulation of extracellular glutamate causes cytotoxicity. For instance, mice with genetic deletion in glutamate transporter-1 exhibit reduced glutamate clearance, and consequently display abnormal cell death in motor neurons, reminiscent of amyotrophic lateral sclerosis (Staats and Van Den Bosch, 2009).

In Alzheimer's disease (AD) mouse models, reactive astrocytes are detected near β-amyloid plaques (Nagele et al., 2004). Although chronic rise in $[Ca^{2+}]_i$ is a well-known phenomenon in reactive astrocytes in AD, its underlying mechanisms remain unclear. A recent study demonstrated that purinergic signaling through Cx43 hemichannels and P2Y1 receptors mediated the hyperactivity of astrocytes in AD (Delekate et al., 2014). Consistent with this finding, upregulation of Cx43 hemichannels was observed in an AD mouse model (Mei et al., 2010), and AD patients displayed higher levels of ATP in brain regions surrounding β-amyloid plaques (Mecheri et al., 1997; Mandal et al., 2012).

Gliotransmitter release from astrocytes is also required for correct development of neuronal circuits. In particular, glial-neuronal communication through NMDA receptors is an essential process for proper dendritic morphogenesis and establishment of synaptic connections (Rabacchi et al., 1992; Sin et al., 2002; Espinosa et al., 2009). Although NMDA receptors are activated by both glutamate and D-serine, recent discoveries suggest that D-serine plays an important role in dendritic development and long-term potentiation (Henneberger et al., 2010; Devito et al., 2011; Balu and Coyle, 2012; Diniz et al., 2012). Mice with a deletion of serine racemase showed reduced levels of brain D-serine and brain-derived neurotrophic factor, and loss of glutamatergic neurotransmission, and consequently had less complex dendrites (Morita et al., 2007; Balu and Coyle, 2012). Because NMDA receptor malfunction has been considered to be responsible for schizophrenia, deficiency in D-serine secretion from astrocytes can be a potent schizophrenia risk factor (Van Horn et al., 2013). Indeed, association studies of schizophrenia patients revealed several mutations in genes for serine racemase, as well as D-amino acid oxidase and its interacting protein G72 (Boks et al., 2007; Morita et al., 2007; Müller et al., 2011; Caldinelli et al., 2013).

INJURY AND INFECTION

Acute brain insults, caused by ischemia or infection, affect neuronal circuitry through direct inflammatory responses in neurons and through signals from glial cells (Vesce et al., 2007; Calì et al., 2009). Astrocytes under acute inflammatory conditions undergo reactive astrogliosis similarly to those in CNS diseases, albeit with differences in gene expression and cell structure (Khakh and Sofroniew, 2015). Upon injury or ischemia, damaged neurons, endothelial cells and glial cells are known to release considerable amounts of ATP (Cook and McCleskey, 2002; Wang et al., 2004; Davalos et al., 2005; Nedergaard et al., 2010). Increased levels of extracellular ATP activate purinergic receptors on astrocytes, particularly P2Y1 (Domercq et al., 2006), thereby inducing $[Ca^{2+}]_i$ elevation and release of glutamate, as well as ATP (Domercq et al., 2006; Nedergaard et al., 2010). Furthermore, inflammatory molecules including TNFα, interleukin-1β, and PGs, are profoundly engaged in these responses. Not only the activated microglia converge to the site of injury and secrete cytokines; astrocytes themselves synthesize TNFα and PGs (Domercq et al., 2006; Santello et al., 2011). TNFα and PGs either interact with certain processes in the stimulus–secretion coupling machinery within astrocytes (Domercq et al., 2006; Santello et al., 2011), or bind to TNFα and PGs receptors on astrocytes after secretion (Bezzi et al., 2001; Vesce et al., 2007).

CHRONIC AND PRENATAL EXPOSURE TO CHEMICALS

Increasing evidence shows significant correlations between environmentally deleterious chemicals and the risk of neurodevelopmental disorders (Feng et al., 1990; Leonardsson and Ny, 1997). Previous studies have focused on the effects of toxic substances on neurons, but recently it was suggested that astrocytes are also involved in the pathogenesis of those conditions.

Owing to their close connections with microvascular units via BBB, astrocytes tend to be chronically exposed to noxious molecules in circulation. Probably because of their interactions with environmental toxins, astrocytes possess more resilient and adaptive machinery against toxic molecules compared with neurons (Pentreath and Slamon, 2000; Calabrese, 2008). These protective systems include the glutathione system, superoxide dismutase, and hemeoxygenase (Dwyer et al., 1995; Huang and Philbert, 1995; Blaauwgeers et al., 1996; Pentreath and Slamon, 2000). Nevertheless, excessive passage of harmful substances across the BBB seriously affects astrocyte homeostasis and functionality.

The toxicological effects of heavy metals (e.g., mercury, zinc, manganese, and aluminum) on neurons and glial cells have been studied for decades (Calabrese, 2008; De Keyser et al., 2008). However, it is unclear how these metals affect gliotransmitter release. Some studies have shown that lead and manganese induce cytotoxic cell death by impairing glutamate uptake in astrocytes (Normandin and Hazell, 2002; Struzynska et al., 2005). However, pathological effects on gliotransmission by lifestyle-associated factors, such as smoking, drinking, and insufficient

sleep, are becoming the focus of growing interest. Because nicotinic acetylcholine receptors are expressed on astrocytes, they exhibit nicotine sensitivity and $[Ca^{2+}]_i$ increase (Oikawa et al., 2005; Delbro et al., 2009). Ethanol causes reactive oxygen species production, and $[Ca^{2+}]_i$ increase and glutamate secretion from astrocytes (Salazar et al., 2008). Astrocytes exposed to ethanol also exhibit alterations in Golgi complex morphology, secretory vesicle biogenesis, and expression levels of Rab GTPases and motor proteins (Tomas et al., 2005), which may be an additional factor for the dysfunction of brain development caused by ethanol.

Because adenosine plays a critical role in the control of sleep-wakefulness (Thakkar et al., 2003), and chronic alcoholism is frequently accompanied by sleep disorders (Brower, 2001), changes in sleep pattern may also induce alteration in gliotransmitter release. Interestingly, hypothalamic astrocytes from rats following sleep deprivation exhibited different proteome profiles, and the expression of VAMP2, which is an essential protein for vesicular exocytosis (Kim et al., 2014), was significantly increased. These findings suggest a strong association between alcohol intake, sleep disorders, and astrocytic gliotransmission.

Additionally, certain ambient ultrafine particles, which are defined as particulate substances with a diameter less than 100 nm, are emerging as another toxic substance that may deleteriously affect brain function (Block and Calderón-Garcidueñas, 2009; Loane et al., 2013). In a recent study, ultrafine carbon black, a surrogate for ultrafine particles, was shown to induce the release of glutamate and ATP from astrocytes by activating Cx43 and pannexin-1 hemichannels (Wei et al., 2014).

Recent epidemiological and experimental studies have demonstrated that children born from mothers who are exposed to infections or are addicted to alcohol or drugs have a higher risk of neuronal disorders and abnormal behaviors (Jacobsen et al., 2006; Stringari et al., 2008; Boksa, 2010; Brolese et al., 2015). However, the effects of these agents on astrocytes still remain largely unknown. Some studies have shown that prenatal exposure to lipopolysaccharides or nicotine together with postnatal high-fat/cholesterol diet result in enhancement of Cx43 hemichannel activity, and consequently increases the release of glutamate and ATP (Orellana et al., 2014; Avendano et al., 2015).

CONCLUSIONS

Over several decades, researchers have attempted to understand the properties and pathologies of the CNS by focusing solely on neurons; however, recent improvements in molecular and cellular imaging techniques are increasingly indicating that this neurocentric approach needs to be revised. In addition to neurons, glial cells including astrocytes are important elements for brain functions. Astrocytes are located in close morphological and functional relationships with blood vessels and neurons, and various genetic or environmental factors are implicated in gliotransmission impairment. Considering these characteristics of astrocytes, further studies will provide new insight on the significance of gliotransmitter release for fetal neurodevelopment. Thus, new therapies can be developed to overcome environmental chemical-induced neurodevelopmental disorders.

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KH, TK, and TT wrote the paper.

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Estrogenic Effects of Several BPA Analogs in the Developing Zebrafish Brain

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Important set of studies have demonstrated the endocrine disrupting activity of Bisphenol A (BPA). The present work aimed at defining estrogenic-like activity of several BPA structural analogs, including BPS, BPF, BPAF, and BPAP, on 4- or 7-day post-fertilization (dpf) zebrafish larva as an *in vivo* model. We measured the induction level of the estrogen-sensitive marker *cyp19a1b* gene (Aromatase B), expressed in the brain, using three different *in situ/in vivo* strategies: (1) Quantification of *cyp19a1b* transcripts using RT-qPCR in wild type 7-dpf larva brains exposed to bisphenols; (2) Detection and distribution of *cyp19a1b* transcripts using *in situ* hybridization on 7-dpf brain sections (hypothalamus); and (3) Quantification of the *cyp19a1b* promoter activity in live *cyp19a1b*-GFP transgenic zebrafish (EASZY assay) at 4-dpf larval stage. These three different experimental approaches demonstrated that BPS, BPF, or BPAF exposure, similarly to BPA, significantly activates the expression of the estrogenic marker in the brain of developing zebrafish. *In vitro* experiments using both reporter gene assay in a glial cell context and competitive ligand binding assays strongly suggested that up-regulation of *cyp19a1b* is largely mediated by the zebrafish estrogen nuclear receptor alpha (zfERα). Importantly, and in contrast to other tested bisphenol A analogs, the bisphenol AP (BPAP) did not show estrogenic activity in our model.

Keywords: 17β-estradiol, aromatase, cyp19a1b, bisphenol, BPA, hypothalamus, endocrine disruption

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INTRODUCTION

Estrogens play important roles in many developmental and physiological processes (Boon et al., 2010) and references therein, including for brain development (Hojo et al., 2008; Hill and Boon, 2009; Bondesson et al., 2014; Coumailleau et al., 2015). Bisphenol A (BPA) is a well-known chemical compound that mimic and interfere with the actions of endogenous estrogens and thus act as an endocrine disruptor (Krishnan et al., 1993; Gould et al., 1998; Paris et al., 2002; Kitamura et al., 2005; Richter et al., 2007; Wetherill et al., 2007; Vogel, 2009; Grignard et al., 2012). BPA is a chemical used in many industrial and commercial applications including in the production of polycarbonate plastics and epoxy resins, present in a variety of consumer products such as food-packaging materials, toys, thermal and recycled papers, compact discs, impact-resistant safety equipment, and medical devices to name a few (Vinggaard et al., 2000; Vandenberg et al., 2010; Liao and Kannan, 2011; Staples et al., 2011; Huang et al., 2012; Liao et al., 2012a,c). BPA rapidly became one of the most produced and used chemicals worldwide (about 3.4 million tons per year).

The widespread use of BPA has resulted in its detection in environment (Huang et al., 2012; Liao et al., 2012b), in food (Schecter et al., 2010; Liao and Kannan, 2013), and in human biological sample (Sun et al., 2004; Calafat et al., 2005, 2009; Vandenberg et al., 2010; Zalko et al., 2011; Fenichel et al., 2012; Liao et al., 2012a; Vandenberg et al., 2012). Epidemiological studies, along with laboratory studies in many species including primates, provide increasing support that environmental BPA exposure can be harmful to humans and is associated with a wide range of effects in humans, rodents, and wildlife. Indeed, BPA exposure is linked to numerous adverse health concerns including development, diabetes, obesity, cardiovascular, reproductive disorders, behavioral troubles, chronic respiratory and kidney diseases, and carcinogenesis, likely linked to the endocrine disrupting effects (Vandenberg et al., 2012; Rochester, 2013; Rezg et al., 2014). Owing those potential health concerns, Canada (2009), USA (2010), and the European Union (2011) prohibited the use of BPA in the manufacture of polycarbonate feeding bootles for infants. In France, since January 2015, BPA is forbidden in any food or beverage packaging.

Such restrictions on BPA usage recently led manufactories to use alternative bisphenols. Such alternatives include among others bisphenol AF, bisphenol F, and bisphenol S (Liu et al., 2012). For instance, BPS is found in canned soft drinks, canned foods (Vinas et al., 2010; Gallart-Ayala et al., 2011) and in thermal receipt papers (Becerra and Odermatt, 2012; Liao et al., 2012c). BPAF is also incorporated into the production of fluoropolymers, fluoroelastomers, and in a variety of polymers that are used in the manufactoring of electronic devices and plastic optical fibers (Yang et al., 2012). Recent large-scale quantitative studies have identified, in addition to BPA, increasing concentrations of various bisphenols such as BPAF, BPAP, BPF, BPS, BPB, BPZ, and BPP in food products in the United States (Liao and Kannan, 2013). In addition, BPB, BPF, and BPS were also detected in indoor dust in the USA and in several Asian countries (Liao et al., 2012b). However, despite the increasing use of BPA analogs, there is limited information on potential toxicity and endocrine-disrupting activities of these molecules. However, few BPA analogs present some endocrine disrupting activity, as assessed by *in vitro* analysis. For instance, it was shown that BPS and BPAF can bind to estrogen receptors and subsequently exert estrogenic activity at the transcriptional level using cell culture and binding assays (Hashimoto et al., 2001; Kitamura et al., 2005; Kuruto-Niwa et al., 2005; Matsushima et al., 2010; Grignard et al., 2012). Although the estrogenic potential of few BPA analogs have been demonstrated *in vitro*, the *in vivo* potential endocrine-disrupting activity of these compounds remains largely unknown. Recent physiological studies suggest that at least a few BPA analogs have the potential to interfere and disrupt the normal functions of endocrine system in various organisms (Feng et al., 2012; Ji et al., 2013; Naderi et al., 2014; Yang et al., 2014; Eladak et al., 2015). A growing number of studies have shown that BPA has a negative impact on neural development and on the onset of neurological disorders, likely associated to its endocrine-disrupting activities (reviewed in Kajta and Wojtowicz, 2013; Leon-Olea et al., 2014; Negri-Cesi, 2015). To our knowledge, very limited work has assessed estrogenic activity of BPA analogs during brain development, and/or in adult brain. A recent study suggests that exposure to BPS might cause hyperactivity and brain changes in developing zebrafish (Kinch et al., 2015).

In the present study, we assessed the potential *in vivo* estrogenic activities of various BPA analogs and their effects on the central nervous system using the developing zebrafish brain. The developmental pattern of the zebrafish is particularly well-studied (Briggs, 2002) and the species is a widely used model to evaluate the potential adverse effects of chemicals present in the environment and to define the mechanisms underlying the endocrine-disrupting activities (Segner, 2009).Indeed, numerous estrogen-sensitive proteins have been identified in zebrafish, including the liver-produced yolk proteins Vitellogenin 1 and 3 (encoded by vtg1 and vtg3 genes), and the brain-specific aromatase B (AroB), encoded by the brain specific *cyp19a1b* gene, and change in their expression can be used as biomarker for estrogen or xenoestrogen exposure (Kausch et al., 2008; Ruggeri et al., 2008; Levi et al., 2009; Chung et al., 2011; Lam et al., 2011; Hao et al., 2013). We and others have shown that the *cyp19a1b* gene is specifically expressed in a very specific brain population, the radial glial cells, that serves as progenitors during embryonic and adult neurogenesis (for review see Diotel et al., 2010; Coumailleau et al., 2015; Pellegrini et al., 2015). In addition, the presence of functional estrogen response elements in *cyp19a1b* proximal promoter region allows for a strong transcriptional upregulation by estrogens (E2) and xenoestrogens such as ethinyl estradiol (EE2) and BPA (Le Page et al., 2006; Sawyer et al., 2006; Chung et al., 2011; Brion et al., 2012). Thus, the *cyp19a1b* gene can be used *in vivo* as a biomarker of xenoestrogen effects on the central nervous system in developing and adult zebrafish.

In the present work, we investigated the effects of various BPA analogs on *cyp19a1b* expression in developing zebrafish brain exposed from 0 to 1 day post-fertilization (0–1 dpf) to 4–7 dpf. We used 3 different *in situ/in vivo* approaches: (1) quantitative RT-PCR to monitor the expression levels of *cyp19a1b* in wild type larvae (7 dpf); (2) *cyp19a1b in situ* hybridization to precisely analyse the induction and distribution of *cyp19a1b* transcripts in wild type 7-dpf larvae, and (3) the quantification of the brain fluorescence of *cyp19a1b*-GFP transgenic 4-dpf larvae as an *in vivo* assay (EASZY assay). We demonstrate that the majority of the tested bisphenol A analogs (BPS, BPF, and BPAF) induces *in vivo* significant expression of *cyp19a1b* in the brain of zebrafish at early developmental stages.

MATERIALS AND METHODS

Chemicals

Bisphenol analogs, including bisphenol A [BPA; 2,2-bis(4-hydroxyphenyl)propane; 99%), bisphenol F [BPF; 4,4′-dihydroxydiphenyl methane; 98%), bisphenol AF [BPAF; 2,2-bis(4-hydroxylphenyl)hexafluoropropane; 98%), bisphenol S [BPS; bis-(4-hydroxyphenyl)sulfone; 98%), bisphenol AP [BPAP; 4,4′-(1- phenylethylidene)bisphenol; 98%], were purchased from Sigma-Aldrich (St. Louis, MO) and TCI America (Portland, OR). E2 [17β-estradiol] and EE2 [17α-ethinylestradiol] were purchased from Sigma Aldrich (St. Louis, MO, USA). ICI 182

780 was purchased from Tocris Bioscience. Stock solutions were prepared in dimethyl sulfoxide (DMSO; Sigma) and kept at −20°C. Dilution series were freshly prepared before each experiment. The maximum volume of the solvent did not exceed 0.1% (v/v).

Zebrafish Maintenance and Embryo/Larva Exposure

Animals were treated in agreement with the European Union regulations concerning the protection of experimental animals. This study was approved by the ethics committee (CREEA: Comité Rennais d'Ethique en matière d'Expérimentation Animale) under permit number EEA B-35-040. Zebrafish embryos were raised in our facilities (IFR 140, INRA SCRIBE, Rennes, France) in recirculated water kept at 28.5°C and spawned under standard conditions. Embryos were collected 2 h post-fertilization (hpf), and examined under a binoccular. Those embryos that had developed normally were selected and kept in several Petri dishes (zebra.s- c.edu/guides.html) in an incubator at 28.5°C (kept on a 14-h light, 10-h dark cycle).

For subsequent RT-qPCR and *in situ* hybridization analysis with wild type zebrafish, groups of 80 embryos (for each condition) were placed after 1 day post-fertilization (1-dpf) in a large glass flask containing 100 ml of embryonic medium (Mouriec et al., 2009b). Chemical treatments were performed by adding either DMSO alone (negative control), EE2 (positive control), or one of the tested bisphenols (BPA, BPS, BPF, BPAF, BPAP) diluted in water at indicated concentrations, thereby creating 7 experimental treatment conditions. The embryos were held in the exposure flasks until 7-dpf larva stage. For studies with the *cyp19a1b-GFP* transgenic zebrafish line (Tong et al., 2009; Brion et al., 2012), groups of 20 embryos (for each condition) were placed 2 h post-fertilization in crystallization dishes containing 25 ml medium and at indicated concentration of tested bisphenols analogs, EE2, or DMSO. Embryos were maintained in the exposure dishes until 4-dpf larva stage. During the treatment period, 100% of the exposure medium was renewed every 24 h (for both wild type and transgenic zebrafish). No mortality was observed for any treatments during the exposure period.

Quantitative Real-Time PCR

After exposure, approximately 70 wild type heads were collected at 7 dpf (i.e., 6 days of exposure) for each experimental conditions (DMSO, EE2, BPA, BPS, BPF, BPAF, and BPAP) into 1.5 ml Eppendorf tubes, and frozen in liquid nitrogen. Tissue was sonicated (10 s, 3 times) in 250 μL Trizol Reagent (Invitrogen) and RNA extractions were carried out according to the manufacturer's protocol. Reverse transcription was carried out by incubating 2μg total RNA with 1μg of random primer oligonucleotides, 2.5mM dNTPs, and 50 U MMLV-RT (Promega) in the appropriate buffer for 10 min at 65°C and 60 min at 37°C. Quantitative Polymerase chain reaction (qPCR) experiments were performed in an iCycler thermocycler coupled to the MyiQ detector (Bio-Rad. Hercules, CA, USA) using iQ SYBR-Green Supermix (Bio-Rad) according to the manufacturer's protocol. The following primers were used: *ef1* (fw) 5′-AGCAGCAGCTGAGGAGTGAT-3′; *ef1* (rev) 5′-CCGCATTTGTAGATCAGATGG-3′; *cyp19a1b* (AroB; fw) 5′-TCGGCACGGCGT- GCAACTAC-3′; *cyp19a1b* (AroB; rev) 5′-CATACCTATGCATTGCAGACC-3′. For each condition, the RT-PCR experiment was run in triplicates. Expression levels of *ef1* mRNA were used to normalize *cyp19a1b* expression levels. Melting curve and PCR efficiency analyses were performed to confirm correct amplification. For quantification of PCR results, the threshold cycle (Ct) was determined for each reaction. Ct values for each gene of interest were normalized with the housekeeping gene *ef1*, using the ΔΔCt method. Normalized values were used to calculate the degree of induction or inhibition expressed as a "fold difference" compared to normalized control values.

Brain Sections and *In situ* Hybridization

Larvae used for *in situ* hybridization experiments originated from the same exposition groups than RT-qPCR experiments. After exposure to the different conditions, 10 wild type 7-dpf larvae (for each treatment) were fixed overnight at 4°C in 4% paraformaldehyde, before embedding in parrafin. Serial thin transverse sections (8 μM) were placed on cryofrost slides and subjected to *in situ* hybridization experiments. Sense and antisense digoxigenin-labeled riboprobes for the *cyp19a1b* gene were transcribed using the Digoxigenin RNA labeling kit in accordance with the manufacturer's instructions (Roche, Mannheim, Germany) and as previously described (Menuet et al., 2005). The brain sections were processed for *in situ* hybridization using stringent conditions as previously published (D'Amico et al., 2011, 2013). After NBT/BCIP revelation, sections were counterstained with DAPI, and mounted in a drop of vectashield (Vector Laboratories). All sections were photographed with an Olympus PROVIS AX70 microscope with a digital camera (Olympus SP71), or a Nikon multizoom AZ100 macroscope with a DS-Ri1 color camera.

In vivo Imaging with the EASZY Assay

Quantification of fluorescence in transgenic *cyp19a1b*-GFP zebrafish larva brain was performed according to Brion et al. (2012). In this assay, estrogenic activity is detected in living 4 days-old larvae (treated or not with an estrogen mimic compound) from the observation of the reporter gene fluorescence in the radial glial cells. After exposure conditions (see above), live tg (*cyp19a1b*-GFP), 4 dpf larvae (20 specimens per condition) were observed in dorsal view and each specimen was photographed using a Zeiss AxioImager.Z1 fluorescence microscope equipped with a AxioCam Mrm camera (Zeiss GmbH, Göttingen, Germany). All photographs were taken using the same parameters: only the head was photographed under a X10 objective, with a 134 ms exposure time and maximal intensity. Photographs were analyzed using the Axiovision Imaging software and fluorescence quantification was performed using the ImageJ software (http://rsbweb.nih.gov/ij/). For each picture, the integrated density was measured, i.e., the sum of the gray-values of all the pixels within the region of interest. A gray-value of 290 was defined as background value. For each micrograph the fluorescence fold induction of fluorescence

was calculated comparing the integrated pixel density with the average fluorescence induction obtained in the control group.

Plasmid Constructions

The zfER-α, zfER-β1, and zfER-β2 expression vectors correspond to Topo-pcDNA3 expression vector (Invitrogen, San Diego, CA, USA), containing the coding regions of each zebrafish estrogen receptor cDNA as previously described (Menuet et al., 2002). The *cyp19a1b*-Luciferase plasmid consists of 500 bp of the proximal promoter region of zebrafish *cyp19a1b* gene, containing an ERE, coupled to the luciferase reporter gene (Menuet et al., 2002).

Glial Cell Culture and Transfection Experiments

Human U251-MG glial cells were maintained in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Saint Aubin, France) supplemented with 10% fetal calf serum (Biowest, Nuaillé, France), 4 mM L-Glutamine (Gibco, Carlsbad, CA, USA) and 1 mM Na-Pyruvate (Life Technologies, Saint Aubin, France) and kept at 37°C and 5% CO_2 atmosphere (Le Page et al., 2006). The medium was also supplemented with 20 U/mL penicillin, 20 μg/mL streptomycin, and 50 ng/mL amphotericin B (Gibco). For transfections, cells were plated in 24-well plates at a density of 25,000 cells/ml in the same medium, except the fetal calf serum was charcoal-treated and used at a concentration of 2.5%. Cells were transfected with 25 ng/well either the vector expression containing or not the the zfERα, zfERβ1, zfERβ2 coding region and cytomegalovirus [CMV]−β-Galactosidase, and 150 ng/well of the *cyp19a1b*-Luciferase reporter plasmid, using JetPEI as a transfection reagent (Polyplus Transfection, Illkirch, France). One day after transfection, U251-MG cells were treated with chemicals using same concentrations as *in vivo* experiments (10–9 M and 10–6 M, for EE2 and bisphenols, respectively) and DMSO as a vehicle (1/10.000). Luciferase activity was measured 24 h later (Luciferase assay system, Promega, Madison, WI, USA). β-Galactosidase activity was used to normalize transfection efficiency. Chemicals were tested in at least 3 independent experiments and each experiment was performed in triplicate.

Zebrafish Estrogen Receptor Competitive-Binding Assays

We also performed a competitive binding assay to test the binding properties of our compounds of interest with the three zfERs (Blair et al., 2000). The three zebrafish estrogen receptor proteins were synthesized using the Topo-pcDNA3 expression vector containing the coding region of zfERα, zfERβ1, and zfERβ2 (Menuet et al., 2002). The TNT Quick Coupled Transcription/Translation Systems kit (Promega, Madison, WI, USA) was used for synthesis of zfER proteins by adding 1 μg of each ER expression vector and according to the manufacturer's protocol. Efficiency of translation was assessed by SDS-PAGE (data not shown). After *in vitro* synthesis, 5 μl of zfERα, zfERβ1, or zfERβ2 were incubated overnight at 4°C with 10^{-9} M [^{3}H]-E2 in absence or presence of increasing concentrations of radioinert E2 (10^{-11} M, 10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M), BPA, BPF, BPS, BPAF, or BPAP (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M). The relative binding affinity for each compound was analyzed by their efficiency to move [^{3}H]-E2 from the zfER binding site. Results were expressed as a percentage of displaced [^{3}H]-E2 binding. The 10^{-7} M E2 containing 100-fold excess of radioinert E2 compared to [^{3}H]-E2 was considered as the non-specific binding (Blair et al., 2000). IC50 were calculated using GraphPad Prism, version 6.07.

RESULTS

In this study, we tested the estrogenic potentials of various bisphenols (BPA, BPS, BPAF, BPF, and BPAP) using the estrogen sensitive biomarker *cyp19a1b* gene in zebrafish brain. Three different experimental approaches were used to validate our results: RT-qPCR on whole brain extract and *in situ* hybridization on brain sections in wild type zebrafish larvae (7-dpf), and the EASZY assay on *cyp19a1b*-GFP zebrafish larvae (4-dpf). In addition, we performed two *in vitro* assays including a transfection experiment in a glial cell context and a competitive binding assay, in order to identify the nuclear estrogen receptors mediating up-regulation of the *cyp19a1b* gene.

Establishment of a Proper BPA Concentration Inducing cyp19a1b Gene in the Brain of 7-dpf Zebrafish Larvae

We first performed real-time RT-qPCR to determine the expression level of *cyp19a1b* mRNAs in the brain of 7-dpf zebrafish larvae exposed or not to bisphenol A (BPA) or ethinyl estradiol (EE2), a weak and a strong synthetic estrogenic compounds, respectively. Three different concentrations of BPA (0.1, 1, or 10 μM) were tested to identify the dose of BPA allowing the best *cyp19a1b* gene response, and compared to EE2 (1 nM) or control DMSO-treated larvae To overcome potential individual variations, we pooled 70 heads in each treatment group (DMSO, EE2, BPA 10, BPA 1, and BPA 0,1). In all treatment groups, no toxic or teratogenic effect was identified. Treated animals survived until 7-days larval stage and were identical to untreated larvae regarding morphology and motility (data not shown). After exposure, quantitative RT-PCR on brain extracts was then performed in triplicate as described in Materials and Methods. As shown in **Figure 1A**, exposure to EE2 induced a very strong overexpression (over 60-fold) of the *cyp19a1b* gene in the brain of 7-dpf larvae, in comparison to control larvae treated with DMSO only. A weaker induction, compared to EE2, was detected in larvae exposed to BPA ranging from 0.1 to 10 μM (**Figure 1A**). Interestingly, BPA concentration of 1 μM induced about 20-fold *cyp19a1b* transcripts levels, whereas 0,1, and 10 μM BPA concentrations had lower effects on *cyp19a1b* transcripts (9- and 12-fold inductions, respectively). As BPA was capable of stronger induction at a 1 μM concentration and did not caused notable toxicological effects, we therefore selected this specific concentration to analyze the effects of BPA analogs in subsequent experiments (RT-qPCR, *in situ* hybridization and EASZY assays). This concentration is also either below or similar to the bisphenols concentrations commonly used in studies on zebrafish embryos (Sun et al., 2009; Chung et al., 2011; Lam et al.,

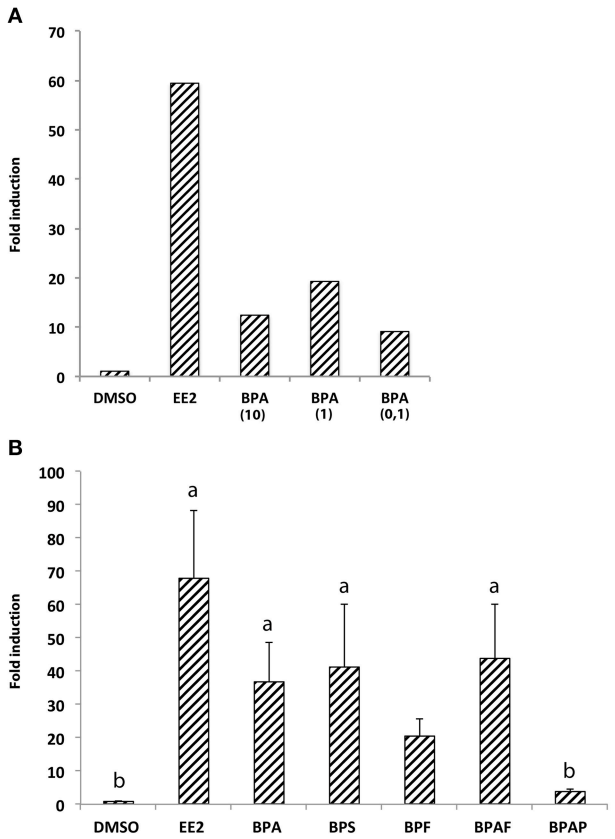


FIGURE 1 | (A) Expression of *cyp19a1b* gene in 7-dpf larvae brains upon treatments with EE2 (1 nM) and three different concentrations of BPA (0.1, 1, or 10 μM). RNA levels were measured in triplicate by real-time quantitative RT-PCR of total RNA prepared from pooled animals (70 per condition). Fold induction was expressed relative to the solvent (DMSO); **(B)** Expression of *cyp19a1b* gene in 7-dpf larvae brains upon treatments with EE2, BPA, and four individual BPA analogs (BPS, BPF, BPAF, and BPAP). For each chemical the concentration used is 1 μM excepted for EE2 (1 nM) and the vehicle (DMSO). RNA levels were measured in triplicate by real-time quantitative RT-PCR of total RNA prepared from pooled animals (70 per condition). Fold induction was expressed relative to the solvent (DMSO); Data in **(B)** are presented as mean ± SEM of six separate egg exposures derived from six independent spawns. **a**: $p < 0.05$ vs. DMSO; **b**: $p < 0.05$ vs. EE2.

2011; Staples et al., 2011; Wu et al., 2011; Keiter et al., 2012; Ji et al., 2013; Saili et al., 2013; Tse et al., 2013; Wang et al., 2013; Naderi et al., 2014).

Bisphenol A Analogs Induce cyp19a1b Gene Expression in Wild Type 7-dpf Larva Brain

The ability of BPA analogs to stimulate or not the expression of *cyp19a1b* estrogenic marker gene was then tested *in vivo* in the brain of wild type 7-dpf larvae. BPS, BPF, BPAF, and BPAP were tested at 1 μM on groups of 70 larvae. In addition, BPA (1 μM) and EE2 (1 nM) groups were used as positive controls, and a DMSO group as a negative control. Due to possible variations between egg batches, the entire experiment was repeated on six independent layings using the same standardized exposure protocols. Importantly, 1 μM treatments of BPA analogs did not affect the survival rate and the motility of exposed larvae (data not shown). In addition, no teratogenicity was observed all along the treatment and prior to the RT-qPCR analysis (data not shown). **Figure 1B** shows data obtained in the six independent experiments along with statistical analysis (Kruskal-Wallis). We found a general effect of the bisphenol analogs treatment on aromatase expression ($H = 26.95$, $p < 0.0001$). Dunn's *post-hoc* analysis showed a significant *cyp19a1b* induction by BPA, BPS, and BPAF in 7-dpf larvae (about 36-, 41-, and 43-fold induction compared to DMSO-treated larvae, respectively; **Figure 1B**). Although BPF exposure lead to an apparent increase in aromatase expression, this 20-fold up-regulation did not reach statistical significance. In contrast, BPAP had no effect on *cyp19a1b* gene.

In vivo Detection of cyp19a1b Promoter Activity Using cyp19a1b-GFP Transgenic Larvae Exposed to Bisphenols

To confirm the results obtained above, we tested the estrogenic activity of BPA analogs using the *cyp19a1b*-GFP transgenic zebrafish line, also named tg (cyp19a1b-GFP) (Tong et al., 2009). The use of tg (cyp19a1b-GFP) larvae was previously shown to be a very sensitive and fast assay (EASZY assay) to detect estrogenic activity (Brion et al., 2012; Petersen et al., 2013; Fetter et al., 2014). As described in Materials and Methods, experimental groups of 20 transgenic embryos were exposed for 4 days (from 2 h to 4 days post-fertilization) with 1 μM BPA, BPS, BPF, BPAF, or BPAP. In addition, control groups of transgenic embryos were exposed to either EE2 (1 nM) or DMSO alone. **Figure 2A** shows examples of the GFP signal generated in the whole brain by the different bisphenols together with the positive (EE2) and negative (DMSO) controls. Larvae treated only with DMSO (**Figures 2Ag,B**) show a basal GFP fluorescence, equivalent to those treated only with water (data not show). GFP induction occurs mostly in the midline of the brain, at the preoptic area level (arrow in **Figure 2Ag**). All other GFP fluorescence induction values were normalized in relation to this signal, considered as the basal activity of the *cyp19a1b* promoter (**Figure 2B**). Quantification of the signal and one-way ANOVA analysis revealed a significant overall effect of the treatment [$F_{(6, 85)} = 35.43$, $p < 0.0001$]. As expected, exposition of larvae to the synthetic estrogen EE2 (1 nM) strongly increased the GFP fluorescence intensity in the brain, attesting an important activity of the *cyp19a1b* promoter in the presence of EE2 (**Figures 2Aa,B**). An intense GFP signal is detected in the radial glial cells together with a much wider distribution from the telencephalon to the caudal hypothalamus (**Figure 2A**, compared **Figure 2Aa** and **Figure 2Ag**). As shown in **Figure 2B**, there was 31-fold induction of the GFP fluorescence in EE2-treated animals compared to DMSO-treated animals. The BPA also significantly increased the GFP fluorescence intensity (**Figures 2Ab,B**), albeit to a lower level compared to EE2 (16-fold induction compared to the DMSO control). Using this *in vivo* experiment, we also demonstrated that BPA analogs such as BPS, BPF, and BPAF also increased the GFP fluorescence in the

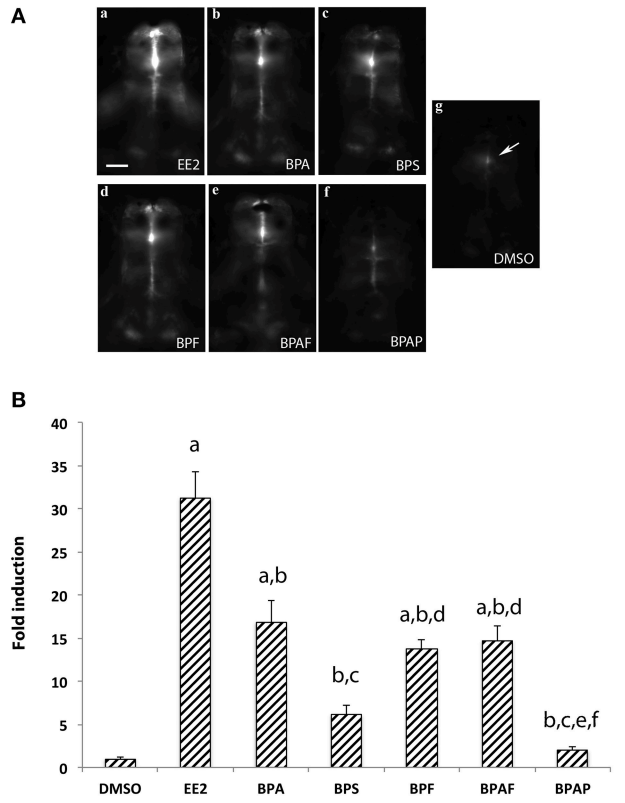


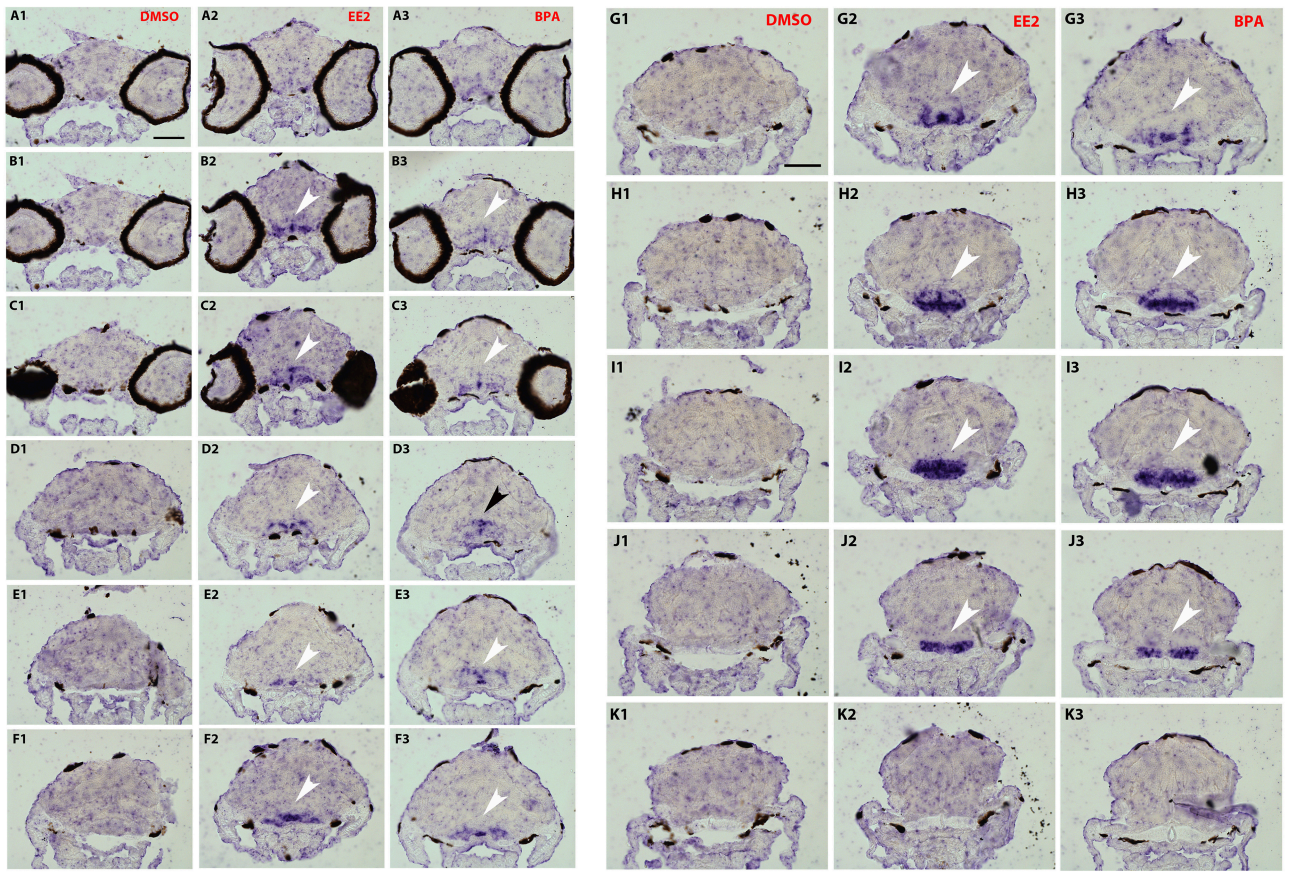
FIGURE 2 | (A) *In vivo* imaging of 4-dpf old live transgenic *cyp19a1b*-GFP zebrafish larvae exposed to EE2, BPA, and various bisphenol A analogs.For each chemical the concentration used is 1 μM excepted for EE2 (1 nM) and the vehicle (DMSO). Dorsal views of the brains. Anterior is to the top. Bar = 20 μm. **(B)** Quantification of GFP fluorescence in zebrafish larvae exposed to the various compounds. Results are expressed as fold induction above control. Data re presented as mean ± SEM of 20 specimens per condition ($n = 20$). **a**: $p < 0.05$ vs. DMSO; **b**: $p < 0.05$ vs. EE2, **c**: $p < 0.05$ vs. BPA, **d**: $p < 0.05$ vs. BPS, **e**: $p < 0.05$ vs. BPF, **f**: $p < 0.05$ vs. AF.

brain (**Figures 2Ac–e**). Quantifications of the GFP signal reveals that fluorescence intensity for BPAF and BPF (14- and 13-fold inductions, respectively) was similar to BPA and significantly higher than the control (**Figure 2B**). The 6-fold induction by BPS did not reach statistical significance (**Figure 2B**). In contrast, larvae exposed to BPAP did not affect GFP fluorescence signal (**Figures 2A,B**). Taken together, these data show a correlation between the induction of the *cyp19a1b* promoter activity in the transgenic zebrafish line (trough measuring GFP fluorescence) and the *cyp19a1b* gene expression that we observed in RT-qPCR analysis in wild type larvae with BPA, BPS, BPF, and BPAF (**Figure 1B**). In addition, we also confirm that BPAP did not significantly induce *cyp19a1b* gene expression.

Effects of Bisphenols on cyp19a1b Transcripts Distribution in the Developing Zebrafish Brain

To analyze a possible change in the distribution of *cyp19a1b* transcripts in the brain following bisphenol treatment, we performed *in situ* hybridization on serial and thin transverse sections of wild type exposed larva brains (7-dpf). We first compared *cyp19a1b* expression patterns in larvae exposed to EE2, BPA or DMSO alone. As shown in **Figures 3**, **4**, *cyp19a1b* transcripts cannot be detected on thin sections along the rostral-caudal axis of brains that were only treated with DMSO (**Figures 3**, **4A1-K1**). In contrast, EE2-treated larvae show a massive over-expression of the *cyp19a1b* transcripts in several and specific regions of the brain, notably in posterior telencephalon, preoptic area (arrows in **Figures 3B2–D2**) and caudal hypothalamus (lateral and posterior ventricular recesses; arrows in **Figures 4G2–J2**). Importantly, BPA-treated larvae also displayed strong presence of *cyp19a1b* transcripts in virtually identical regions than EE2-treated larvae (arrows in **Figures 3**, **4A3–K3**). These *in situ* hybridization data confirm and extend previous studies showing that both estrogen and xenoestrogens increase *cyp19a1b* RNA levels in these brain regions (Menuet et al., 2005; Lassiter and Linney, 2007; Mouriec et al., 2009a; Tong et al., 2009; Chung et al., 2011).

As the strongest induction of *cyp19a1b* transcripts along the rostro-caudal axis of the brain was observed in the caudal hypothalamus, notably in the area of the nucleus recessus posterioris (nrp) (**Figures 4I2,3**), we therefore focused our analysis on this brain particular region for the assessment of estrogenic activities of BPA analogs. As shown in the **Figure 5**, we confirmed that no *cyp19a1b* transcript was detectable in the nrp of DMSO-treated larva (**Figures 5A,C**), whereas strong levels of *cyp19a1b* transcripts were found in BPA-treated larva (**Figures 5B,D**). Most importantly, a similar high detection of *cyp19a1b* transcripts was also observed in this hypothalamic region with other bisphenols such as BPS, BPAF, and BPF (**Figures 5E–H**). These *in situ* data are in perfect agreement with the above RT-qPCR and EASZY assays (**Figures 1**, **2**). For BPAP-treated larva, there was also an increase in *cyp19a1b* transcripts in the nrp region (**Figure 5H**) albeit to a lower levels compared to specimens treated with other bisphenols (BPA, BPS, BPF, and BPAF). Although no significant increase of *cyp19a1b* transcripts was observed in BPAP-treated animals using RT-qPCR and EASZY assays (**Figures 1**, **2**), we decided to analyze in close details the distribution of *cyp19a1b* transcripts in the whole brain of BPAP-treated larva. As clearly shown in **Figure 6**, BPAP induces expression of *cyp19a1b* transcripts in only a few (xeno) estrogen-sensitive *cyp19a1b* expression sites. In particular, *cyp19a1b* transcripts were not observed in the anterior regions of the brain, corresponding to the posterior telencephalon and the preoptic area (**Figures 6B2–E2**). For BPS-, BPF-, and BPAF-treated animals, the *cyp19a1b* transcripts distribution patterns in the whole brain were identical to EE2- and BPA-treated animals, including in brain regions other than the nrp (dat not shown). Taken together, the absence of *cyp19a1b* transcripts in anterior regions of the brain and the weak detection of *cyp19a1b* transcripts in the caudal hypothalamus argue and confirm that BPAP has almost no estrogenic activity in the brain compared to other tested bisphenols (BPA, BPF, BPAF, and BPS).



FIGURES 3, 4 | Distribution of *cyp19a1b* transcripts in 7-dpf old zebrafish brains after treatments with EE2 (A2–K2) and BPA (A3–K3) and compared to the DMSO control (A1–K1). Images of transverse sections through the rostrocaudal axis of brains. Arrowheads highlight areas of labeling. For all images, dorsal is to the top. Scale bar = 50 μm.

Bisphenols Induce cyp19a1b Activity through Estrogen Receptors in a Glial Cell Model

To investigate in more detail the mechanism of bisphenols-induced transcription of *cyp19a1b* in radial glial cells of the brain, we performed functional *cyp19a1b*-luciferase reporter gene assay in a reconstituted glial cell line model (Le Page et al., 2006). We tested the impact of each bisphenol on the transcriptional activity of the three distinct zebrafish estrogen nuclear receptors (ERα, ERβ1, and ERβ2) transfected in U251MG cells, an ER-negative human glial cell line. In this assay, the zebrafish *cyp19a1b* promoter upstream of the luciferase is used as the reporter gene. We evaluated *trans*-activation properties of the different zebrafish estrogen nuclear receptors (ERα, ERβ1, and ERβ2) upon individual bisphenol treatments (BPA, BPS, BPF, and BPAF), EE2 and DMSO in six independent transfection experiments. As shown in **Figure 7A**, a priori analysis using a one-tailed *t*-test confirmed previous studies (Le Page et al., 2010) showing a strong induction of the *cyp19a1b*-luciferase reporter gene upon EE2 treatments in cells transfected with either ERα ($t = 3.01$ $df = 10$, $p < 0.01$), ERβ1 ($t = 1.927$ $df = 10$, $p < 0.05$) or ERβ2 ($t = 2.495$, $df = 10$, $p < 0.05$) compared to DMSO treatment. EE2-dependent activation of the *cyp19a1b* promoter was about 2- to 3-fold more efficient with ERα compared to ERβ1 and ERβ2. No reporter gene activity was found in absence of estrogen receptors (**Figure 7A**; empty plasmid), confirming that the transcriptional activity detected upon EE2 treatment is mediated by the presence of an estrogen nuclear receptor. We found a significant effect of the treatment [$F_{(6,82)} = 12.69$, $p < 0.0001$], estrogen receptor subtype [$F_{(3,82)} = 24.24$, $p < 0.0001$], and an interaction [$F_{(18,82)} = 3.53$, $p < 0.0001$] on luciferase expression. More precisely, *post-hoc* analysis revealed a significant stimulation of *cyp19a1b* promoter activity by BPA and BPAF in ERα-containing cells. BPF also increased luciferase activity in ERα cells, but the stimulation did not reach statistical significance. In contrast, no significant luciferase activity was found in cell transfected with subtype receptor ERβ1 or ERβ2 upon stimulation with any bisphenols. Independently of the estrogen receptor sub-type, BPAP, and BPS did not stimulate the reporter gene (**Figure 7A**). To further confirm that stimulation of *cyp19a1b* promoter upon BPA, BPF, and BPAF treatments was mediated by ERα–dependent transcription, we repeated the transfection experiments with ERα in presence or absence of

ICI 182 780, an antagonist of estrogen nuclear receptors. As shown in **Figure 7B**, simultaneous treatment with ICI completely abolished BPA, BPF, and BPAF stimulations found in ERα-expressing cells (**Figure 7A**). Taken together, the reporter gene assays in a glial cell context provide evidence that BPA, BPF, and BPAF are ERα agonists whereas BPS is likely to work through other mechanisms.

Bisphenols Binding to Zebrafish Estrogen Nuclear Receptors

Using *in vitro* competition assays strategy, we examined the receptor-binding affinity of BPA and BPA analogs (BPS, BPA, BPF, and BPAF) relative to [3H]17beta-estradiol for the three *in vitro* translated zebrafish estrogen receptors (ERα, ERβ1, and ERβ2). As expected, E2 show a high binding activity with the three zebrafish estrogen receptors (IC50: 1.5, 1.1, and 1.2 nM for ERα, ERβ1, and ERβ2, respectively; **Figures 8A–C**). In perfect agreement with reporter gene assays, we found that BPA, BPF, and BPAF bind *in vitro* to ERα receptor (**Figure 8A**). BPAF showed the highest affinity, followed by BPA and BPF (IC50; 0.076, 2.8, 10.6 μM, respectively). In addition, these receptor-binding activities were clearly reduced with ERβ1 and ERβ2 sub-type receptors (**Figures 8B–C**). In the competition assay with BPAF, the IC50 was almost 10 times stronger for ERβ1 [IC50 = 0.66 μM] than for ERα. Importantly, BPS displayed almost no binding affinities with any ER receptors (**Figures 8A–C**). These results suggest that the weak *cyp19a1b*-luciferase activity observed in the transfection experiment for BPS was probably due to the absence of binding to ERα.

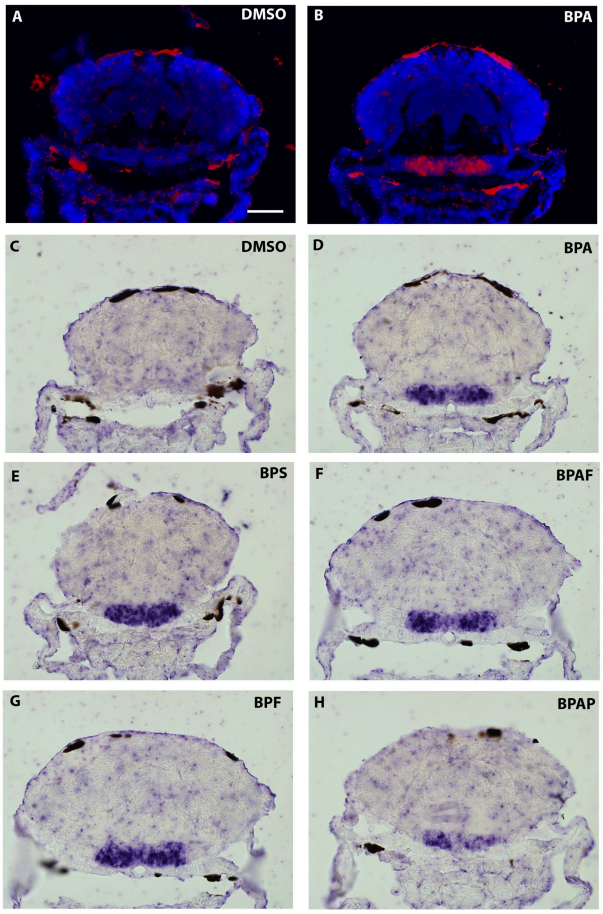


FIGURE 5 | *In situ* expression of *cyp19a1b* at the level of the caudal hypothalamus (7-dpf old larvae) after treatments with various BPA analogs (E–H) and comparison with negative (A,C) and positive (B,D) controls.

DISCUSSION

The present work investigated the effects of several bisphenol A analogs on *cyp19a1b* gene regulation, coding for aromatase B, a well-known target of (xeno) estrogen signaling pathways in the fish brain. We show here that BPA, BPS, BPF, and BPAF are able to up-regulate the aromatase B in the brain of developing zebrafish, using three different *in vivo* and *in situ* methods, i.e., RT-qPCR, *in situ* hybridization and the transgenic *cyp19a1b*-GFP.

The BPA exposure of zebrafish larvae led to a 36-fold over-expression of *cyp19a1b* gene in the brain compared to control 7-dpf larvae, as demonstrated by RT-qPCR (**Figure 1B**). This over-expression was confirmed by the EASZY assay with which we found a BPA-inducing activity of the *cyp19a1b* promoter by quantification of the GFP fluorescence in transgenic *cyp19a1b*-GFP brains in 4-dpf larvae (**Figure 2B**; 16-fold induction). The level of *cyp19a1b* induction is in agreement with previous RT-qPCR experiments carried out on whole zebrafish larvae that were exposed to BPA between 3 and 4-dpf developmental stages (Chung et al., 2011) and with previous experiments performed in our laboratory (Brion et al., 2012). Additionnally, *in situ* hybridization experiments on larva brain sections confirmed that BPA caused a strong expression of the *cyp19a1b* gene in specific areas of the brain, in particular in the hypothalamus (**Figures 3–5**). Thus, we show here that the combination of the three different techniques (RT-qPCR, EASZY assay, and *in situ* hybridization) is a valid strategy to investigate estrogenic properties of bisphenol compounds.

Our study indicates that BPA analogs, BPS, BPAF, and BPF, stimulate *cyp19a1b* expression *in vivo*. Indeed, in larvae treated with one of these three bisphenols, we observed a clear induction of the endogeneous *cyp19a1b* gene expression in the brain of wild type 7-dpf old zebrafish (**Figures 1B**, **5**) and up-stimulation of the *cyp19a1b* promoter in radial glial cells of GFP transgenic zebrafish larvae (4 dpf; **Figure 2**). The pattern of up-regulation of *cyp19a1b* by the three BPA analogs is similar to the modulation observed after BPA exposure, and therefore, strongly suggest that BPS, BPAF, and BPF exert estrogenic effects on developing zebrafish brain. It is also likely that numerous other estrogen sensitive responses, in the brain but also in other tissues, will also be directly affected by the presence of the bisphenol analogs. Moreover, the up-regulation of brain aromatase will lead to an elevated local enzymatic activity and therefore,

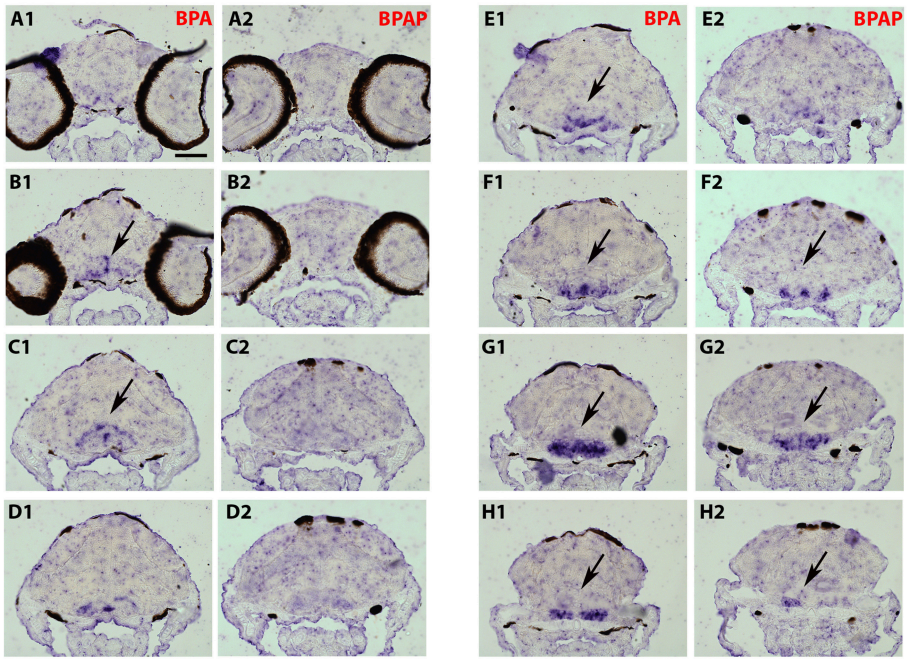


FIGURE 6 | Distribution of *cyp19a1b* transcripts in a 7-dpf old zebrafish brain after treatment with BPAP (A2–H2) and compared to a BPA-treated brain (A1–H1). Images of transverse sections through the rostrocaudal axis of brains. Arrowheads highlight areas of labeling. For all images, dorsal is to the top. Scale bar = 50 μm.

elevated levels of locally produced endogenous estrogen might be expected, adding to the direct estrogenic effect of bisphenol. This abnormal increase in estrogenic activity, considered as endocrine disrupting activity, will likely affect brain development at molecular, cellular, organ, and functional levels, as previously shown for BPA (Kajta and Wojtowicz, 2013; Rochester, 2013; Leon-Olea et al., 2014; Negri-Cesi, 2015). *In utero* or perinatal exposures to BPA in mammals leads to permanent disruptions in behavior, including increased levels of aggression and anxiety, and alterations in learning, memory, exploration, and emotional responsiveness (see for example, refereces Farabollini et al., 2002; Miyatake et al., 2006; Rubin et al., 2006; Kawai et al., 2007; Palanza et al., 2008; Tian et al., 2010; Galea and Barha, 2011; Wolstenholme et al., 2011; Xu et al., 2011). There is little information on potential *in vivo* effect of BPA analogs on brain development and function. However, studies at peripheric level provide evidence that BPA analogs can have adverse effects by interfering with the endocrine system. For instance, the balance of sex steroid hormones and normal reproduction was significantly affected in adult zebrafish following early (Naderi et al., 2014) or late (adult stage) exposure to BPS (Ji et al., 2013). In the later study, BPS exposure led to a significant increase of 17β-estradiol and decrease of testosterone in the plasma of male zebrafish, and these alterations were accompanied by an up-regulation of central and peripheral aromatase expression (both *cyp19a1a* and *cyp19a1b* genes). Similarly to BPS, BPAF exposure can also disrupt sex hormone levels and vitellogenin expression in zebrafish (Yang et al., 2014) and, in adult male rats, BPAF was associated with testosterone reduction by directly affecting testis function (Feng et al., 2012). In human fetal testis explants, low dose of BPS, or BPF is also sufficient to decrease basal testosterone secretion (Eladak et al., 2015). In the brain, it was recently shown that low-dose exposure to BPA and BPS might cause hyperactivity and brain changes in the developing zebrafish due to precocious hypothalamic neurogenesis (Kinch et al., 2015). Interestingly, such brain effects were paralleled with an increase of *cyp19a1b* expression. In *C. elegans*, BPA, and BPS exposure during early embryogenesis also affect neural functionality at adult stage (Mersha et al., 2015) and, in juvenile female rats, BPA, BPF, and BPS can affect 5α-reductase expression and dopamine-serotonin innervations in the prefrontal cortex (Castro et al., 2015).

The preoptic area and the hypothalamus are key integrative centers in the brain that play pivotal functions in the neuroendocrine regulation of homeostasis, reproduction, sexual behavior and stress response (Zohar et al., 2010 and references therein). The detailed analysis of the distribution of *cyp19a1b* transcripts on thin serial sections corresponding to the whole brain (7 dpf) provided evidence that exposure to xenoestrogenes, such as BPA or EE2, strongly induced expression of *cyp19a1b* transcripts in specific brain regions, including posterior telencephalon, preoptic area and caudal hypothalamus (**Figures 3**, **4**). Increase in *cyp19a1b* promoter activity in these specific brain regions was also detected in transgenic larvae (EASZY assays; **Figure 2**). The different brain regions where *cyp19a1b* was upregulated following bisphenol exposure were

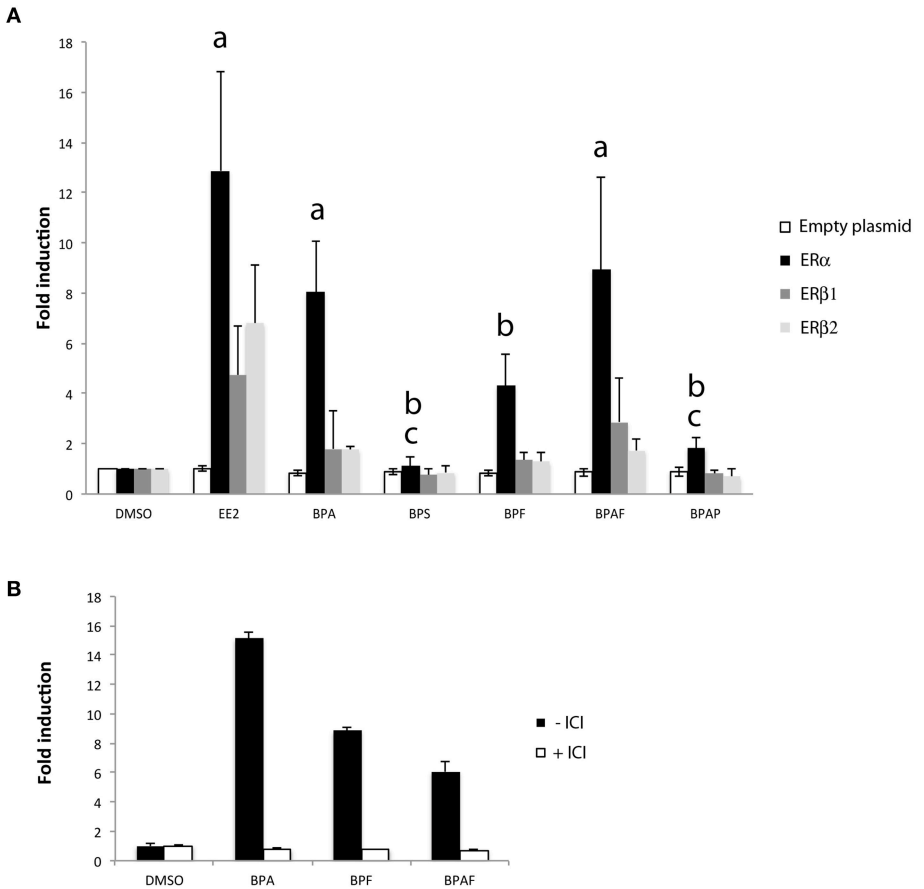


FIGURE 7 | (A) *Cyp19a1b*-luciferase assay in a glial cell context expressing either ERα, ERβ1, or ERβ2. Luciferase activity was measured in triplicate for each condition following treatment with EE2 or bisphenol analogs. Data are presented as mean ± SEM of five independent cellular treatment experiments. **a**: $p < 0.05$ vs. DMSO in ERα–containing cells; **b**: $p < 0.05$ vs. EE2 in ERα-containing cells; **c**: ERα vs. BPA. **(B)** *Cyp19a1b*-luciferase assay in a glial cell context expressing the ERα sub-type in presence or absence of ICI. Luciferase activity was measured in triplicate for each condition.

previously identified as sites of estrogen-induced *cyp19a1b* expression (Menuet et al., 2005; Lassiter and Linney, 2007; Tong et al., 2009; Mouriec et al., 2009a; Chung et al., 2011). In addition, we demonstrated that the caudal hypothalamus, and more precisely the lateral and posterior ventricular recesses (nrp), contains the highest amount of *cyp19a1b* transcripts after BPA or EE2 exposures. Importantly, BPS, BPF and BPAF were also able to strongly induce *cyp19a1b* expression in the nrp in the caudal hypothalamus, with a similar intensity compared to BPA and EE2 (**Figure 5**). Since various xenoestrogens can strongly stimulate *cyp19a1b* expression in similar and specific brain regions, this raise concern about the consequences of their combined actions on hypothalamic development and functioning.

Three distinct nuclear estrogen receptors (zfER) are characterized in zebrafish: ERα, ERβ1, and ERβ2, corresponding to *esr1*, *esr2b*, and *esr2a*, respectively. The three receptors can bind estradiol and are strongly expressed in the anterior and posterior preoptic area, and in the caudal hypothalamus (Menuet et al., 2002). As shown in this study, these two neuroendocrine regions are also the major sites of bisphenol-induced *cyp19a1b* expression (**Figures 3–6**). To define which estrogen receptors was implicated in the up-regulation of *cyp19a1b* gene, we used zebrafish *cyp19a1b* promoter luciferase reporter gene assay in a reconstituted glial cell context expressing one zebrafish ER subtype at a time (as previously reported (Menuet et al., 2005; Le Page et al., 2006). We showed here that BPA, used at a 10^{-6} M concentration, activates *cyp19a1b* promoter via its interaction with ER alpha, while the presence of either ER beta subtypes does not allow BPA to activate the promoter. BPF and BPAF are also able to stimulate *in vitro* the *cyp19a1b* promoter activity via the activation of ERα. This up-regulation was inhibited by the presence of the specific estrogen receptor antagonist ICI 182 780. (**Figure 7**). *In vitro* ligand competition assays confirmed that BPF and BPAF, in addition to BPA, physically bind zebrafish estrogen receptor alpha (**Figure 8**). Previous *in vitro* studies (reporter gene and ligand binding assays) performed in other cellular models also showed the ability of BPF and BPAF to act as an estrogen mimic that binds to estrogen receptors and subsequently exert *trans*-activation activities (Kitamura et al., 2005; Cabaton et al., 2009; Matsushima et al., 2010; Li et al.,

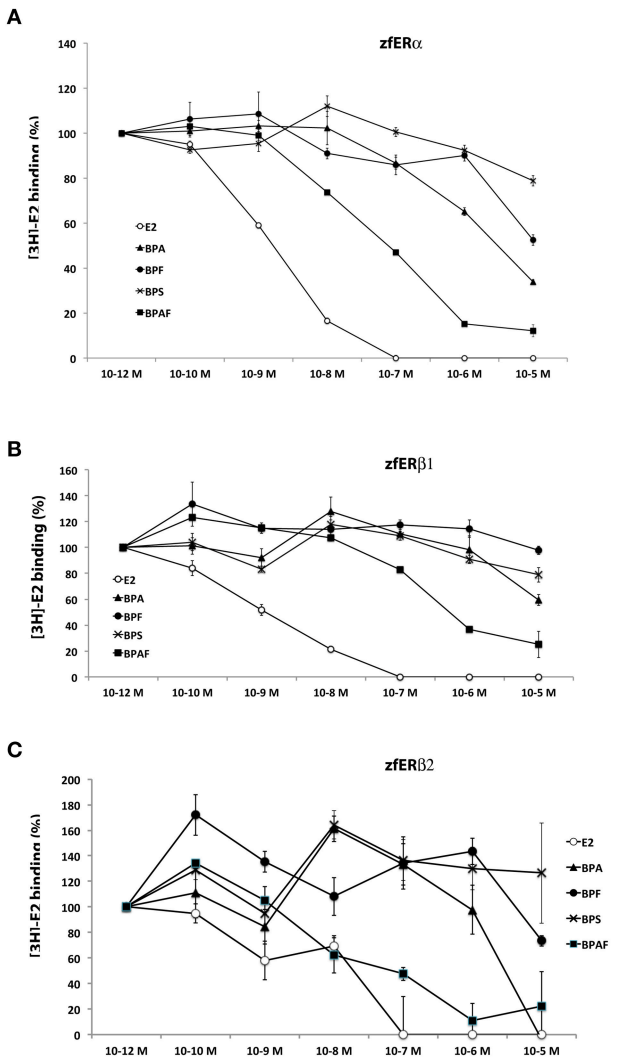


FIGURE 8 | *In vitro* bisphenols-and E2-binding activity to zebrafish estrogen receptors. (A–C) Competitive binding assays relative to [3H]17β-estradiol on ERα **(A)**, ERβ1 **(B)**, and ERβ2 **(C)**. Binding activity was measured in triplicate for each condition and data are presented as mean ± SEM of these 3 radioactivity countings. IC50 are provided in the Results section.

2012). In yeast assays, estrogenic activity for these BPA analogs was also reported (Hashimoto et al., 2001; Ruan et al., 2014).

As stated above, we showed that BPS significantly stimulated the expression of *cyp19a1b in vivo* but, interestingly, this effect did not involve the bisphenol-dependent activation of estrogen receptors in our *cyp19a1b*-luciferase reporter gene in the functional glial cell assay (**Figure 7**). This is in stark contrast with the data obtained with BPA, BPF, and BPAF, acting on *cyp19a1b* up-regulation via ERα subtype (**Figure 7**). It is possible that BPS has only a very weak affinity for the zebrafish estrogen receptors. This hypothesis was clearly reinforced by the ligand competition assays that showed almost no binding activity for BPS compared to other tested bisphenols (**Figure 8**). Previous *in vitro* studies have shown for BPS, lower (Chen et al., 2002; Kitamura et al., 2005; Rosenmai et al., 2014), similar (Hashimoto et al., 2001; Kitamura et al., 2005; Kuruto-Niwa et al., 2005; Grignard et al., 2012; Kang et al., 2014), or higher (Molina-Molina et al., 2013) estrogenic activity than BPA, depending on the experimental model. In the reporter gene assay, the lack of estrogenic activity for BPS could be linked to the absence of key transcriptional co-factors required for efficient estrogen receptor-dependent *trans*-activation. Alternatively, *in vivo* BPS-induced estrogenic activity could be mediated through a different pathway than estrogen receptors. BPS acts as an estrogen mimic in certain conditions, but can also antagonize androgen receptor (Hashimoto et al., 2001; Kitamura et al., 2005; Kuruto-Niwa et al., 2005; Grignard et al., 2012). Recent *in vitro* studies provide evidence that BPA analogs have a clear effect on androgen receptor activity as well as on steroid hormone synthesis (Rosenmai et al., 2014), suggesting that these compounds may interfere with the endocrine system through several modes of action. In addition, in the case of BPAF and in human breast cells, estrogenic activity has been proven to be mediated through both genomic (ERα) and nongenomic pathways (Li et al., 2014).

We did not observe a significant induction of the expression of *cyp19a1b* gene expression in the brain of larvae treated with BPAP, using RT-qPCR and EASZY strategies (**Figures 1**, **2**). These data were confirmed in the glial cell context *cyp19a1b*-luciferase reporter gene assay (**Figure 7**). The detailed analysis of *cyp19a1b* transcripts distribution carried out in the whole brains of BPAP-treated larvae revealed that *cyp19a1b* transcripts were, indeed, not detected in telencephalon or preoptic area and very weakly in the caudal hypothalamus (**Figure 6**). Taken together, these data suggest that BPAP might have no or a very weak estrogenic activity in the brain *in vivo*, compared to other bisphenols tested here (BPA, BPS, BPF, and BPAF). To the best of our knowledge, there is only one study that reports low estrogenic activities for BPAP in a recombinant gene yeast assay (Zhang et al., 2009). Our data suggest that BPAP might be a safer alternative to BPA and to other BPA analogs currently used. Presently, BPAP use in industry is not (yet) a common BPA substitute as its environmental concentrations, as measured in food or in indoor dust, is very low compared to other bisphenols (Liao et al., 2012b; Liao and Kannan, 2013).

In conclusion, this work shows that BPA, BPF, BPAF, and BPS exhibit estrogenic activity on the *cyp19a1b* gene (aromatase B), a brain specific gene, which is considered as one of the most, if not the most, E2-sensitive gene in fish (Brion et al., 2012; Lee et al., 2012; Petersen et al., 2013). Thus, BPA analogs, because of their widespread use and their potential to persist in the environment, may be equally as harmul as BPA to developing brains. In contrast, BPAP appears to have no estrogenic activity in the brain of zebrafish. To confirm that BPAP could be a safer alternative to BPA, studies investigating its effects on other tissues and signaling pathways will be required. A replacemant of BPA by any of these coumpounds should be considered with caution and further studies are clearly required to clarify the precise *in vivo* effects of BPA analogs and their mechanisms of actions.

AUTHOR CONTRIBUTIONS

PC, designed the study, developed the methodology, conducted experiments and wrote the manuscript. JC, developed methodology and conducted experiments. CV, maintained embryo and larva development and helped with qPCR analysis. TC, helped with the statistics and editing the article. OK, EP, helped with the design of the study and editing the article.

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In Utero Bisphenol A Exposure Induces Abnormal Neuronal Migration in the Cerebral Cortex of Mice

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Bisphenol A (BPA) has been known to have endocrine-disrupting activity to induce reproductive and behavioral abnormalities in offspring of laboratory animal species. However, morphological basis of this abnormality during brain development is largely unknown. Cerebral cortex plays a crucial role in higher brain function, and its precisely laminated structure is formed by neuronal migration. In the present study, transfecting a plasmid (pCAG-mCherry) by *in utero* electroporation (IUE), we visualized developing neurons and investigated the possible effects of *in utero* BPA exposure on neuronal migration. Pregnant mice were exposed to BPA by osmotic pump at estimated daily doses of 0, 40 (BPA-40), or 400 (BPA-400) μg/kg from embryonic day 14.5 (E14.5) to E18.5. IUE was performed at E14.5 and neuronal migration was analyzed at E18.5. Compared with the control group, neuronal migration in the cortical plate was significantly decreased in the BPA-40 group; however, there was no significant difference in the BPA-400 group. Among several neuronal migration-related genes and cortical layer-specific genes, TrkB in the BPA-400 group was found significantly upregulated. In conclusion, *in utero* exposure to low BPA dose was found to disrupt neuronal migration in the cerebral cortex in a dose-specific manner.

Keywords: bisphenol A, low dose, brain development, cerebral cortex, environmental chemicals, neuronal migration

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Ling W, Endo T, Kubo K, Nakajima K, Kakeyama M and Tohyama C (2016) In Utero Bisphenol A Exposure Induces Abnormal Neuronal Migration in the Cerebral Cortex of Mice. Front. Endocrinol. 7:7. doi: 10.3389/fendo.2016.00007

INTRODUCTION

Bisphenol A (BPA, 4,4′-dihydroxy-2,2-diphenylpropane) is a monomer used worldwide for manufacturing plastics, such as polycarbonates and epoxy resins. Humans are widely exposed to BPA *via* leaching from plastic bottles, sealants for canned food, and other environmental sources. Low

Abbreviations: BDNF, brain-derived neurotrophic factor; BPA, bisphenol A; BrdU, 5-bromodeoxyuridine; Cdk5, cyclin-dependent kinase 5; Cux2, cut-like homeobox 2; DISC1, disrupted in schizophrenia 1; E, embryonic day; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IUE, *in utero* electroporation; Kitl, kit ligand; Lhx2, LIM homeobox protein 2; MAP2, microtubule-associated protein 2; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% Triton X-100; PFA, paraformaldehyde; PND, postnatal day; Slitrk1, SLIT and NTRK-like family, member 1; Tle3, transducin-like enhancer of split 3, homolog of Drosophila E; TrkB, neurotrophic tyrosine kinase, receptor, type 2.

doses of BPA exposure during the perinatal period can result in numerous effects on health, ranging from reversible physiological responses to more long-term adverse effects. Exposure to BPA early in life has been reported to be associated with behavioral problems in children (1–3), presumably due to their limited capacity to metabolize BPA and to the fact that the blood–brain barrier is not fully developed. BPA has also been suggested to have adverse effects on neuronal development in human infants (4). Furthermore, animal studies have shown that offspring born to dams exposed to low doses of BPA during gestation and the early postnatal period had abnormal brain morphologies (5–9). However, the mechanisms by which maternal BPA exposure affects embryonic brain development are still largely unknown.

The laminated structure of the cerebral cortex is formed by highly tuned neuronal migration. Perturbations of this neuronal migration result in neurological and developmental abnormalities. Although neuronal migration has been suggested as a target of chemical exposure, it has not been widely studied in the context of developmental neurotoxicity. A few studies have reported that neuronal migration can be interrupted by environmental chemicals, such as methylmercury (10, 11) and toluene (12). The aim of the present study was to examine the possible effects of prenatal exposure to low doses of BPA on the process of neuronal migration. For this, we used *in utero* electroporation (IUE), a gene-transfer technique that enabled us to introduce fluorescent protein expression vectors into neuronal progenitor cells and visualize the process of migration (13, 14). We found that prenatal exposure to BPA interrupted neuronal migration in the cerebral cortex in a dose-specific manner.

MATERIALS AND METHODS

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 at a temperature of 22–24°C, humidity at 40−60%, and under 12-h light/12-h dark cycles (lights on and off at 0800 and 2000 hours, respectively). Food (Labo MR Stock, Nosan, Yokohama, Japan) and water were provided *ad libitum*. Pregnant mice were exposed to BPA (Wako Pure Chemical Ind., Osaka, Japan) at a daily dose equivalent to 0, 40, or 400 μg/kg b.w. from E14.5 to E18.5 by implanting an osmotic pump (Alzet, Micro-Osmotic Pump, Model 1007D, Cupertino, CA, USA) into the peritoneal cavity. According to the doses given to the dams, the control and BPA exposed groups were named as Control, BPA-40, and BPA-400, respectively. The experimental protocols for the animal experiments were approved by the Animal Care and Use Committee of the University of Tokyo.

A plasmid (pCAG-mCherry, a kind gift from Dr. Masanori Matsuzaki at the National Institute for Basic Biology, Okazaki, Japan) was purified using the EndoFree Plasmid Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's protocol. The purified plasmid was diluted with phosphate-buffered saline (PBS) to a final concentration of 3 μg/μl before use.

In utero electroporation was performed at E14.5, as described previously (13, 14). Briefly, time-pregnant mice were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital solution (Dainippon Sumitomo Pharma, Osaka, Japan) at a dose of 50 mg/kg b.w. After the uterus was carefully pulled out from the abdominal cavity, an aliquot (approximately 1 μl) of plasmid solution colored by 0.01% fast green was injected into the lateral ventricle of the embryo and was transfected by electroporation (30−35 V, 50 ms, four pulses) using a square wave electroporator (CUY21SC, Nepa Gene Co., Chiba, Japan) with a forceps-type electrode (CUY650P5). The uterus was returned to the abdominal cavity, and an osmotic pump was implanted in the peritoneal cavity, followed by a closure of the abdomen with sutures. In this study, plasmids were successfully transfected into more than half of embryonic brains.

Mice were sacrificed at E18.5. Embryonic brains were collected, fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4°C, and immersed consecutively in 20 and 30% sucrose in PBS at 4°C. Then, brains were embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan), and stored at −80°C until analysis. Frozen brains were cut into 20 μm thick coronal sections by cryostat (CM3050S, Leica Microsystems K.K., Tokyo, Japan).

To visualize the cortical plate (CP) boundary, microtubule-associated protein 2 (Map2) immunostaining was performed. Briefly, brain tissue sections were washed in PBS containing 0.05% Triton X-100 (PBST) and fixed in 4% PFA for 10 min. After blocking with 3% bovine serum albumins in PBST at room temperature for 1 h, the brain sections were incubated with anti-MAP2 antibodies that were conjugated to Alexa Fluor 488 (Merck Millipore Japan Headquarters, Tokyo, Japan) for 3 h. Following additional washing with PBST, the sections were mounted with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). Images were acquired using a Leica microscope (DM6000 B, Leica Microsystems K.K.), processed with Neurolucida (MBF Bioscience) and Image-J (NIH) software.

Cell distribution in the CP was evaluated using a bin analysis, as described previously (15). The CP was equally divided into 10 bins in which the bin closest to the ventricle was numbered as Bin 1 and the bin closest to the pia mater was numbered as Bin 10. In each bin, the number of fluorescent cells (mCherry-positive cells) was estimated as a percentage of the total number in all 10 bins, using Image-J software (National Institute of Health, Bethesda, MD, USA). Embryonic brains that were successfully transfected with fluorescent protein vectors were selected for cell migration analysis. One to three brains from each litter were randomly selected, and total two to four litters in each dosed group were analyzed.

For body weight and mRNA analyses, another set of BPA-exposed pregnant mice was used. At PND 0, we checked the litter size and body weight and randomly selected one male from each litter, followed by the analysis of a total of six pups in each dosed group. Forebrains were dissected, snap frozen in liquid nitrogen, and stored at −80°C until analysis.

Total RNAs were extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA synthesis was performed using the PrimeScript RT reagent Kit (Takara, Otsu, Japan). Quantitative real-time PCR was performed using the Thunderbird qPCR mix (Toyobo, Osaka, Japan) and LightCycler (Roche Diagnostic Co., Tokyo, Japan). Primer design and specificity check were performed by Primer-BLAST (NCBI, Bethesda, MD, USA). The mRNA expression of the target gene

in each sample was normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

For statistical analysis, one-way ANOVA (for litter size, body weight, and mRNA expression) and two-way ANOVA (for neuronal migration) followed by the Tukey–Kramer's *post hoc* test were used. All data are expressed as mean ± SEM. p-Values <0.05 were considered statistically significant.

RESULTS

There was no significant difference in litter size between control dams and BPA-exposed dams. In addition, no statistical differences in body weight were observed between groups for either sex at PND 0 (**Table 1**).

To investigate whether *in utero* BPA exposure affects neuronal migration, IUE was performed at E14.5 to introduce a fluorescent protein expression vector (pCAG-mCherry) into neural progenitor cells, and the distribution of mCherry-positive neurons was analyzed at E18.5 in the three groups (Control, BPA-40, and BPA-400) (**Figure 1A**). At E18.5, mCherry-positive cells were found in ventricular zone (VZ), subventricular zone (SVZ), intermediate zone (IZ), and CP (**Figures 1B,C**). Beneath the subplate (SP), not only mCherry-positive cells but also axons of projection neurons overlapped together exhibiting intense mCherry fluorescent signals, and it was very difficult to differentiate each single mCherry-positive cell from mCherry signals in IZ, so only the mCherry-positive cells migrated into the CP were subjected to a bin analysis to examine the cell distribution in CP (**Figure 1B**). In the control group, the majority of mCherry-positive neurons were located in layers II/III (corresponding to Bins 8 and 9) of the cerebral cortex. In the BPA-40 group, the percentage of mCherry-positive neurons in Bin 9 was significantly lower than that in the control group or the BPA-400 group. There were no significant differences in the distribution of mCherry-positive neurons in the CP between the BPA-400 group and the control group (**Figure 1D**). These data show that prenatal exposure to BPA suppresses neuronal migration in a dose-specific manner.

Neuronal migration is guided by various molecular cues. Thus, we examined the forebrain mRNA expression of genes that are known to be important for neuronal migration and layer formation, such as brain-derived neurotrophic factor (BDNF), neurotrophic tyrosine kinase receptor type 2 (TrkB), Reelin, cyclin-dependent kinase 5 (Cdk5), and disrupted in schizophrenia 1 (DISC1), and the neocortical layer II/III laminar-specific genes, such as transducin-like enhancer of split 3, homolog of Drosophila E (Tle3), kit ligand (Kitl), LIM homeobox protein 2 (Lhx2), cut-like homeobox 2 (Cux2), and SLIT and NTRK-like family, member 1 (Slitrk1) (**Figure 2**). TrkB, a receptor for neurotrophins, was significantly increased in the BPA-400 group

TABLE 1 | Litter size and pup body weight after *in utero* BPA exposure[a].

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

[a]*Data are shown as mean ± SEM.*
[b]*n = 6 dams per treatment.*

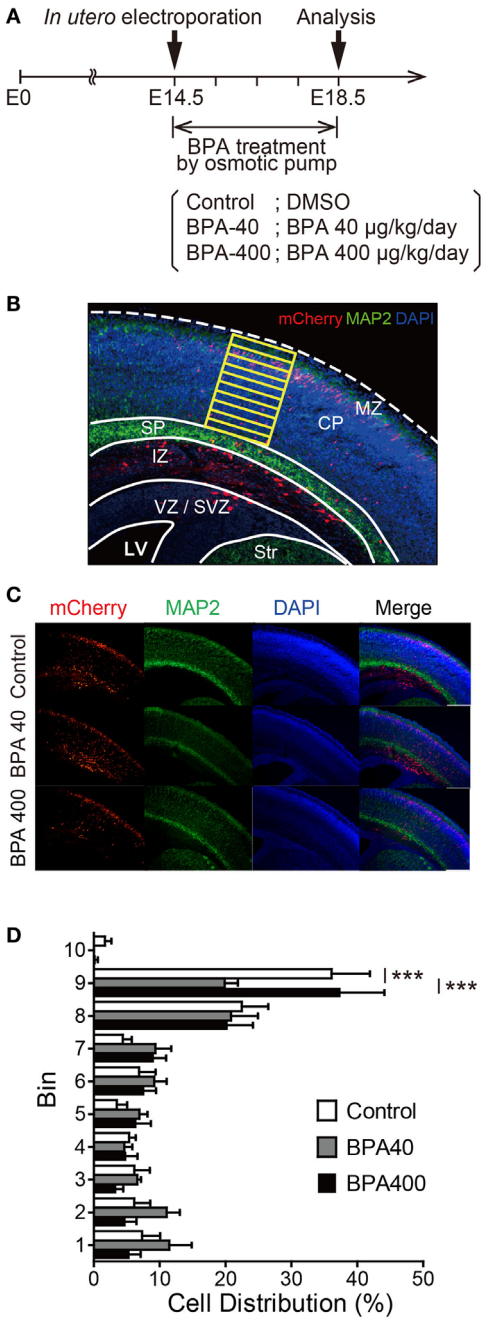

FIGURE 1 | Effects of prenatal BPA exposure on neuronal migration in the developing cerebral cortex. (A) Diagram of the experiment design. **(B)** Bin analysis was performed to evaluate differences in cell distribution. MZ, marginal zone; CP, cortical plate; SP, subplate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone; LV, lateral ventricle; Str, striatum. **(C)** Representative photographs of brain sections in the Control, BPA-40, and BPA-400 groups. **(D)** Distribution of mCherry-positive neurons in the CP at E18.5. Data are shown as mean ± SEM, $n = 4$ (control), $n = 5$ (BPA-40), $n = 5$ (BPA-400), $^{***}p < 0.01$.

compared with the control group. No significant difference in the mRNA expression of other genes was observed among the control, BPA-40, and BPA-400 groups.

DISCUSSION

In this study, we used IUE to transfect neural progenitor cells with plasmids that express fluorescent protein, and found that *in utero* low doses of BPA exposure significantly perturbed neuronal migration in the embryonic cerebral cortex in mice. The neurons born in VZ at E14.5 are mostly excitatory projection neurons that undergo radial migration in the cerebral cortex before reaching their final destination in layers II/III. Neurons located in layers II/III are commissural projection neurons extending axons to the opposite hemisphere across the corpus callosum. It has been reported that abnormalities in higher brain function observed in various neurological diseases are considered to be associated with the inappropriate positioning of neurons, which in turn leads to inaccurate projections and impairment of synaptogenesis (16, 17). Thus, it is possible that abnormal neuronal migration observed after low doses of BPA exposure could be a cause of abnormal brain function, but the direct link between delayed neuronal migration and high brain functions warrant future studies.

A previous study reported that BPA exposure from E0.5 at a daily dose of 20 μg/kg b.w. significantly decreased the number of 5-bromodeoxyuridine (BrdU)-positive cells in the VZ at E14.5 and E16.5 and increased the number of BrdU-positive cells in the CP at E14.5 compared with the control group, indicating that prenatal BPA exposure accelerates neuronal migration (18). However, the same research group also reported that when BrdU-positive cells were labeled at E14.5 in mice exposed to BPA *in utero*, there was subsequently a significant increase in labeled cells in cortical layers V and VI and a decrease in labeled cells in layer IV when the brains were examined at postnatal week 3 (19). Another *in vitro* study reported that BPA treatment increased the tangential migration of interneurons in cortical slices (20). In cerebral cortex, inhibitory neurons and excitatory neurons are born at different time periods and different places. Inhibitory neurons are born in the ganglionic eminence and migrate tangentially, whereas excitatory neurons are born in the VZ and migrate radially. The differences between results from previous studies and our study may be due to different exposure durations, doses, and neuronal types studied. The present study showed that the prenatal BPA exposure suppressed radial migration of excitatory neurons in the developing cerebral cortex.

The daily doses of 40 and 400 μg/kg used in this study are low doses compared with 5 mg/kg/day, which is regarded as the cutoff dose for low-dose effects regardless of the exposure route and duration (21). The observation of the present study, an abnormal neuronal migration in the mouse embryonic brain, can be added to the list of toxic phenotypes induced by low doses of BPA. Our study provides an example of a dose-specific response to BPA, as shown by the distinct disturbance of neural migration in the BPA-40 group that was absent in the BPA-400 group. A number of previous studies have investigated the effects of low doses of BPA and have shown that the dose–response curve has an inverted U-shape (22, 23). BPA can bind to the estrogen receptor and

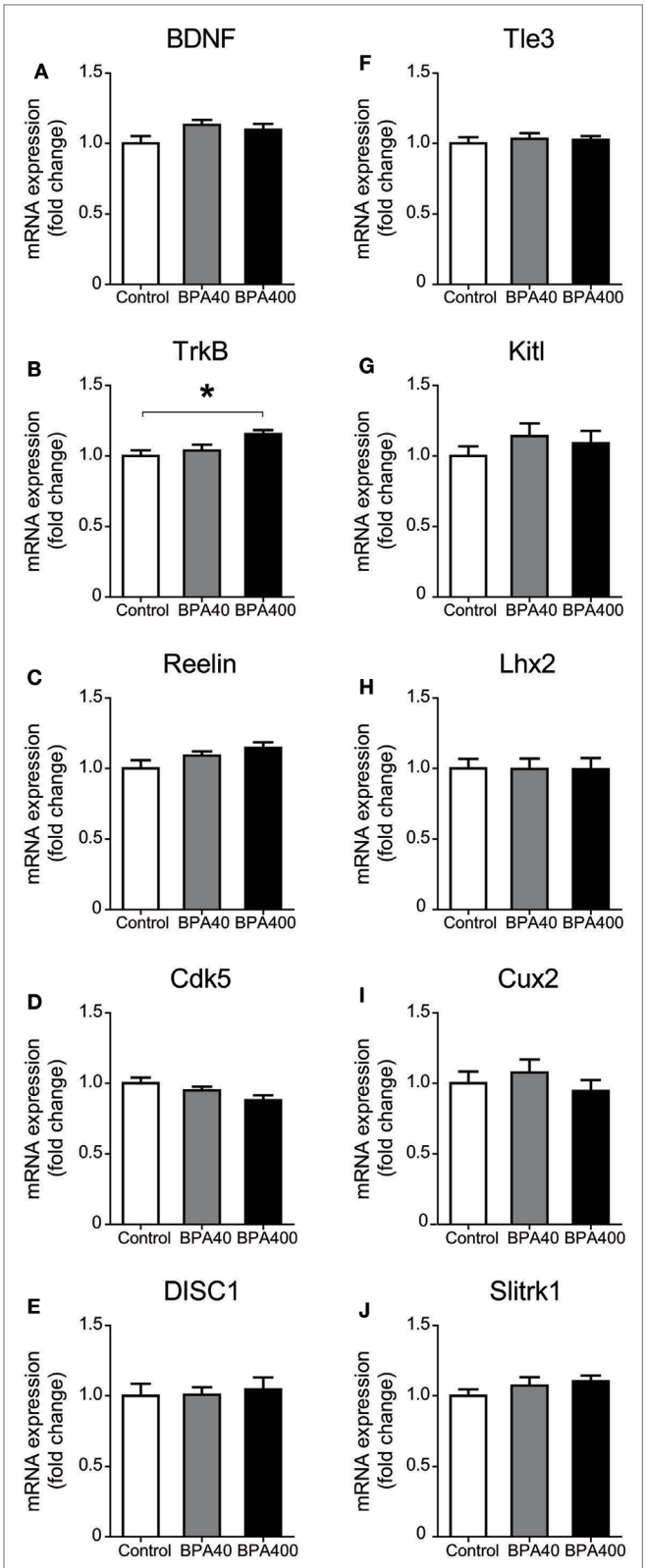

FIGURE 2 | mRNA expression of neuronal migration-related genes [BDNF (A), TrkB (B), Reelin (C), Cdk5 (D), and DISC1(E)] and cortical layer-specific genes [Tle3 (F), Kitl (G), Lhx2 (H), Cux2 (I), and Slitrk1 (J)] in mouse forebrain. Data are shown as mean ± SEM, $n = 6$ per treatment, $^*p < 0.05$.

has been shown to have estrogenic properties. As hormones are known to act in a non-monotonic dose–response manner, the low-dose-specific response to BPA may be regulated by interactions between BPA and hormone receptors (22, 23). For further study, the mode of action of BPA needs to be studied extending a dose range that includes multiple lower BPA doses.

In order to investigate the molecular basis of BPA-induced impairment of neuronal migration observed in the developing brain, we analyzed several migration guidance genes and neuron-specific markers for layer II/III. We identified a significantly enhanced gene expression of TrkB upon high dose BPA exposure. TrkB is a receptor for neurotrophins, which mediate neuronal migration, differentiation, and survival through beneficial trophic effects (24). Therefore, a plausible explanation for the dose-specific effects of BPA on neuronal migration would be that compensatory mechanisms may have been triggered in the BPA-400 group, such that higher TrkB expression minimized the effects of BPA exposure on neuronal migration. However, in order to elucidate mechanisms of BPA-induced abnormal neuronal migration, gene expressions of other neurotrophins (NGF, NT3, and NT4/5) and receptors (Trk A and Trk C) in specific brain regions, such as CP, need to be investigated in future studies. Migration guidance genes, such as Reelin, and several layer II/III-specific genes did not show any significant differences in expression between the control and BPA-40 groups. Because of the limited time point of the determination of mRNA levels on PND 0, it can be speculated that BPA may have disrupted expression of layer-specific genes in later time points. A recent study showed that perinatal exposure to BPA in mice at levels relevant to those exposed to humans transgenerationally altered behaviors and gene expression in brains, including expression of genes for several estrogen receptors, oxytocin, and vasopressin (25). Another recent study (9) showed reduction in overall length and branching number of basal dendrites of hippocampal CA1 pyramidal neurons in 3-week-old mouse pups, and spine densities in aged mice, both of which were born to dams administered BPA (40 or 400 μg/kg per day) during gestation. However, in the present study, we did not find any altered expression in genes, except TrkB as described above, that are relevant to neuronal migration or morphogenesis in BPA-exposed groups. The link of micromorphologically altered neuronal development with the molecular basis warrants prospective studies.

AUTHOR CONTRIBUTIONS

WL, TE, MK, and CT conceived this study. WL and TE performed experiments. WL analyzed experimental data. WL, TE, MK, and CT interpreted the data. K-iK and KN provided guidance and technical supports on IUE. WL and CT wrote the manuscript.

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REVIEW
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An Emerging Role of micro-RNA in the Effect of the Endocrine Disruptors

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Endocrine-disrupting chemicals (EDCs) are diverse natural and synthetic chemicals that may alter various mechanisms of the endocrine system and produce adverse developmental, reproductive, metabolic, and neurological effects in both humans and wildlife. Research on EDCs has revealed that they use a variety of both nuclear receptor-mediated and non-receptor-mediated mechanisms to modulate different components of the endocrine system. The molecular mechanisms underlying the effects of EDCs are still under investigation. Interestingly, some of the effects of EDCs have been observed to pass on to subsequent unexposed generations, which can be explained by the gametic transmission of deregulated epigenetic marks. Epigenetics is the study of heritable changes in gene expression that occur without a change in the DNA sequence. Epigenetic mechanisms, including histone modifications, DNA methylation, and specific micro-RNAs (miRNAs) expression, have been proposed to mediate transgenerational transmission and can be triggered by environmental factors. MiRNAs are short non-coding RNA molecules that post-transcriptionally repress the expression of genes by binding to 3′-untranslated regions of the target mRNAs. Given that there is mounting evidence that miRNAs are regulated by hormones, then clearly it is important to investigate the potential for environmental EDCs to deregulate miRNA expression and action.

Keywords: micro-RNA, endocrine disruptors, environment

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INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are diverse natural and synthetic chemicals that may alter various mechanisms of the endocrine system and produce adverse developmental, reproductive, metabolic, and neurological effects in both humans and wildlife (Henley and Korach, 2006). To date, close to 800 chemicals are known or suspected to be capable of interfering with hormone receptors and/or hormone synthesis and then play a larger role in the causation of many endocrine diseases and disorders (WHO | State of the science of endocrine disrupting chemicals, 2012). Excretion of EDCs is dependent on the nature of the chemical substances. If the substance is non-persistent it is usually predicted that they are metabolized by the liver then finally eliminated from the body through feces and urine. Persistent endocrine disruptors are accumulated especially in adipose tissue and they can be released slowly. One way of excretion of these persistent endocrine disruptors is thought to be from mother to child through breast feeding. It is observed in many studies that the daily intake of breast milk containing organic pollutants may exceed the

tolerable limit. It has been established that some EDCs can act directly on hormone receptors as hormone mimics or antagonists. Others can act directly on proteins that control the delivery of a hormone to its target cell or tissue. In addition, EDCs may act synergistically and produce additive effects. Most studies on EDCs have focused on chemicals that affect the reproductive and thyroid axis. However, several studies have suggested that environmental chemicals could affect several physiological systems that lead to metabolic disorders or central nervous system dysfunctions (Casals-Casas and Desvergne, 2011). For instance, neurobehavioral disorders have been associated with hypothalamic-pituitary-adrenal (HPA) axis disruption induced by hydroxyl-polychlorinated biphenyl (PCB; Kimura-Kuroda et al., 2007).

It is particularly difficult to highlight only one mechanism of action shared by the set of EDCs. In fact, the main problem is that there are many and diverse EDCs including industrial chemicals, pesticides, pollutants, and plastic industry compounds. Nevertheless, research on EDCs has revealed that they use a variety of both nuclear receptor- and non-receptor-mediated mechanisms to modulate different components of the endocrine system. For instance Vinclozolin (VCZ), a widely used fungicide with antiandrogenic effects in mammals, is a competitive antagonist of androgen receptor (AR) ligand binding (Kelce et al., 1997). Several studies showed that exposure to VCZ induce masculinized females and feminized males in rodents (Buckley et al., 2006). Interestingly, some of the effects of VCZ have been observed to pass on to subsequent unexposed generations, which can be explained by the gametic transmission of deregulated epigenetic marks (Anway et al., 2005; Stouder and Paoloni-Giacobino, 2010; Guerrero-Bosagna et al., 2012; Skinner et al., 2013). Epigenetic mechanisms, including histone modifications, DNA methylation, and specific micro-RNAs (miRNAs) expression, have been proposed to mediate such transgenerational transmission (Reik et al., 2001; Del-Mazo et al., 2013).

This review provides an insight into the toxicological effects of EDCs and particularly new molecular mechanisms, i.e., miRNAs, involved in the EDCs induced endocrine disruption.

THE DIFFERENT TYPES OF EDCS

The term endocrine disruptors were first introduced by the group of Soto in 1993 that showed that EDCs induced developmental abnormalities (Colborn et al., 1993). The International Program on Chemical Safety (IPCS) in 2002 and World Health Organization in 2013 defined EDCs as "*...an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations. A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub) populations.*" To date, EDCs include a large variety of chemical classes such as pesticides [methoxychlor, chlopyrifos, and dichlorodiphenyltrichloroethane (DDT)], pharmaceutical agents [diethylstrilbestrol (DES)], plastic packaging compounds [Bisphenol A (BPA), phthalates], and other industrial products that are used in daily life as fungicides VCZ or solvents/lubricants (dioxins). Some of them but not all are exposed in this paragraph.

A large number of chemicals are used as pesticides. The most important pesticides are organochlorines pesticides (OCPs), organophosphates, or triazines. The most emblematic of the banned OCPs is DDT and the exposure to it persists. The pesticides are involved in a large number of diseases including cancer, diabetes but also neurodegenerative disease as Parkinson or Alzheimer (Mostafalou et al., 2012; Mostafalou and Abdollahi, 2013).

The dioxins are a general name for a family of organochlorines including the polychlorinated dibenzodioxins (PCDDs), the polychlorinated dibenzofurans (PCDFs), and the polychlorinated biphenyls (PCBs). Dioxins are produced by various industrial processes and are commonly regarded as highly toxic compounds that are environmental pollutants and persistent organic pollutants. Among the PCDDs, the 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent and toxic compound and became known as a contaminant in Agent Orange, a herbicide used as a weapon in the Vietnam War (Schecter et al., 2006). TCDD was also released into the environment during the Seveso disaster (Sweeney and Mocarelli, 2000). The TCDD and the other toxins have been shown to be involved in different diseases including cancers, thyroid dysfunction, and nervous system degeneration but also type 2 diabetes (Pelclová et al., 2006; Schecter et al., 2006; Mostafalou et al., 2012; Mostafalou and Abdollahi, 2013).

An important number of EDCs are found in plastic products. World plastic production exceeded 300 million tons in 2010 (Halden, 2010). Most abundant of these plastics are phthalates and BPA. These are two most common EDCs and are associated with parental and social behavioral disturbances but also endocrine disease. Phthalates are mainly used as plasticizers in a wide range of common products, and are released into the environment. Phthalate exposure may be through direct use or by indirect means through leaching and general environmental contamination (Aurela et al., 1999). Food products are believed to be the main source of di-(2-ethylhexyl) phthalate (DEHP) and other phthalates in the general population. Fatty foods such as milk, butter, and meats are a major source. In several studies in human and rodents, high and even low doses of phthalates have been shown to change hormone levels as T3, T4, and thyroid-stimulating hormone and cause birth defects (Gayathri et al., 2004; Heudorf et al., 2007; Meeker et al., 2009). BPA is one of the other emblematic plastics used in polycarbonate plastic and polystyrene resins. Interestingly, it has been shown that BPA is detected in 95% of urine sample from a reference population of 394 adults in the United States (Calafat et al., 2005). This higher level of BPA in urine is associated with cardiovascular disease, sterility, and other reproductive diseases but also diabetes and liver abnormalities (Takeuchi et al., 2004; Sugiura-Ogasawara et al., 2005; Lang et al., 2008).

THE TOXICOLOGICAL EFFECTS OF EDCs ON ENDOCRINE AXIS

Reproductive Axis

In the last few years, it has been noticed that the incidence of certain diseases of the reproductive axis has increased (WHO | State of the science of endocrine disrupting chemicals, 2012). It is well-established that estrogen and androgen are involved in sexual differentiation. In this context, EDCs may act as estrogen and or androgen antagonists and induce different sexual disorders in males and females (Diamanti-Kandarakis et al., 2009; Sweeney et al., 2015; Toppari et al., 2016). For instance, DES and phthalates exposure to rats are associated with cryptorchidism or micropenis (Fisher et al., 2003; Li et al., 2003). In human, it has been shown that breast milk dioxin concentration correlated positively with the risk of cryptorchidism in Denmark (Main et al., 2007). It has also been shown that perinatal exposure to low doses of dioxin can permanently reduce sperm quality (Mocarelli et al., 2011). In humans, exposure to PCBs caused a defect in the development of the reproductive system (Staessen et al., 2001). Recently, epidemiological, study suggested that prenatal exposure to PCBs may be also associated with increased risk for cryptorchidism (Koskenniemi et al., 2015).

EDCs are associated with some types of female reproductive axis disorder including polycystic ovarian syndrome (PCOS). PCOS is a problem in which a woman's hormones are out of balance. It can disrupt the menstrual cycle and makes it difficult to become pregnant. If it isn't treated, over time it can lead to serious health problems, such as diabetes and heart disease. Most women with PCOS grow many small cysts on their ovaries. Interestingly, women with PCOS have higher levels of BPA and increased testosterone in these women is consistent with decreased clearance of BPA (Takeuchi et al., 2004, 2006). The cause of PCOS is not fully understood, but the EDCs as well as BPA could play a role in the onset of PCOS. Female rats exhibited sexual precocity as a consequence of exposure to DTT (Rasier et al., 2007).

It has also been shown in the hypothalamic GT1-7 cell line that organochlorine pesticides such as methoxychlor and chlopyrifos altered gonadotropin-releasing hormone (GnRH) gene expression and biosynthesis (Gore, 2002) suggesting that EDCs could affect the different levels or reproductive axis. Interestingly, it has been revealed that the BPA-mediated inhibition of GnRH neuronal activity occurred independent of estrogen receptors via a non-canonical unknown pathway (Klenke et al., 2016).

Thyreotropic Axis

Thyroid hormones (T3 and T4) are important for brain development, for the modulation of metabolism and are associated with many aspects of normal adult physiology. For these reasons, thyreotropic axis disruption induced a large scale of perturbation in adult physiology, development, and metabolism. It has been reported that numerous EDCs can directly affect the normal functioning of the thyroid gland. In numerous studies, it has been shown that different EDCs such as PCBs, BPA, or DTT have thyroid-disrupting effects in animals and humans (Patrick, 2009; Molehin et al., 2016).

The EDCs can affect the thyroid system at different levels such as the transport and/or biosynthesis of the thyroid hormones. It has been shown that PCBs have a high affinity with thyroxin specific binding protein which can affect the thyroid hormone transport (Rickenbacher et al., 1986; McKinney et al., 1987; Darnerud et al., 1996). More precisely, treatment of mice during gestation with PCB as 3,3′, 4,4′-tetrachlorobiphenyl (CB-77) leads to a decrease of free and total T4 in fetal plasma (Darnerud et al., 1996). More recently, the group of Seegal examined the effects of a mixture of PCBs and polybrominated diphenyl ethers (PBDEs) coexposure from gestational day 6 through postnatal day 21, alone and in combination, on T4 levels in rat offspring (Miller et al., 2012, 201). They observed that PCBs and PBDEs induces similar reductions in T4 levels and that coexposure to a mixture of PCBs and PBDEs has additive effects on T4 level in male and female offspring (Miller et al., 2012). In the study of Schmutzler et al., rats (female, ovariectomized) were treated for 12 weeks with different EDCs and an alteration in thyrotropin (TSH) and thyroid hormones (T4, T3) serum levels were observed (Schmutzler et al., 2004). In another set of studies, exposure to phthalates induced thyroid function alterations (Mitchell et al., 1985; Hinton et al., 1986; Price et al., 1988). Interestingly, the treatment of rats for periods of 3 months with di-(2-ethylhexyl) phthalate increased the number and size of lysosomes, hypertrophy of the Golgi apparatus, and dilation of the rough endoplasmic reticulum in thyroid cells and these changes are consistent with persistent hyperactivity in the gland (Price et al., 1988). It has also been shown that EDCs can alter deiodinase activity which is the peroxidase enzyme that is involved in the activation or deactivation of thyroid hormones (Meerts et al., 2002; Viluksela et al., 2004; Noyes et al., 2013).

In human, there is now growing evidence that PCBs but also BPA and phthalates have thyroid-disrupting effects (Boas et al., 2012; Campos and Freire, 2016). For instance, the group of Yoshinaga showed that exposure to hydroxylated-PCBs at environmental levels during the first trimester of pregnancy can affect neonatal thyroid hormone status (Hisada et al., 2014). It has also been shown that early exposure to certain environmental chemicals with endocrine-disruption activity as pesticides may interfere with neonatal thyroid hormone status (Freire et al., 2011).

Central Nervous System

There is strong evidence that there is a correlation between the increasing prevalence of neurodevelopmental disorders and the increase in exposure to pollutants over the past several decades (Weiss and Landrigan, 2000; Landrigan and Goldman, 2011a,b). For instance, since the 1970s, there have been dramatic increases in previously rare neurodevelopmental disorders such as autism which is characterized by some degree of impaired social behavior, communication and language, and a narrow range of interests and activities that are both unique to the individual and carried out repetitively. In the 1970s, autism's prevalence was estimated to be between 4 and 5 in 10,000

children (Wing et al., 1976) but today this value is estimated to be 1 in 110 children (Rice et al., 2007). In a review of the literature performed by de Cock et al., a positive association was found for autism in relation to exposure to different chemicals investigated, which included hazardous air pollutants, pesticides, and BPA (de Cock et al., 2012). In the same study, a relationship between attention deficit hyperactivity disorders and different EDCs including BCPs and pesticides such as chlorpyrifos has been done (de Cock et al., 2012).

The function of central nervous system (CNS) can be affected by EDCs and these effects can be induced by different mechanisms. The most important is the effects of EDCs on different endocrine axis important for CNS functions and development. Evidence that prenatal estrogen exposure is important in neuronal correct development emerged from reports of psychosis in patients prenatally exposed to the synthetic estrogen DES (Katz et al., 1987; Brown, 2009; Inadera, 2015; Negri-Cesi, 2015). Interestingly, several researches indicate that BPA is an estrogenic EDC that alters or interferes with normal endocrine development in various vertebrate and invertebrate species (vom Saal et al., 2007) suggesting a role of BPA in CNS disease. For instance, prenatal exposure to low dose of BPA disturbed neocortical histogenesis in mice (Nakamura et al., 2006, 2007).

As exposed above, BPA is a well-known xenoestrogen (Kuiper et al., 1998; Delfosse et al., 2014; Inadera, 2015). BPA has complex action in the CNS but primarily BPA was exhibited to bind both estrogen receptors α and β (ERα and ERβ) and has also been shown to act as an anti-androgen (Kuiper et al., 1998; Wolstenholme et al., 2011). Interestingly, it has been described endocrine and neuroendocrine abnormalities in schizophrenia (Marx and Lieberman, 1998; Stevens, 2002). In fact, estrogen has been associated with a neuroprotective effect but lower plasma levels of estrogens induced schizophrenia-like syndrome in males and females (Huber et al., 2001; Kaneda and Ohmori, 2005; Segal et al., 2007). Furthermore, neuronal disorders have also been associated with an impairment of HPA axis. For instance, the increase of glucocorticoid concentrations induced hippocampal nerve damage and schizophrenia (Cotter and Pariante, 2002). In rat, corticosterone exposures also lead to degeneration of the prefrontal cortex causing impairments in executive functions such as behavioral flexibility and working memory (Cerqueira et al., 2005). It has been established in baboons that HPA is potentially affected by estrogen (Pepe and Albrecht, 1998; Albrecht et al., 2005). In addition, it has been recently shown that perinatal exposure to low-dose of BPA caused HPA axis dysfunctions (Panagiotidou et al., 2014; Chen et al., 2015; Zhou et al., 2015). Particularly, the administration of low doses of BPA (2 μg/kg.day) to female breeders from gestation day 10 to lactation day 7 induced obvious anxiety/depression-like behaviors in the offspring (Chen et al., 2015). Notably, significant increase in serum corticosterone and adrenocorticotropin, and corticotropin-releasing hormone mRNA were detected in BPA-exposed rats before or after the mild stressor (Chen et al., 2015). Altogether these different observations strongly suggest that BPA and other EDCs could be associated to schizophrenia pathogenesis (Brown, 2009).

Metabolic Disorders

In addition to the reproductive and neuronal developmental effects, there is also evidence that metabolic disorders may be linked to EDCs (Casals-Casas et al., 2008; Newbold et al., 2008). Obesity, diabetes and metabolic syndrome are due to disruption of the energy storage balance endocrine system and thus are potentially sensitive to EDCs. This hypothesis is supported by different epidemiological and animal studies that have shown that a variety of EDCs can influence adipogenesis and obesity (Baillie-Hamilton, 2002; Casals-Casas et al., 2008; Elobeid and Allison, 2008; Newbold et al., 2008; Chen et al., 2009). For instance, the administration of DES to neonatal mice induced overweight associated with an increase of abdominal body fats and inflammatory biomarkers (Newbold et al., 2007). In rats, perinatal exposure to low doses of BPA increased adipogenesis and body weight in adult females (Somm et al., 2009). EDCs are also involved in glucose homeostasis defects. In accordance with this fact, epidemiological studies report that exposure to EDCs may affect the risk of type 2 diabetes (Remillard and Bunce, 2002; Huang et al., 2015; Song et al., 2016). Very low doses of BPA induced hyperinsulenemia and type 2 diabetes (Alonso-Magdalena et al., 2010). In the same way, low doses of BPA and dioxins altered α-cell function and glucagon release which lead to glucose homeostasis defect (Alonso-Magdalena et al., 2005). Interestingly, it has been established that EDCs such as BPA or dioxins are accumulated by adipose tissue and that they are released slowly and have induced glucose homeostasis impairment (Alonso-Magdalena et al., 2011). When administrated to mother mice, BPA induces metabolic disorders in adult male offspring such as an age-related change in food intake, an increase in body weight and liver weight, abdominal adipocyte mass, number and volume, and in serum leptin and insulin, but a decrease in serum adiponectin and in glucose tolerance (Angle et al., 2013). Furthermore, mother mice treated with BPA during gestation, at environmentally relevant doses, exhibit profound glucose intolerance and altered insulin sensitivity as well as increased body weight (Alonso-Magdalena et al., 2015).

MICRO-RNAs AND EDCs

EDCs often act via more than one mechanism. The target cells of the hormones bear receptors specific to a given hormone and will be activated by either a lipid-soluble (permeable to plasma membrane) or water-soluble hormone (binds cell-surface receptor; Casals-Casas and Desvergne, 2011; Wolstenholme et al., 2011; Maqbool et al., 2016). Lipid-soluble hormones (steroid hormones and hormones of the thyroid gland) diffuse through the plasma membrane to enter the target cell and bind to a nuclear receptor (NR) protein that will in turn activates expression of specific genes that influence specific physiological cell activities. Water-soluble hormones (such as insulin) bind to a receptor protein on the plasma membrane of the cell which leads to specific cellular transduction pathways (Casals-Casas and Desvergne, 2011; Maqbool et al., 2016; Wolstenholme et al.,

2011). Because many EDCs are small lipophilic compounds, they can directly interact with a given NR, which presumably perturbs or modulates downstream gene expression.

In parallel with these classical pathways, it appears that EDCs not only involve genetics but also epigenetic mechanisms. Epigenetics is broadly defined as those heritable changes in the genome not dependent upon changes in genetic sequences (e.g., DNA methylation or histone modification). These epigenetic processes control tissue development by controlling gene expression. Thus, a major route by which hormones act during development is by changing the epigenome. These different epigenetic mechanisms also include miRNAs which are short non-coding RNA molecules that post-transcriptionally repress the expression of genes by binding to 3′-untranslated regions (3′UTR) of the target mRNAs. Recently, it appears that miRNAs can be involved in the action of EDCs (Cameron et al., 2016; Klinge, 2015). This part of the review focuses on the regulation of miRNAs by the EDCs which appear as a new molecular mechanism involved in endocrine disruption.

Biogenesis and Action of miRNAs

The miRNAs are short non-coding RNA with a size of 21–26 nucleotides that suppress target gene expression through the inhibition of gene translation and the increase of the degradation of target mRNAs (Bartel, 2004). These small regulatory molecules are involved in a large range of biological processes such as development, cell proliferation, apoptosis, synaptic plasticity, and energy metabolism (Bartel, 2004). The gene regulation and processing as well as the mode of action of miRNAs are conserved over the evolution of a species (Stricklin et al., 2005; Landgraf et al., 2007; Ruby et al., 2007b). In recent decades, research on miRNAs has deepened our understanding of their mechanisms of action and their biological functions. These regulatory RNAs are predicted to modulate the expression of ∼30% of protein-coding genes (Lewis et al., 2005). The miRNA can affect translation and mRNA stability by means of RNA-RNA interactions. A number of algorithms allow the identification of the potentially targeted mRNA by miRNA and conversely miRNA modulator of mRNA. Although the regulation of genes by miRNAs is an active area of research, few targets of miRNAs have been experimentally validated in a physiological context.

In parallel with the discovery of new miRNA, the identification of the components of the miRNA maturation and processing machinery is an active area of research (**Figure 1**). The miRNA genes are located throughout the genome, within introns of protein-coding genes and rarely in exons (Rodriguez et al., 2004). Despite the small number of cases studied, it seems that the promoters of miRNAs have the same characteristics as those genes encoding proteins. The genes encode primary RNA (pri-miRNAs) conformation stem-loop, with one or two sequences which produce mature miRNAs (Hutvágner et al., 2001; Lagos-Quintana et al., 2001; Lau et al., 2001). The transcription machinery involves a RNA polymerase II (Lee et al., 2004; Bortolin-Cavaillé et al., 2009). The pri-miRNA is cleaved and polyadenylated at 3′ and 5′ capped in the same manner as the mRNAs (**Figure 1**; Cai et al., 2004). The steps of the pri-miRNA maturation require two endonucleases before they become functional miRNAs (Lee et al., 2003). The first step involves an RNA binding protein, the DiGeorge Syndrome Critical Region 8 (DGCR8) also called Partner of Drosha (PASHA) associated with Drosha (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004). Drosha cleaves sequences on either side of the stem-loop of the pri-miRNA and gives the precursor miRNA (pre-miRNA). Pre-miRNA is exported from the nucleus to the cytoplasm by a karyopherin known as Exportin 5 (Yi et al., 2003; Bohnsack et al., 2004; Lund et al., 2004). In the second step, endonuclease DICER cleaves the pre-miRNA loop region in the cytoplasm, thereby releasing a double-stranded RNA of about 20 nucleotide pairs containing the mature miRNA (Bernstein et al., 2001; Grishok et al., 2001; Hutvágner et al., 2001; Ketting et al., 2001). Like Drosha, DICER is associated with an RNA binding-protein, the human immunodeficiency virus Transactivating Response RNA-Binding Protein (TRBP; Chendrimada et al., 2005; Gregory et al., 2005; Haase et al., 2005). One of the two strands is recognized by a protein of the family of the Argonautes (AGO), most commonly AGO2, which in turn recruits other elements of the RNA-induced silencing complex (RISC; Sontheimer, 2005). The other strand called "star strand" is degraded. An asterisk is associated with the name of the miRNA that is not incorporated into the RISC complex (e.g., miR-488∗). However, for some miRNAs, both strands may be incorporated into the RISC complex. In this case, the end of the strand 5′ of the stem-loop is called "5p" and that of strand 3′ is called "3p" (e.g., miR-384-5p and miR-384-3p). In fact, new data indicates that a small fraction of the star strand is incorporated into the RISC complex for most miRNA families (Yang et al., 2011). For these reasons, the nomenclature scheme "–5p/–3p" is increasingly used instead of the terminology "mature/star." The RISC complex/mature miRNA (miRISC) recognizes the target mRNA and induces degradation and/or inactivation of the latter (**Figure 2**).

However, various studies reveal that some families of miRNAs undergo non-canonical pathway maturation. Importantly, some studies described miRNAs called mirtrons which are located in the short sequence of introns. The mirtrons undergo a first processing step, independent of Drosha, by the splicing machinery to give miRNA with a lariat structure. The introns are then processed by the lariat-debranching enzyme to give the pre-miRNAs which carry-on its maturation by the canonical pathway (Okamura et al., 2007; Ruby et al., 2007a). It has also been reported in one case (miR-451) that the cleavage step by DICER is substituted with AGO2 (Cheloufi et al., 2010; Cifuentes et al., 2010).

The miRNA-mRNA Interactions

The action of miRNAs depends on their specific interaction with their targets. In plants, miRNAs bind to their targets with perfect complementarity of bases, which induces a rapid cleavage of the transcript by the ribonuclease activity of AGO (Baumberger and Baulcombe, 2005). In metazoans, the majority of miRNAs partially bind to their targets primarily through a region of so-called seed sequence, located at positions 2–7 from the miRNA 5′-end (Doench and Sharp, 2004; Brennecke et al., 2005). This region binds perfectly on the 3′UTR via complementary

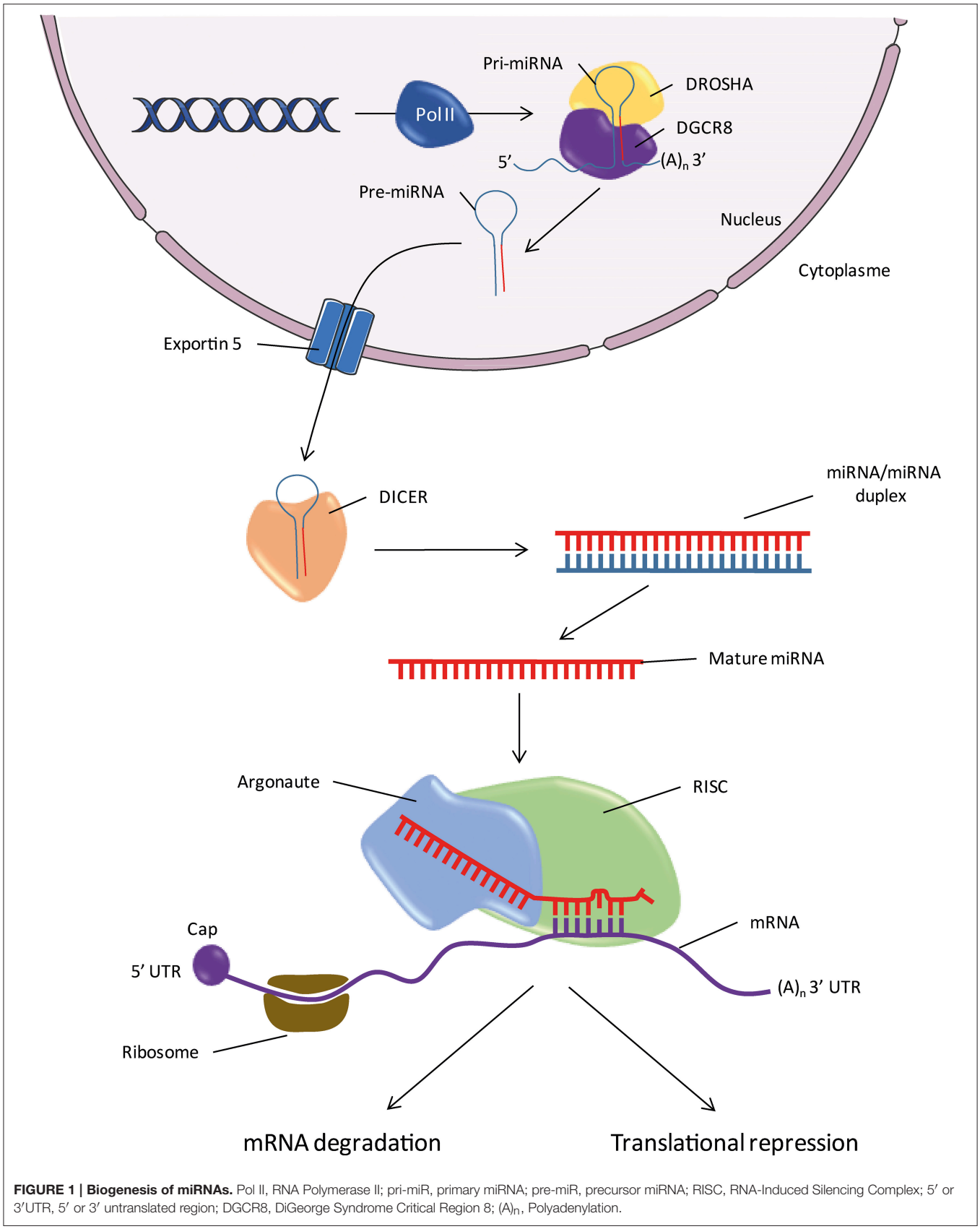

FIGURE 1 | Biogenesis of miRNAs. Pol II, RNA Polymerase II; pri-miR, primary miRNA; pre-miR, precursor miRNA; RISC, RNA-Induced Silencing Complex; 5′ or 3′UTR, 5′ or 3′ untranslated region; DGCR8, DiGeorge Syndrome Critical Region 8; $(A)_n$, Polyadenylation.

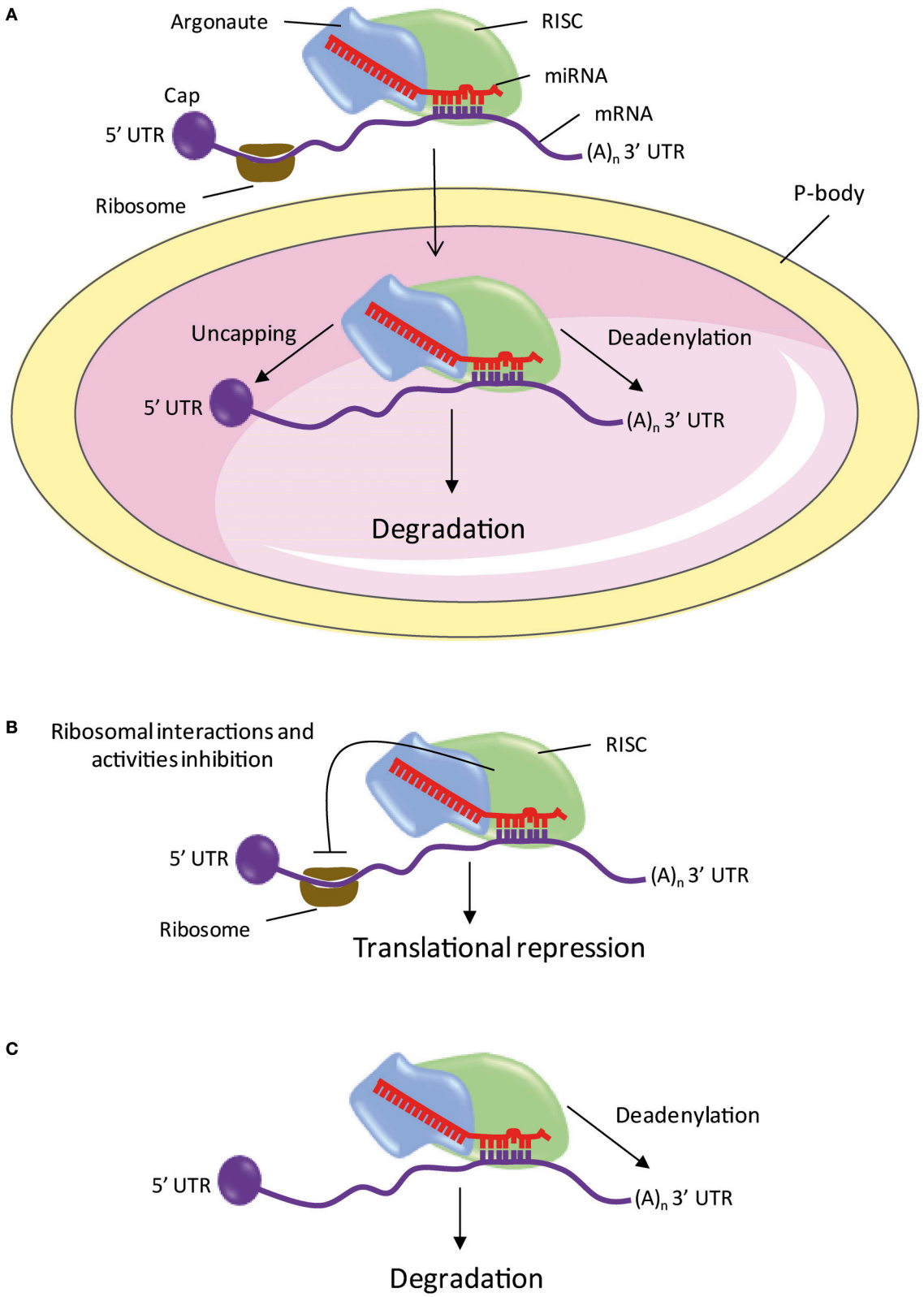

FIGURE 2 | The different mechanisms of mRNA repression or degradation by miRNA. (A) The processing bodies. **(B)** Action on the initiation of translation and repression in post-initiation steps. **(C)** Deadenylation. miRNA, microRNA; P-bodies, processing bodies; RISC, *RNA-Induced Silencing Complex;* 5′ or 3′UTR, 5′ or 3′ untranslated region; $(A)_n$, Polyadenylation.

base interactions. These interactions induce inhibition of the expression of the target mRNA through a blocking of the translation or a degradation of the transcript. The different mechanisms of mRNA repression or degradation by miRNA are briefly described below.

Processing bodies (P-bodies) are cytoplasmic foci containing mRNA degradation enzymes and trinucleotide repeat-containing gene 6A protein (TNRC6A or GW182 for Drosophila). These are involved in the catabolism and/or storage of untranslated mRNA (**Figure 2A**; Eystathioy et al., 2002, 2003; Ingelfinger et al., 2002; van Dijk et al., 2002; Sheth and Parker, 2003). The GW182 proteins are also found in the miRISC complex where they play a key role in the repression induced by miRNAs (Jakymiw et al., 2005; Liu et al., 2005; Eulalio et al., 2008). In addition, the AGO and GW182 proteins, miRNAs and targeted mRNAs are found in the P-bodies (Ding et al., 2005; Liu et al., 2005; Pillai et al., 2005; Sen and Blau, 2005). These studies suggest that targeted mRNAs are repressed or degraded in the P-bodies.

The mechanism by which miRISC inhibits translation is controversial. Several studies indicate a blocking of the initiation of translation, while other studies suggest a repression in post-initiation steps (**Figure 2B**). Indeed, it has been shown that the miRNA targeted mRNAs are associated with fewer ribosomes during elongation than in mRNAs controls (Humphreys et al., 2005; Pillai et al., 2005; Bhattacharyya et al., 2006; Huang et al., 2007; Ding and Grosshans, 2009). The initiation is stopped by the blocking by miRISC of the interaction of the translation ribosomal subunit 60S with mRNA (Chendrimada et al., 2007; Wang et al., 2008). In addition, GW182 recognizes the 5′ cap of the mRNA and prevents the initiation of translation (Eulalio et al., 2008). In the other studies, two mechanisms inducing translation repression after initiation have been described. It has been shown that miRISC promotes the release of ribosomes during elongation, thus blocking translation (Petersen et al., 2006). Another study suggests that the elongation process is maintained without peptide production when mRNA is targeted by a miRNA (Nottrott et al., 2006). The authors suggest that the complex-related proteases miRISC could degrade the native peptides.

Studies showed that repression of many miRNA targets is associated with a deadenylation and degradation (**Figure 2C**; Lim et al., 2005; Giraldez et al., 2006; Wu et al., 2006; Wakiyama et al., 2007; Eulalio et al., 2009). Comparative analysis of large scale proteomic and transcriptomic changes, following overexpression or inhibition of a miRNA in mammalian cells show that the vast majority of targets repressed by a miRNA have decreased their level of mRNA reflecting a lower presence of protein (Baek et al., 2008; Selbach et al., 2008; Hendrickson et al., 2009; Guo et al., 2010). These studies show that repression induced by miRNAs predominantly results in mRNA degradation.

Modulation of miRNA Expression by Hormones

Numerous studies clearly indicated that different hormones modulate miRNA expression in different organs (Hu et al., 2013; Cameron et al., 2016; Derghal et al., 2015; Klinge, 2015). For instance, the treatment with thyroid hormones of hepatocytes cells AML 12 over-expressing miR-206 resulted in decreased miR-206 expression, and a significant increase in two predicted target genes (i.e., Mup1 and Gpd2; Dong et al., 2010).

It has also been shown that estradiol actively controls miRNA production in various tissues such as mammary and ovarian cells (Gupta et al., 2012). More precisely, estrogens modulate miRNA transcription by inactivating RNA polymerase II and precursor miRNA biogenesis by blocking Drosha-mediated processing (Gupta et al., 2012). It also been shown that estrogen regulates miRNA expression in brain and particularly in the hippocampus, the amygdala and paraventricular nucleus (Rao et al., 2013). Recently, it has been established that miR-27a/b and miR-494 regulate tissue factor pathway inhibitor α (TFPIα) expression suggesting a possible role of these miRNAs in the estrogen mediated downregulation of TFPIα involved in breast cancer (Ali et al., 2016).

Several studies indicate that gonadotropins as estrogen can affect miRNA expression (Cohen et al., 2016). In accordance with this, it has been observed variability in miRNA expression profiles in estrogen receptor-positive and -negative breast cancer phenotypes (Iorio et al., 2005; Mattie et al., 2006). As described recently miR-136-3p expression levels were increased after the administration of human chorionic gonadotropin to ovarian cells (Kitahara et al., 2013). Direct action of estrogen on miRNAs expression has been demonstrated in different studies. For instance, an aberrant miRNA expression has been characterized in estrogen-induced rat breast carcinogenesis (Kovalchuk et al., 2007). Using the microarray approach, it has been shown that estrogen can modulate the profile of miRNAs expression in zebrafish model and in human MCF-7 and ZR-75 breast cancer cells (Cohen et al., 2008; Bhat-Nakshatri et al., 2009; Maillot et al., 2009; Ferraro et al., 2012).

Altogether, these different observations suggest that the link between hormones, miRNAs and mRNA targets will lead to an improved understanding of how EDCs affect the different endocrine axis.

Modulation of miRNA Expression by EDCs

A few recent studies report the effect of several EDCs on the expression of miRNAs in fish, animals, or cell lines (Collotta et al., 2013; Vrijens et al., 2015). These disturbances of miRNAs expression profile by EDCs are associated with diseases of the CNS and reproductive axis as well as metabolic disorders (Vrijens et al., 2015).

In humans, it has been shown that several EDCs as DTT or BPA decreased the expression of miR-21 which has a key role in cancer especially in breast cancer development (Tilghman et al., 2012; Sicard et al., 2013). In addition, decreased expression of let-7f is also associated with breast cancer (Sakurai et al., 2012). In the work led by Tilghman et al., DTT (10 μM) or BPA (10 μM) activate ERα in MCF-7 breast cancer cells which down-regulated the expression of miR-21, let-7a-f, miR-15b, and miR-28b and increased the expression miR-638, miR-663, and miR-1915 (Tilghman et al., 2012). In addition, it has been exhibited an important role of miR-19 in BPA-mediated MCF-7 cell proliferation (Li et al., 2014). The xenoestrogens DES also

showed a decrease of miR-34b expression in MCF-7 cells (Lee et al., 2011). In rats, the neonatal exposure to the estrogenic analog (i.e., estradiol benzoate) increased the expression of miR-29 in testicular tissue (Meunier et al., 2012). Increased miR-29 expression resulted in a decrease in DNA methyltransferases (DNMT1, 3a and 3b) and antiapoptotic myeloid cell leukemia sequence 1 (Mcl-1) protein levels. Together, the increased miR-29 combined with a subsequent reduction of DNMT and Mcl-1 protein levels may represent a basis of explanation for the adult expression of the germ cell apoptosis phenotype. Interestingly, BPA given to rats at moderate doses is associated with erectile dysfunction, cavernosal lipofibrosis and alterations of global gene transcription including a set of miRNAs expressed in the penile shaft (Kovanecz et al., 2014). In female, prenatal BPA treatment in sheep results in hypergonadotropism and ovarian cycle disruptions (Veiga-Lopez et al., 2013). Interestingly, in this study it has been shown that fetal ovarian miRNAs expression was altered by prenatal BPA with 45 down-regulated (>1.5-fold) at day 65 and 11 down-regulated at day 90 of gestation (Veiga-Lopez et al., 2013). In chicks, several miRNAs (miR-1623, miR-1552-3p, miR-1573, miR-124a, and miR-1764) were down-regulated in the DES-treated chick oviduct compared with control oviduct (Lim and Song, 2015). Interestingly, these miRNAs regulate the expression of vitelline membrane outer layer protein 1, a basic protein present in the outer layer of the vitelline membrane of eggs, plays essential roles in separating the yolk from the egg white (Lim and Song, 2015). There is a growing concern about the potential health effects of exposure to various EDCs during pregnancy and infancy. The placenta is expected to be an effective barrier protecting the developing embryo against some EDCs circulating in maternal blood. However, it has been shown recently that miR-146a was significant overexpressed and correlated significantly with BPA accumulation in the placenta from pregnant women living in a polluted area and undergoing therapeutic abortion due to fetal malformations (De Felice et al., 2015). This observation has been also established in HTR-8 and 3A human placental cells (Avissar-Whiting et al., 2010). These different studies highlight the fact that the EDCs induce miRNA-expression alterations in the reproductive axis.

In the context of CNS disease, Jiang et al. established by *in silico* approach that miR-146a is involved in Alzheimer's disease (Jiang et al., 2013). Interestingly, BPA exposure of human placental cell lines has been shown to alter miRNA expression levels, and specifically, miR-146a was strongly induced by BPA treatment (Avissar-Whiting et al., 2010). Then, miR-146a could be used as a biomarker for Alzheimer's disease after EDCs exposure.

Recently, it has been established that the expression of hepatic miRNA (miR-22b, miR-140, miR-210a, mir-301, miR-457b, and let-7d) is increased in fluoxetine (the active ingredient in Prozac®) exposed female zebrafish (Craig et al., 2014). Interestingly, the miRNAs that were up-regulated were predicted to be responsible for down-regulating pathways such as insulin signaling, cholesterol synthesis, and triglyceride synthesis (Craig et al., 2014). Recently, it was shown that miR-21, 221, 222, and 429 expression levels decreased in the liver of DDT-treated female Wistar rats, whereas increases were observed in cytochrome 1A1 and 2B1 mRNA (Chanyshev et al., 2014; Gulyaeva et al., 2016). By an original approach using DNA-Au bio bar code (DNA-Au) and G-quadruplex-based DNA enzyme, Meng et al. demonstrated that miR-21 expression is increased in BPA-treated human hepatocarcinoma BEL-7402 cells (Meng et al., 2013). In primary mouse hepatocyte, TCCD modulated the expression of miR-503-5p that targeting cyclin D2 which was involved in the discriminative process of p53 signaling and metabolism (Rieswijk et al., 2015). In addition, it also been shown that TCDD regulates the expression of miR-101a and miR-122 and that cyclooxygenase-2, a target gene of miR-101a, plays a significant role in liver damage in mice exposed to TCDD (Yoshioka et al., 2011). Altogether, these observations suggest that the EDCs can induce metabolic disorders through the disturbance of specific miRNAs in the liver.

Altogether, these different studies indicated that miRNAs profile changed in tissue exposed to different EDCs. Potentially, miRNAs can be considered as new biomarkers for EDCs exposure (Vrijens et al., 2015).

CONCLUSION

Despite the high number of studies generated in the past few years on the mechanism of how EDCs act on the different endocrine axis, much still needs to be learnt. To date, very few ecotoxicology studies have considered miRNA in the context of endocrine disruption. In this review, we have seen that exposure to EDCs may lead to modification of miRNAs expression associated with endocrine disruption. However, many questions remain open, for instance (i) what is the impact on the miRNAs expression in different tissues which have suffered chronic low level EDCs exposure, (ii) what are the effects of the exposure either to a single EDC or to a complex mixture of different chemicals. Further, studies are warranted to evaluate if miRNAs may act as a causal link between EDCs exposure and their effect on health or if they can be used as a diagnostic or prognostic tools.

AUTHOR CONTRIBUTIONS

LM and AD wrote the manuscript. MD and JT helped with manuscript preparation.

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Learning and Memory Deficits in Male Adult Mice Treated with a Benzodiazepine Sleep-Inducing Drug during the Juvenile Period

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Gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the mammalian central nervous system, is also known to be important for brain development. Therefore, disturbances of GABA receptor (GABA-R) mediated signaling (GABA-R signal) during brain development may influence normal brain maturation and cause late-onset brain malfunctions. In this study, we examined whether the stimulation of the GABA-R signal during brain development induces late-onset adverse effects on the brain in adult male mice. To stimulate the GABA-R signal, we used either the benzodiazepine sleep-inducing drug triazolam (TZ) or the non-benzodiazepine drug zolpidem (ZP). We detected learning and memory deficits in mice treated with TZ during the juvenile period, as seen in the fear conditioning test. On the other hand, ZP administration during the juvenile period had little effect. In addition, decreased protein expression of GluR1 and GluR4, which are excitatory neurotransmitter receptors, was detected in the hippocampi of mice treated with TZ during the juvenile period. We measured mRNA expression of the immediate early genes (IEGs), which are neuronal activity markers, in the hippocampus shortly after the administration of TZ or ZP to juvenile mice. Decreased IEG expression was detected in mice with juvenile TZ administration, but not in mice with juvenile ZP administration. Our findings demonstrate that TZ administration during the juvenile period can induce irreversible learning and memory deficits in adult mice. It may need to take an extra care for the prescription of benzodiazepine sleep-inducing drugs to juveniles because it might cause learning and memory deficits.

Keywords: sleep-inducing drug, triazolam, zolpidem, GABA receptor signal, behavioral battery test

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INTRODUCTION

Normal brain development requires various neuronal signals must be activated at the appropriate timing and with the proper extent in the developmental brain (Rice and Barone, 2000). Excitatory glutamate receptor (Glu-R) signals and inhibitory gamma-aminobutyric acid (GABA) receptor (GABA-R) signals are the most important neuronal signals in the adult brain. These neuronal signals are also known to be important for brain development. These signals have roles in neuronal

cellular proliferation and differentiation, neuronal migration, the construction of neuronal circuits, and the reorganization of neuronal circuits (Luján et al., 2005). Therefore, the stimulation of these neuronal signals with external factors, such as chemical compounds, may interfere with normal brain development and result in late-onset functional deficits during adulthood. We have previously reported that the transient activation of Glu-R signals in the prenatal mouse brain with domoic acid results in aberrant emotional behavior, as well as learning and memory deficits, as revealed by a mouse behavioral battery tests (BBT) (Tanemura et al., 2009). On the other hand, Haas et al. have shown that prenatal GABA-R signal activation with the anxiolytic drug Diazepam (DZP) leads to the inhibition of neuronal migration and the disruption of cerebral cortex neuronal circuits (Haas et al., 2013). In addition, Shen et al. have shown that neonatal activation of GABA-R signals by DZP results in increased anxiety-like behavior (Shen et al., 2012). However, the behavioral tests applied in these studies were limited, and their results were not analyzed in an integrated manner. We therefore believe that analyses of several behavioral tests during the adult stage following the activation of GABA-R signals during brain development are critically needed.

In this study, we report the results of the BBT that we conducted. These include the open field test, the light/dark transition test, the elevated plus maze test, the contextual/cued fear conditioning test, and the pre-pulse inhibition test. The results of these tests will help us to understand the effects of GABA-R signal activation during brain development with the benzodiazepine (BZD) sleep-inducing drug triazolam (TZ: original brand name "Halcion") or the non-BZD drug zolpidem (ZP: originally marketed as "Ambien" and available worldwide under many brand names) on behavior during the adult stage (Pakes et al., 1981; Holm and Goa, 2000). These sleep-inducing drugs have similar pharmacokinetic and pharmacodynamic effects in humans (Lobo and Greene, 1997). They are preferentially used as drugs for the treatment of insomnia owing to their lack of carryover effects on the next day (Neubauer, 2007). Their reported side effects include drug-dependence, withdrawal symptoms, psychiatric symptoms (excitement stimulation, confusion, aggression, noctambulation, hallucinations, delusions, and agitation), transient anterograde amnesia before and after sleeping, or arousal during sleep (Pakes et al., 1981; Jonas et al., 1992; Toner et al., 2000; Greenblatt and Roth, 2012). Treatment of sleep disorders in children using hypnotic drugs is common (Kahn et al., 1989; Stores, 1996; Owens et al., 2003; Weiss and Garbutt, 2010; Felt and Chervin, 2014), although their safety in children has not been established (FDA, 2008, 2013). These chemicals act by suppressing excitatory neuronal activity by inducing hyperpolarization following the cellular influx of chloride ions when they bind to GABA (A)-R alpha and gamma receptors. GABA (A)-R is found as pentamers of a combination of 19 subunits (α1–6, β1–3, γ1–3, δ, ϵ, θ, π, and ρ1–3). The BZD chemical TZ binds to GABA (A)–R α1, 2, 3, 5, and 6 subtypes in any combination, along with the γ2 subtype. The non-BZD chemical ZP binds only to combinations of GABA (A)–R α1 and γ2 subtypes (Rudolph and Knoflach, 2011).

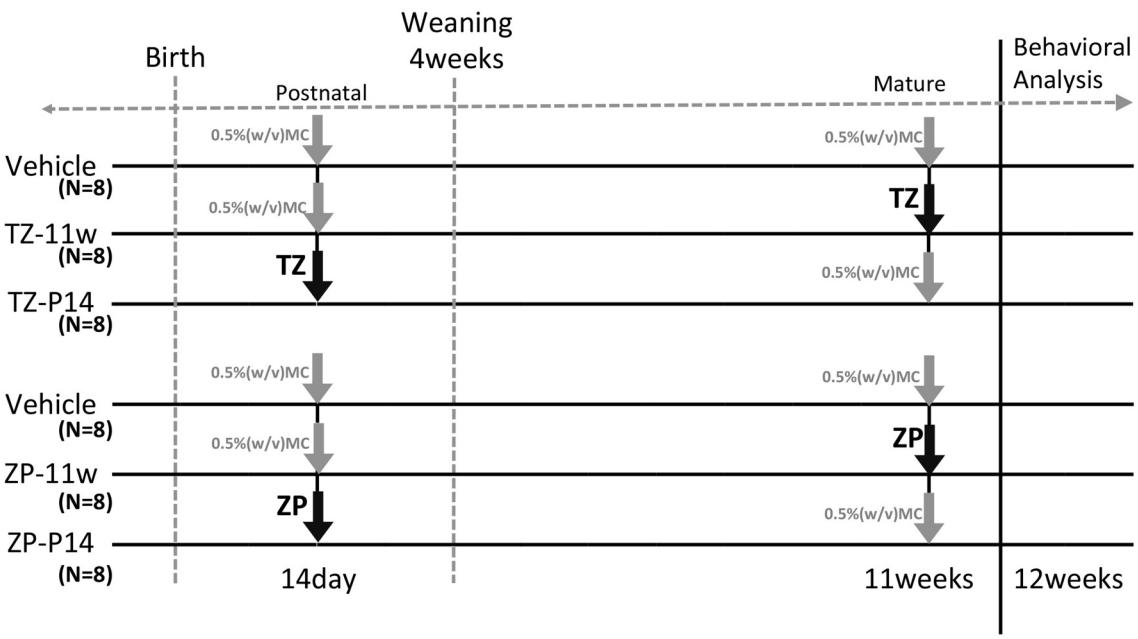

FIGURE 1 | Experimental schedule. This figure describes the experimental schedules for triazolam (TZ) and zolpidem (ZP) treatment. The gray arrow indicates vehicle and the black arrows indicate TZ 1 mg/kg or ZP 50 mg/kg. All mice received the drug three times by gavage. The two ages of 2 weeks (2 w) and 11 weeks (11 w) were selected. TZ and ZP were administered to the mice at one of the two time points and vehicle was administered at the other two time points. The mice were weaned at 4 weeks. Male mice were selected and were housed in new cages (4 mice per cage). A behavioral battery tests (BBT) was carried out starting at 12 weeks of age. Each group consisted of eight mice (6 group, 48 mice total).

MATERIALS AND METHODS

Animal Experiment

All animal experiments were conducted with permission from the Animal Ethics Committee at the National Institute of Health Sciences. Pregnant female C57BL/6NCrSlc mice at embryonic day 11 were purchased from Japan SLC Inc. (Shizuoka, Japan). The mice were housed in plastic cages and maintained under a 12-h light/12-h dark cycle with water and chow (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) provided *ad libitum*. Triazolam (TZ: 8-chloro-6-(2-chlorophhenyl)-1-methyl-4H-1, 2, 4 -triazolo[4,3-a]-1, 4-benzodiazepine; Sigma Aldrich Co. Steinheim, Germany), and Zolpidem (ZP: N,N,6-Trimethyl-2-(4-methylphenyl)-imidazo[1,2-a]pyridine-3-acetamide; Sigma Aldrich Co., Steinheim, Germany) were dissolved in 0.5% (w/v) methyl cellulose solution (Wako Pure Chemical Industries, Ltd. Osaka, Japan) and administered by gavage at the doses of 1 and 50 mg/kg. In the vehicle group, 0.5% (w/v) methyl cellulose solution (MC) was administered by gavage. Administration and experimental schedules are shown in **Figure 1**. Mice in the Vehicle group (8 mice per experimental group, 16 mice total) were treated with 0.5% (w/v) MC at 2 weeks and 11 weeks of age. The TZ-2w group (8 mice) was treated with TZ at 2 weeks of age and with 0.5% (w/v) MC at 11 weeks of age. The ZP-2w group (8 mice) was treated with ZP at 2 weeks of age and with 0.5% (w/v) MC at 11 weeks of age. The TZ-11w group (8 mice) was treated with 0.5% (w/v) MC at 2 weeks of age and with TZ at 11 weeks of age. The ZP-11w group (8 mice) was treated with 0.5% (w/v) MC at 2 weeks of age and with ZP at 11 weeks of age. Subsequently, a series of BBT were conducted at 12 weeks of age. After the BBT, the brains of the mice were dissected, and the hippocampus was removed for biochemical analysis at 13 weeks of age.

Mouse Behavioral Battery Test

We conducted a behavioral battery test (BBT), including the open field test (OF), the light/dark transition test (LD), the elevated plus maze test (EP), the contextual/cued fear conditioning test (FZ), and the additional pre-pulse inhibition test (PPI). For the OF, LD, EP, and FZ methods details, refer to the Tanemura et al. (2009). In the present study, we added the PPI further for information processing analysis. Experimental apparatuses and image analysis software was obtained from O'Hara & Co., Ltd., Japan. Image analysis software (Image OF4, Image LD2,

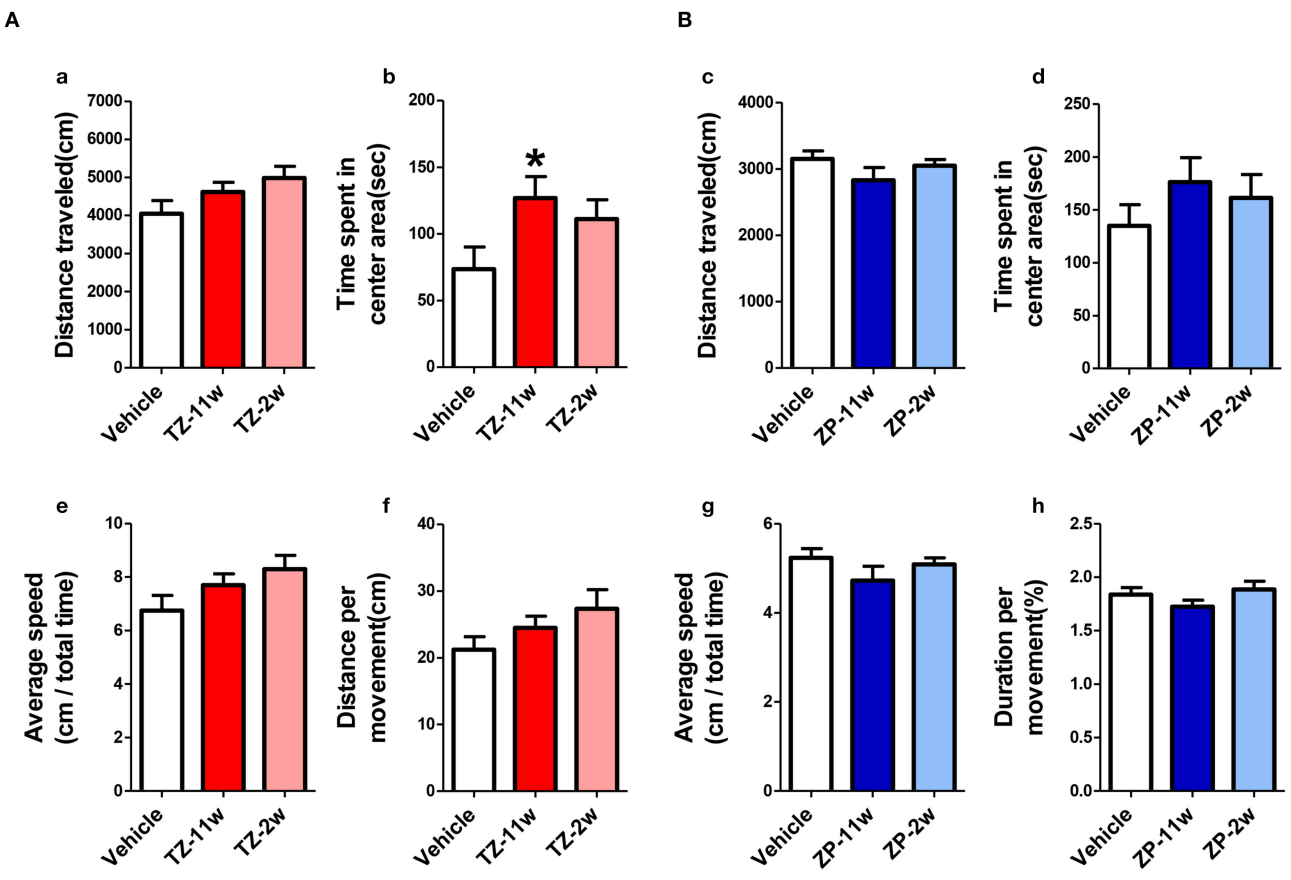

FIGURE 2 | Results of the open field (OF) test. (A,B) The scores of the OF test (total test time, 600 s) are shown. **(Aa,Bc)** Distance traveled (cm) during the test period (600 s) is shown. **(Ab,Bd)** Time spent in the center area (seconds) is shown. **(Ab)** Statistically significant increases were detected in the TZ-11w group. **(Ae,Bg)** Average speed (cm/total time) is shown. **(Af,Bh)** Distance per movement (cm) is shown. $n = 8$, mean ± SEM. Asterisk (*) indicates a statistically significant difference (*$p < 0.05$, Student's t-test) compared to the Vehicle group.

Image EP2, and Image FZ2) were developed using the public domain ImageJ program. All experiments were performed with 8 mice per group (TZ experimental: 3 groups, ZP experimental: 3 groups). We thus had 6 groups for a total of 48 mice. The experimental tests were conducted between 13:30 and 16:30. The level of background noise during BBT was about 50 dB. After each trial, the apparatus was wiped and cleaned. The pre-pulse inhibition test apparatus consists of a light source and a sound system, and a startle measurement load cell. These are set into a soundproof box. The software for the operation of the apparatus and the data analysis is the SR-9040 (O'Hara & Co., Ltd., Tokyo, Japan). The white background noise level is set to 70 dB in the soundproof box. The mouse is put into a plastic cylinder and kept there for 90 s before the test. The test schedule consists of three blocks, and the total trial time is 30 min. Breakdown of each block is as follows: 80, 85, 95, 100, 105, and 110 dB pulse × 3 (acclimation block), 120 dB pulse × 10 (acoustic startle response block). The combinations of pre-pulse are 80–120, 85–120, 95–120, 100–120, 105–120 dB, with a delay of 100 ms × 6 (pre-pulse inhibition measurement block). These combinations were presented in a pseudorandom order, such that each trial type was presented once within a block. The inhibition ratio (%) of the startle response is calculated as follows: (1–pre-pulse [80, 85, 95, 100, or 105 dB] startle response value / acoustic startle response value) × 100.

Western Blotting

Hippocampal extracts were dissolved in Tris-buffered saline (pH 7.4) containing protease inhibitors (Nacalai Tesque, Inc., Kyoto, Japan) and phosphatase inhibitors (Nacalai Tesque, Inc., Kyoto, Japan). Equal volume of the total protein solutions were added to 2 × sample buffer solution (Nacalai Tesque, Inc., Kyoto, Japan). The amount of protein was quantified using Qubit protein assay kits (Life Technologies Co., California, USA). The protein samples (30 μg/well) were subjected to SDS-PAGE (7.5% polyacrylamide), and transferred to a nitrocellulose membrane. The membranes were blocked in Blocking-one (Nacalai Tesque, Inc., Kyoto, Japan) at room temperature for 90 min and incubated with primary antibodies, such as those against acetyl-tubulin (sc-23950, Santa Cruz Biotechnology, Inc., California, USA), MAP2 (sc-20172), GluR1 (T9026, Sigma Aldrich Co., Steinheim, Germany), and GluR4 (SAB450126) overnight at room temperature. The membranes were then washed with phosphate buffered saline (pH 7.4) with 0.05% Tween-20 (PBS-T). The membranes were then incubated with peroxidase-conjugated secondary antibodies for 2 h at room temperature. After several PBS-T washes, the membranes were incubated with chemi-lumi one L (Nacalai Tesque, Inc., Kyoto, Japan) for signal production. The signal was photographed using an LAS-3000 (Fujifilm Co., Ltd., Tokyo, Japan). The intensity of each protein band was measured

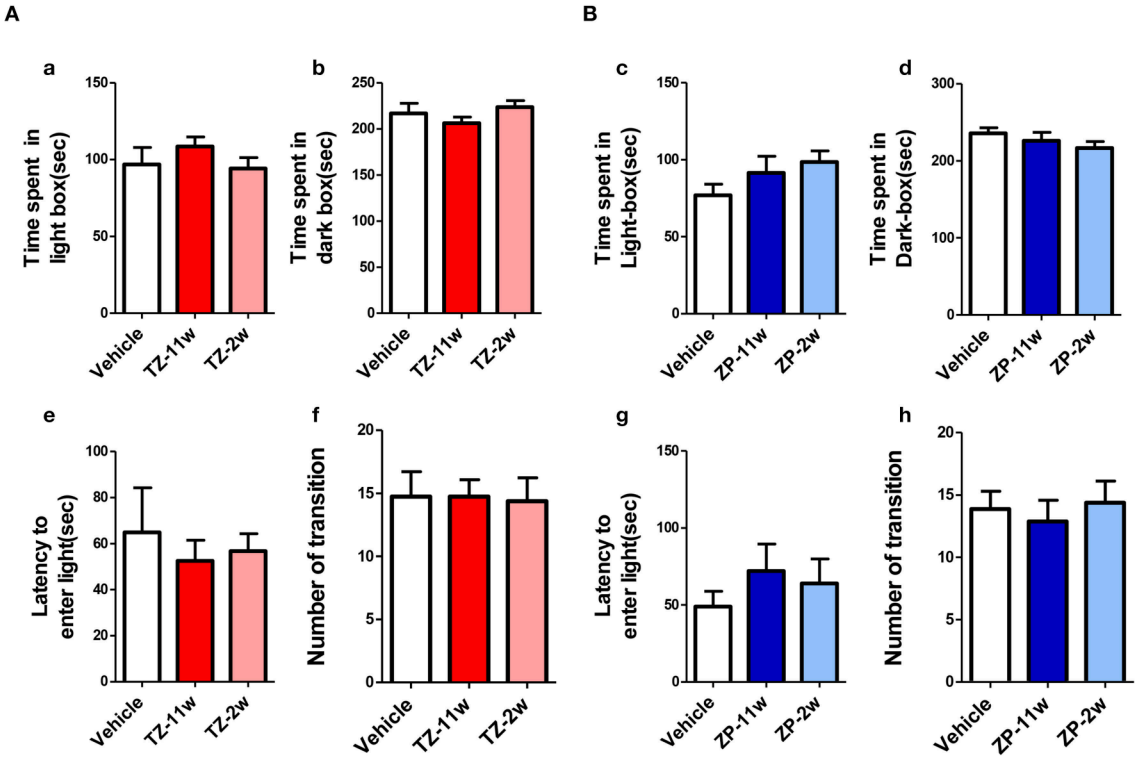

FIGURE 3 | Results of the light/dark transition (LD) test. (A,B) The scores for the LD test (total test time, 360 s) are shown. **(Aa,Bc)** Time spent in the light box (seconds). **(Ab,Bd)** Light box distance traveled (seconds) is shown. **(Ae,Bg)** The number of transitions between the dark box and the light box. **(Af,Bh)** Latency (seconds) to enter the light box for the first time. No significant differences compared with the Vehicle group were detected in the LD. The number of mice per group was eight and the scores are shown as the mean ± SEM.

using the Gel Analyzer program in ImageJ, and normalized to the intensity of the acetyl-tubulin band using the following formula: (intensity of each protein band/acetyl-tubulin band intensity) × 100. We present each normalized band intensity as a percentage of its intensity in the Vehicle group. The changes in protein expression associated with TZ and ZP treatment were calculated as follows: Ratio (%) = (Vehicle, TZ and ZP group individual value/Vehicle group average value) × 100.

Quantitative RT-PCR

Duplicate homogenate hippocampal samples were treated with DNaseI (amplification grade, Invitrogen Corp., Carlsbad, CA, USA) for 15 min at room temperature. They were then incubated with Super-Script II (Invitrogen) for 50 min at 42°C for reverse transcription. Quantitative real time PCR was performed using an ABI PRISM 7900 HT sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq (Takara Bio Inc., Japan) with initial denaturation at 95°C for 10 s followed by 45 cycles of 5 s at 95°C and 60 s at 60°C. Ct values were obtained. The primers were synthesized by FASMAC Co., Ltd., Japan. Expression of genes of interest was normalized to that of *Actb* and presented as fold change over baseline using the delta-delta CT method. Fold changes of relative gene expression levels compared to those of Vehicle animals were calculated (Livak and Schmittgen, 2001).

Primers

Arc: forward 5′-TACCGTTAGCCCCTATGCCATC-3′, reverse 5′-TGATATTGCTGAGCCTCAACTG-3′

c-Fos: forward 5′-ATGGGCTCTCCTGTCAACACAC-3′, reverse 5′-ATGGCTGTCACCGTGGGGATAAAG-3′

Nr4a1: forward 5′- TTAAGAGGTGGGTCGGGTTC -3′, reverse 5′- GCAATCCTTCTCGCACACTA -3′

ActB: forward 5′-GGACTCATCGTACTCCTGCTT-3′, reverse 5′-GAGATTACTGCTCTGGCTCCT-3′.

Statistical Analysis

Statistical analysis was conducted using Prism 5.04 (SAS Institute, California, USA). Data was analyzed using Student's unpaired *t*-tests. Values in graphs are expressed as the mean ± standard error of the mean (SEM).

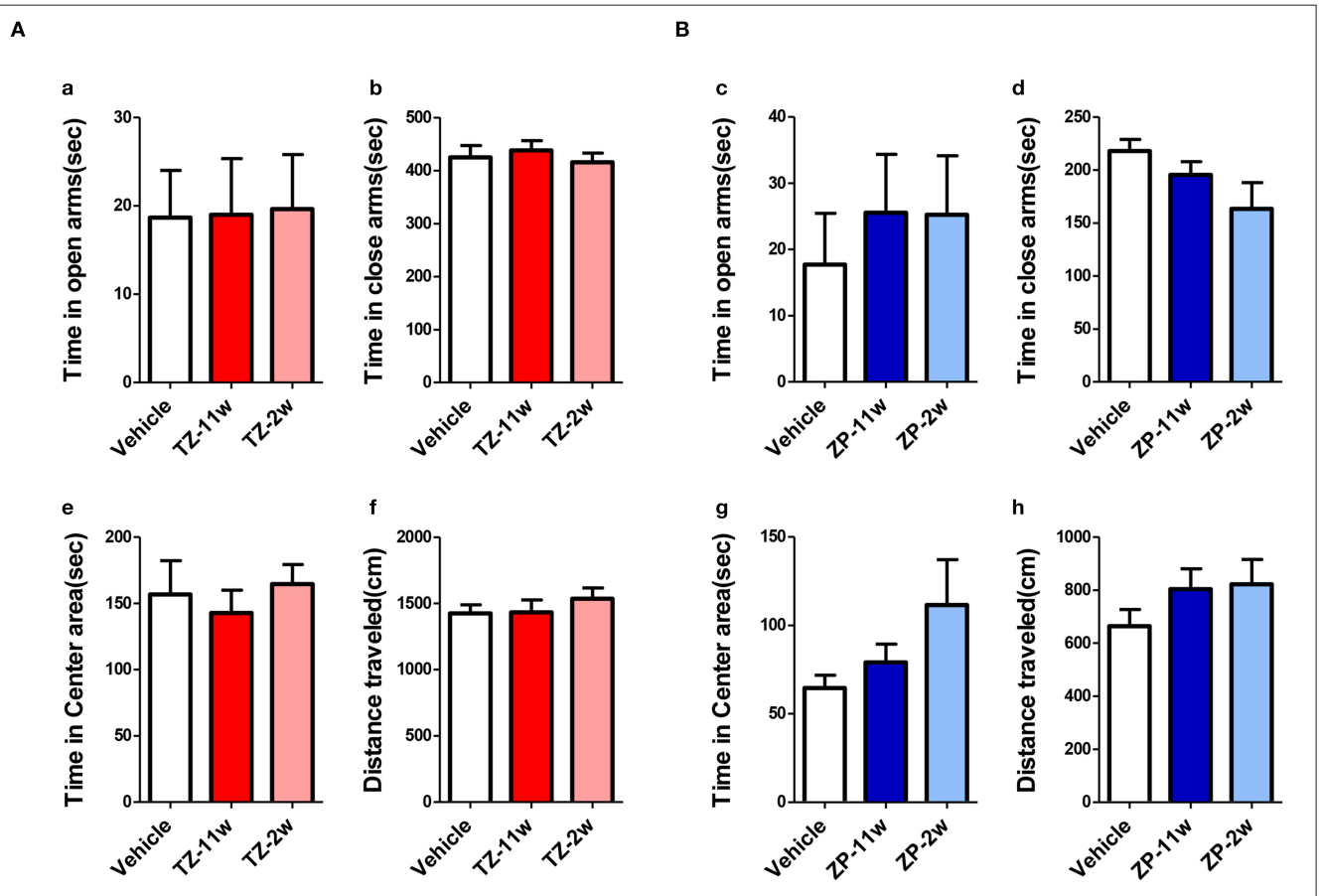

FIGURE 4 | Results of the elevated plus maze (EP) test. EP tests were conducted to analyze emotional behavioral changes in TZ- and ZP-treated mice. **(A,B)** The scores of the EP test (total test time, 600 s) are shown. **(Aa,Bc)** Time spent in the open arm (seconds). **(Ab,Bd)** Time spent in the closed arm (seconds). **(Ae,Bg)** Time spent in the center area (seconds). **(Af,Bh)** Total distance moved (cm). No significant differences with the Vehicle group were detected in the EP. The number of mice per group was eight and the scores are shown as the mean ± SEM.

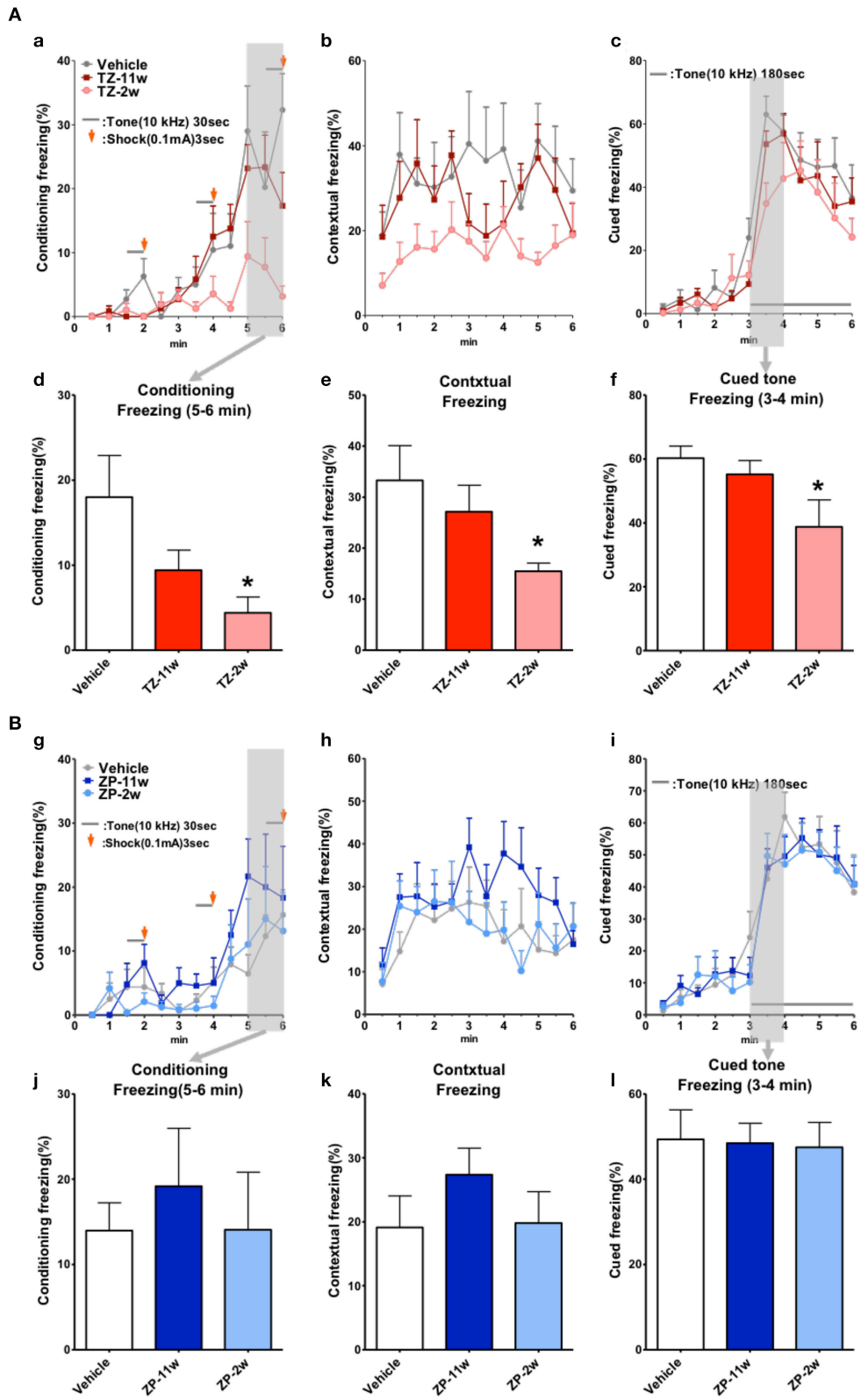

FIGURE 5 | Results of the fear conditioning test. (A,B) Fear conditioning test was conducted to analyze the effects of TZ and ZP on learning and contextual memory, i.e., place (test-box) and sound (cued tone). **(Aa,d,Bg,j)** The total conditioning time was 360 s. Three cycles consisting of a tone (30 s) and a mild foot shock

(Continued)

FIGURE 5 | Continued
(arrows: 0.1 mA, 3 s) were carried out for each mouse after it was allowed to explore the box freely for 90 s. **(Aa,Bg)** The time course of the freezing % scores is plotted for the conditioning test. As the cycles of the conditioning were repeated, the freezing percentages increased in the Vehicle group and in all groups other than the TZ-2w group. This indicates successful conditioning. On the other hand, the freezing % remained low in the TZ-2w group. **(Ad,Bj)** The average freezing % scores during the later period (180–360 s) of the conditioning test are shown. A significant decrease in freezing % was observed in the TZ-2w group. **(Ab,e,Bh,k)** Contextual tests were conducted 24 h after the conditioning test to analyze the effects of triazolam on place memory function. **(Ab,Bh)** The time course of the freezing % scores is plotted for the contextual test. The total time of the test is 360 s. **(Ae,Bk)** The average freezing % scores in the contextual test are shown. A significant decrease in freezing % was detected in the TZ-2w group. **(Ac,f,Bi,l)** The cued test was conducted 24 h after the contextual test to analyze the effects of triazolam on cued memory function. The total time of the test is 360 s. **(Ac,Bi)** The time course of the freezing % scores was plotted for the cued test. The tone was presented to the mice during the later period of the test (180–360 s). **(Af,Bl)** The average freezing % scores for the first one minute period after the tone are presented. The freezing % scores of the TZ-2w group were significantly lower than those of the Vehicle group. The number of mice per group was eight and the scores are shown as the mean ± SEM. Asterisk (*) indicate statistically significant differences (*$p < 0.05$, Student's *t*-test) compared to the Vehicle group.

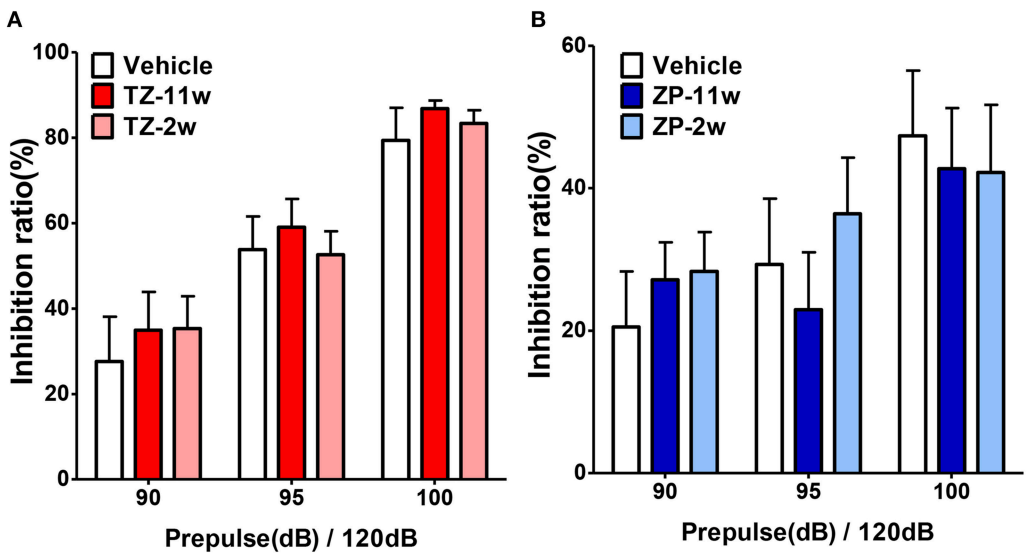

FIGURE 6 | Results of the pre-pulse inhibition (PPI) test. (A,B) The pre-pulse inhibition test was conducted to analyze the effects of sleep-inducing drugs on sensory information processing. The scores indicated the inhibition (%) of the startle response to a 120-dB sound with pre-pulse sounds of 90, 95, 100, and 105 dB when compared to the response to a 120-dB sound without a pre-pulse sound. No significant differences were found in the PPI test. The number of mice per group was eight and the scores are shown as the mean ± SEM.

RESULT

Results of Behavioral Battery Tests in the TZ-11w, TZ-2w, ZP-11w, and ZP-2w Groups

In the open field test (**Figure 2**), the total distance traveled and the time spent in the center region were significantly longer in the TZ-11w group (**Figure 2Ab**). Exposure to either chemical did not lead to any changes in the results of the light/dark transition test (**Figure 3**) or the elevated plus maze test (**Figure 4**). In the fear conditioning test, only the TZ-2w group did not show an increase in freezing % during the later period of the conditioning trial (**Figures 5Aa,d, Bg,j**, $p < 0.05$). In addition, in the contextual test, only the TZ-2w group had significantly lower freezing percentages (**Figures 5Ab,e, Bh,k**, $p < 0.05$). All other groups responded quickly to the tone and showed high freezing responses, while mice in the TZ-2w group had slower responses to the tone, which resulted in lower freezing responses (**Figures 5Ac,f, Bi,l**, $p < 0.05$). These results indicate that the administration of TZ during the juvenile period induces deficits in learning and memory. Exposure to either chemical did not lead to any changes in the results of the pre-pulse inhibition test (**Figure 6**). Significant differences were not observed in any of the tests in our BBT in the ZP-11w and ZP-2w groups. The results of the series of BBT performed on the TZ-11w, TZ-2w, ZP-11w, and ZP-2w groups during the adult stage are summarized in **Table 1**.

Protein Expression in the Adult Hippocampus in the TZ-11w, TZ-2w, ZP-11w, and ZP-2w Groups

We analyzed expression of several proteins in the adult mice hippocampus following juvenile or adult stage exposure to triazolam or zolpidem using western blotting (**Figure 7**). Although, no differences were detected in the TZ-11w, ZP-2w, and ZP-11w groups, MAP2 (**Figures 7A,Bb**, $p < 0.05$) was increased and GluR1 and GluR4 (**Figures 7A,Ba,b**, $p <$

TABLE 1 | A summary for the behavioral change at adult stage with different developmental exposure of triazolam 1 mg/kg B.W. and zolpidem 50 mg/kg B.W.

Test name	Behavioral tasks	Triazolam 1 mg/kg		Zolpidem 50 mg/kg	
		TZ-11w	TZ-2w	ZP-11w	ZP-2w
Open field	Distance traveled	1.14	1.23	0.90	0.97
	Center region time	1.72*	1.51	1.31	1.20
	Average speed	1.14	1.23	0.90	0.97
	Moving speed	1.03	1.09	0.96	0.97
	Moving episode number	0.99	0.98	1.00	0.97
	Total movement duration	1.12*	1.14*	0.93	1.00
	Distance per movement	1.15	1.29	0.91	1.00
	Duration per movement	1.14	1.19	0.94	1.03
Light/Dark transition	Dark distance	0.95	0.96	0.86	0.89
	Light distance	1.08	0.92	1.06	1.18
	Dark time	0.95	1.03	0.96	0.92
	Light time	1.12	0.97	1.19	1.28
	Number of transitions	1.00	0.97	0.93	1.04
	Latency to enter light	0.81	0.87	1.47	1.31
Elevated plus maze	Total distance	1.01	1.08	1.21	1.24
	Total center time	0.91	1.05	1.22	1.73
	Total open area	1.02	1.05	1.45	1.43
	Total close area	1.03	0.98	0.90	0.75
	Total arm select number	0.90	0.97	1.41	1.47
Fear conditioning	Total conditioning freezing %	0.83	0.27	1.57	0.91
	Conditioning 5–6 min freezing %	0.52	0.24*	1.39	1.02
	Total contextual freezing %	0.82	0.47*	1.43	0.72
	Cued tone 3–6 min freezing %	0.89	0.72	0.98	0.96
	Cued tone 3–4 min freezing %	0.92	0.64*	0.92	0.93
Pre-pulse inhibition	Pre-pulse 90 dB	1.07	1.28	1.32	1.38
	Pre-pulse 95 dB	1.19	0.98	0.78	1.24
	Pre-pulse 100 dB	1.07	1.05	0.90	0.89

The number indicates the fold change to vehicle and the asterisk indicates for the tests with the statistically significant difference to vehicle group [$p < 0.05$ (Student's t -test)].*

0.05) were decreased in the TZ-2w group. Both GluR1 and GluR4 belong to the AMPA-type glutamate receptor family and are known to be necessary for long-term potentiation in the hippocampus (Sanderson et al., 2008; Lee and Kirkwood, 2011). Therefore, decreases in the levels of GluR1 and GluR4 may be important mechanisms underlying the learning and memory deficits in mice exposed during the juvenile period.

mRNA Expression of Immediate Early Response Genes (IEGs) in the TZ-2w and ZP-2w Groups

We analyzed mRNA expression levels of IEGs (*Arc*, *c-fos*, and *Nr4a1*) in the hippocampus shortly (8 h) after TZ and ZP administration during the juvenile period. We observed decreased mRNA expression of IEGs in the TZ-2w group, but not in the ZP-2w group (**Figure 8**).

DISCUSSION

In this study, we analyzed the late-onset effects of the stimulation of the GABA-R signal using a behavioral battery tests (BBT) and several biochemical assays. We stimulated the GABA-R signal of mice by the oral administration of the sleep-inducing drugs TZ (1 mg/kg body weight [B.W.]) or ZP (50 mg/kg B.W.) during juvenile (TZ-2w, ZP-2w) and adult stages (TZ-11w, ZP-11w).

The results of the BBT indicate that spontaneous activity, as measured in the open field test, was significantly increased in the TZ-11w group. Reactivity to a novel environment may be changed in mice exposed to TZ during adulthood. On the other hand, deficits in learning and memory were detected by the fear conditioning test only in the TZ-2w group. The freezing response was decreased in all three stages (conditioning test, contextual test, and cued test) of the fear conditioning test. Although, the TZ-11w, ZP-11w, and ZP-2w groups had increases in freezing % as the cycles of tone and mild foot-shock were repeated, the

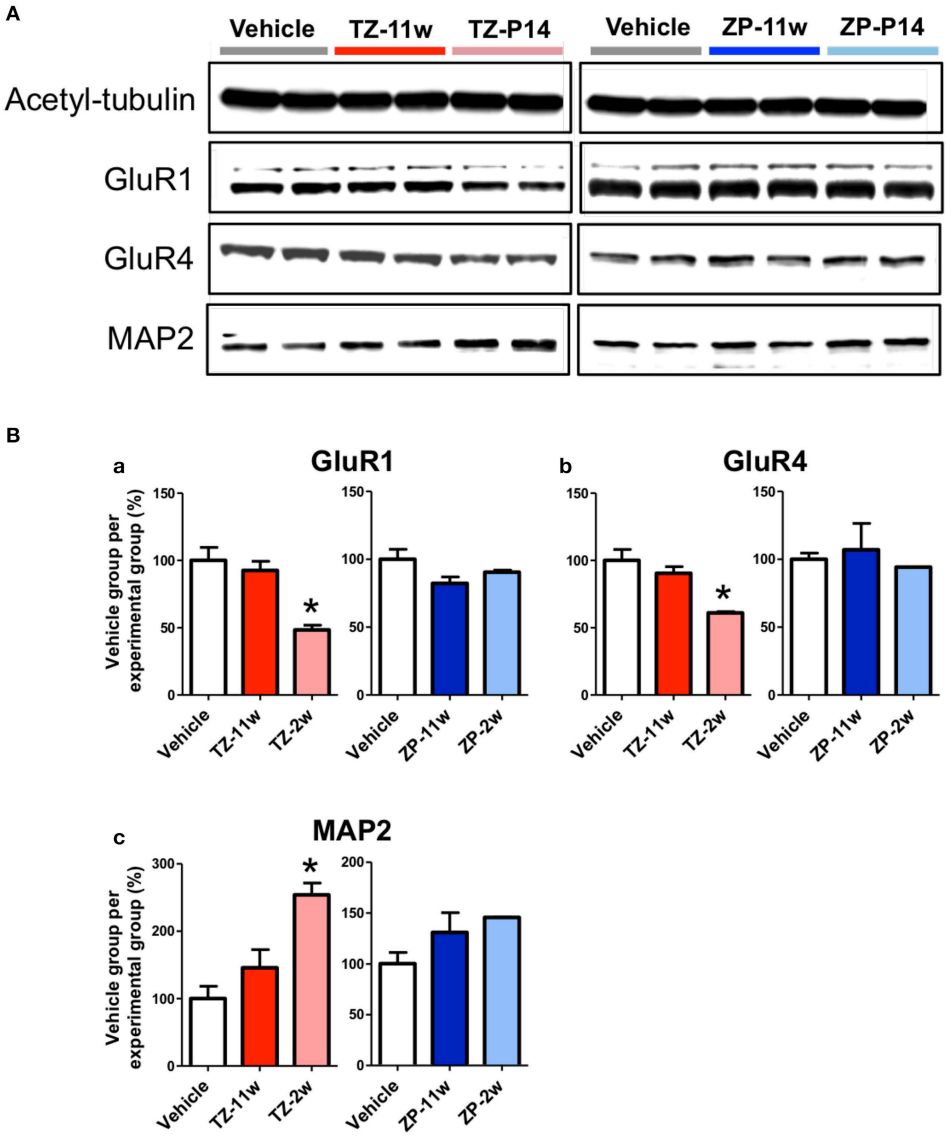

FIGURE 7 | Protein expression in the hippocampus during the adult stage following exposure to TZ and ZP during development. The expression levels of several proteins were analyzed by western blotting to explore the effects of triazolam on the hippocampus during adulthood. **(A)** Acetyl-tubulin and AMPA-type glutamate receptors GluR1 and GluR4, as well as the neuronal cytoskeletal marker MAP2. **(Ba–c)** The expression of each protein was normalized to that of acetyl-tubulin expression and its ratio (%) to the expression in the Vehicle group is shown. The number of samples was two and the value is shown as the mean ± SEM. Asterisk (*) indicate statistically significant differences (*$p < 0.05$, Student's t-test) compared to the Vehicle group.

TZ-2w group did not have an increase in freezing %, even during the later period of the test. These results may indicate that TZ-2w mice lose their ability to make short-term memories, which are needed for quick responses in this situation. In addition, TZ-2w mice had significantly lower scores in the contextual test. This may indicate deficits in spatial memory (Clark and Squire, 1998). In the cued test, a delay of the freezing response to the tone was detected in the TZ-2w group, which may indicate mild deficits in cued memory. We believe that these learning and memory deficits are the most serious effects of TZ treatment in the TZ-2w group.

To identify the molecular mechanisms underlying the late-onset learning and memory deficits, we first analyzed the expressions of several proteins in the hippocampus. We observed late-onset protein expression changes in the TZ-2w group. For example, MAP2 expression was increased in TZ-2w mice. MAP2 is a protein specifically expressed in the dendrites of neurons and plays an important role in the stabilization of tubulin structure in neuronal filaments (Caceres et al., 1983). There are also several reports of decreases in MAP2 levels in Alzheimer's disease and Parkinson's disease (Li et al., 2008; Liu et al., 2011). However, no increases in MAP2 levels have been reported thus

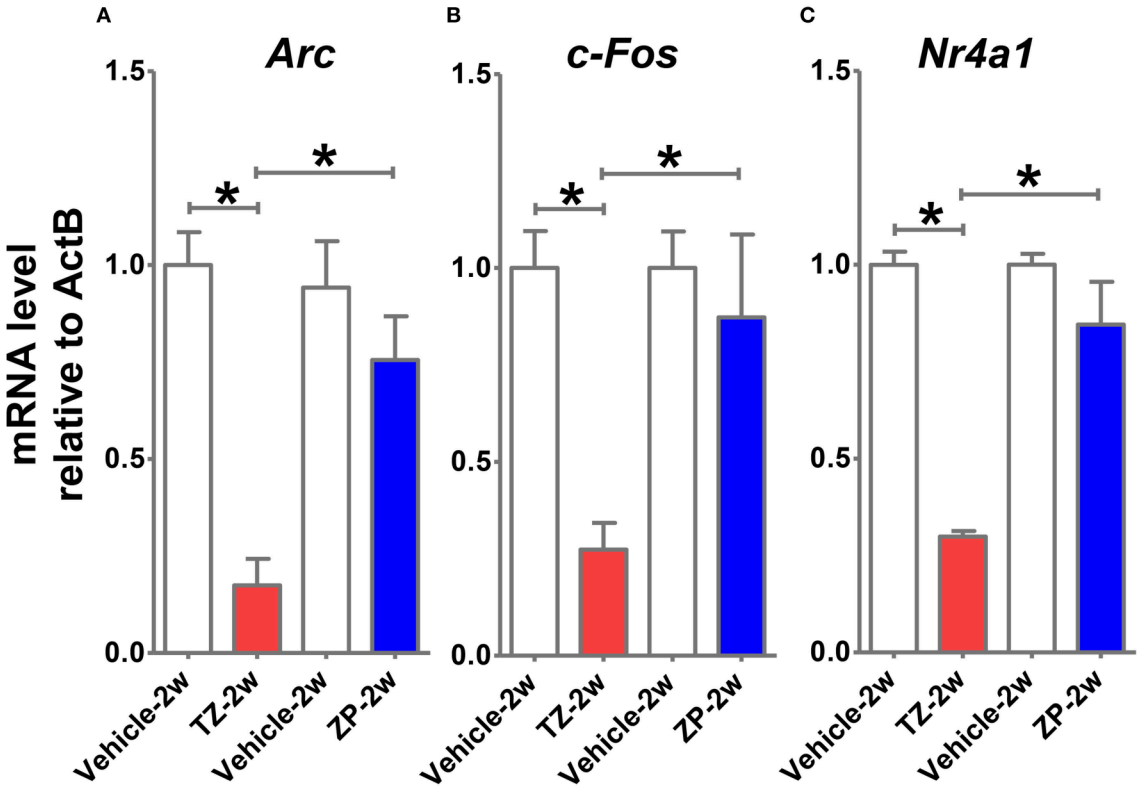

FIGURE 8 | Comparisons of immediate early response gene (IEG) mRNA expression levels in the hippocampus shortly after TZ or ZP exposure. Eight hours after TZ or ZP administration to juvenile (2-week-old) mice, we examined the mRNA expression levels of multiple IEGs in the hippocampus. Significant decreases in the expressions of **(A)** *Arc*, **(B)** *c-Fos*, and **(C)** *Nr4a1* were observed in the TZ-2w group. The number of the samples in each group was three and the values are shown as mean ± SEM. Asterisk (*) indicate statistically significant differences (*$p < 0.05$, Student's *t*-test) compared to the Vehicle group.

far. It is therefore still unclear how this increase in MAP2 levels relates to the deficits induced by triazolam. In addition, the expression levels of GluR1 and GluR4 were decreased in the TZ-2w group. GluR1 and GluR4 are AMPA-type glutamate receptors and are known to be important for learning and memory, as they induce long-term potentiation and synaptic plasticity (Sanderson et al., 2008; Lee and Kirkwood, 2011). Therefore, the deficits of learning and memory in the adult stage in neonatally exposed mice may be related to decreases in GluR1 and GluR4 levels. None of the ZP-treated groups and TZ-11w groups had changes in MAP2, GluR1, or GluR4 protein expression.

We hypothesize that TZ may have greater inhibitory effects on the hippocampus during the juvenile period and that may be a trigger of learning and memory deficits. Therefore, we measured changes in IEG mRNA expression in the hippocampi of juvenile mice shortly after TZ or ZP exposure (8 h). As expected, TZ had a greater inhibitory effect on the juvenile hippocampus than ZP. Indeed, the expression levels of all three IEGs (*Arc*, *c-fos*, and *Nr4a1*) were decreased in response to TZ treatment. IEGs are known to be induced rapidly following neuronal activation and are considered as markers of activated neurons (Sheng and Greenberg, 1990). The TZ-specific suppression of neuronal activity during the juvenile period may thus be responsible for the TZ-specific learning and memory deficits.

In summary, our findings demonstrate that the BZD sleep-inducing drug TZ can lead to learning and memory deficits with juvenile exposure. In contrast, ZP, which is a non-BZD, did not induce deficits in brain functional development. Decreased IEG expression was detected in mice treated with TZ during the juvenile period, but not in mice treated with ZP during the juvenile period. Therefore, we suggest that decreased IEG expression may be one of the triggers for the long-lasting adverse effects of TZ on the brain. The learning and memory deficits induced by TZ may be dependent on the timing of the exposure, as GABA-R signal has different functions in different brain developmental stages and in different brain regions containing GABA-Rs (Rice and Barone, 2000). GABA-R signal is reported to have a critical role in the reconstruction of synapses during the juvenile period (Steward and Falk, 1986; Herschkowitz et al., 1997). Therefore, juvenile exposure to TZ may interfere with synapse reconstruction and affect the proper development of learning and memory.

We have previously reported the aberration of emotional behavior associated with deficits in learning and memory in adult male mice treated with domoic acid (the chemical compound

for the excessive activation of glutamate receptor mediated signal; Glu-R signal) at prenatal period by the administration to pregnant female mice (Tanemura et al., 2009). Nevertheless, we could not find the correlativity with this study using TZ. The following points can be given as that reasons. In previous study, we used mice in prenatal period does not correspond in the degree of maturation of hippocampus from juvenile period (Rice and Barone, 2000; Luján et al., 2005). Moreover, the domoic acid and TZ are completely different mechanism of action. We demonstrated that TZ administration cause the IEGs expression inhibitory in the juvenile period hippocampus. However, the domoic acid administration is known to increase IEG expression in hippocampus (Scallet et al., 2004). For these reasons, current study cannot be directly compared with previous one. We guessed that there might be several differences between the effects induced by disturbances of GABA-R and Glu-R signals, as each neuronal signal has the particular function to construct the neuronal circuits and reorganization depending on brain development (Steward and Falk, 1986; Rice and Barone, 2000; Luján et al., 2005). In addition, we guessed that the various aberrations of behavioral manners might be induced by the various disturbances of neural signals by the neuroactive chemical compounds.

In conclusion, our study indicates that juvenile TZ exposure may lead to learning and memory deficits. GABA-R agonists, such as BZD, are used for the treatment of both sleep disorders and anxiety disorders in both adults and children (Chevreuil et al., 2010; Weiss and Garbutt, 2010; Pelayo and Yuen, 2012). Therefore, considering the possible influence of GABA-R agonists on brain development, their careful prescription to children is warranted.

AUTHOR CONTRIBUTIONS

YF designed and performed experiments, analyzed data, and wrote the paper. KT designed the study, developed the methodology, conducted experiments, and editing the manuscript. KI helped with designed and editing the manuscript. MI helped with behavioral test and western blotting. KA, SK helped with the design of the study. MK, JK for the supervisor and design of the study.

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Neuroendocrinal, Neurodevelopmental, and Embryotoxic Effects of Recombinant Tissue Plasminogen Activator Treatment for Pregnant Women with Acute Ischemic Stroke

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Thrombolysis with recombinant tissue plasminogen activator (rTPA) was the first evidence-based treatment approved for acute stroke. Ischemic stroke is relatively uncommon in fertile women but treatment is often delayed or not given. In randomized trials, pregnancy has been an exclusion criterion for thrombolysis. Physiologic TPA has been shown to have neuroendocrine effects namely in vasopressin secretion. Important TPA effects in brain function and development include neurite outgrowth, migration of cerebellar granular neurons and promotion of long-term potentiation, among others. Until now, no neuroendocrine side-effects have been reported in pregnant women treated with rTPA. The effects of rTPA exposure in the fetus following intravenous thrombolysis in pregnant women are still poorly understood. This depends on low case frequency, short-duration of exposure and the fact that rTPA molecule is too large to pass the placenta. rTPA has a short half-life of 4–5 min, with only 10% of its concentration remaining in circulation after 20 min, which may explain its safety at therapeutically doses. Ischemic stroke during pregnancy occurs most often in the third trimester. Complication rates of rTPA in pregnant women treated for thromboembolic conditions and ischemic stroke were found to be similar when compared to non-pregnant women (7–9% mortality). In embryos of animal models so far, no indications of a teratogenic or mutagenic potential were found. Pregnancy is still considered a relative contraindication when treating acute ischemic stroke with rTPA, however, treatment risk must be balanced against the potential of maternal disability and/or death.

Keywords: rTPA, alteplase, brain development, toxicity, haemorrhagic, intravenous, teratogenic, uterine

NEUROENDOCRINE EFFECTS OF TISSUE PLASMINOGEN ACTIVATOR

Tissue plasminogen activator (TPA) is a serine protease that converts plasminogen into the fibrinolytic enzyme plasmin thus promoting fibrin dissolution in blood clots (Carmeliet et al., 1994). Endothelial cells are the major source of circulating TPA, which is released upon stimulation by factor X-a, bradykinin, fibrin, platelet activating factor, and thrombin (Booyse et al., 1986; Emeis, 1992). Other triggers of TPA released into the bloodstream include among others, anxiety, exercise, surgery, and electroconvulsive therapy, however, these conditions are also coupled to catecholamine release, which may thus be the shared trigger mechanism for both direct TPA release and TPA release from endothelial cells in these conditions. In support of this notion, TPA was demonstrated to be co-expressed and trafficked simultaneously with noradrenaline in the chromaffin cells of the adrenal glands (Parmer et al., 1993). Chromogranin A is one soluble protein that is co-released with TPA and catecholamines. It works as prohormone which, when cleaved into active peptides, inhibits the further release of catecholamines (Parmer et al., 1993). Experiments with knockout mice lacking TPA provided further evidence for a role of TPA in behavioral stress responses and catecholamine release. These mice exhibit deficient stress-induced anxiety behavior (Pawlak et al., 2003, 2005) and show anxiety-like behavior after intracerebroventricular injection of corticotrophin releasing factor (Matys et al., 2004).

TPA-cleaved plasmin regulates proteolysis of among other, laminin, collagen IV, proteoglycans, pro-brain-derived neurotrophic factor (pro-BDNF), and protease activated receptor-1 (Dityatev and Schachner, 2003; Pang et al., 2004). TPA has been shown to directly interact with low-density lipoprotein receptor-related protein (LRP) leading to phosphorylation of mitogen-activated protein kinase (MAPK) 1 and extracellular signal-regulated kinases-1 and 2 (Zhuo et al., 2000; Hu et al., 2006). In turn, serpins (including the CNS variant neuroserpin) and plasminogen activator inhibitor PAI-1 and -2 are the main inhibitors of the serine protease family including TPA, urokinase-type plasminogen activator (uPA), plasmin, and thrombin (Yepes and Lawrence, 2004).

Endogenous TPA is widely distributed in the neuroendocrine system. In the neuroendocrine cells of the hypophysis, the magnocellular neurons of the hypothalamic supra-optic nucleus, the chromaffin cells of the adrenal medulla, thyroid and parathyroid glands, endogenous TPA first enters the endoplasmic reticulum where it binds to a signal peptide and is transported through the Golgi complex. It is then released either via the regulated secretory pathway (vesicular) or the constitutive secretory pathway (direct release) (Kelly, 1985). Although there is one regulated secretory pathway, TPA gets rapidly released from storage vesicles originated from the Golgi complex and appears to be mediated by calcium ion influx (Gualandris et al., 1996). A similar mechanism of TPA co-release with parathyroid hormone has been described in parathyroid cells (Bansal and MacGregor, 1992).

In the brain, wide expression of neuronal TPA and the plasmin inhibitor neuroserpin are found in the developing and adult nervous system and have been shown to play a role in neuronal plasticity (Lee et al., 2015). TPA expression predominate in the lobar hemispheres, thalamus, medulla oblongata, and mesencephalon whereas neuroserpin, although also overlapping with TPA in the lobar hemispheres and mesencephalon, is more abundant in the spinal cord, substantia nigra and Purkinje cells (Teesalu et al., 2004). Moreover, TPA mRNA expression is seen in ventricular ependymal cells and meningeal blood vessel cells (Hashimoto et al., 1998).

In particular, a role of TPA in the neurohypophysis has been proposed for the osmotic regulation of body fluids. The antidiuretic hormone vasopressin (arginine-vasopressin or AVP) is synthesized in the magnocellular neurons of the hypothalamic supra-optic nucleus and paraventricular nucleus, and packed into neurosecretory granules, which are transported through their axons over to the neurohypophysary terminals (Miyata and Hatton, 2002). TPA immunoreactivity was observed at neurosecretory granules of vasopressin-positive magnocellular terminals and that of plasminogen was seen at astrocytes. With electron microscopy, Imamura et al. were able to show a specific localization of TPA at neurosecretory granules containing vasopressin, indicating that TPA is co-released along with the exocytosis of vasopressin and might be an early regulator of vasopressin release (Imamura et al., 2010).

TPA has been implicated in neurite outgrowth of neuronal cultures (Pittman et al., 1989), neuronal regeneration, migration of cerebellar granule neurons (Seeds et al., 1995), and prohormone synthesis (Sappino et al., 1993). TPA is capable of potentiating N-methyl-D-aspartate (NMDA) receptor activation by cleaving the NMDA receptor 1 (NMDAR1) subunit (Nicole et al., 2001). The significance TPA-induced cleavage of NMDAR1, as well as cleavage of pro-BDNF by plasmin is particularly relevant for learning and memory. On the one hand, TPA knockout (KO) mice show reduced maintenance of the long-term potential in the hippocampal CA1 area and exhibit less open-field exploration and poor performance in a context-conditioning task (Calabresi et al., 2000). On the other hand, mice overexpressing TPA show an enhancement of the long-term potential in the hippocampus with improved performance in spatial navigation learning tasks (Baranes et al., 1998; Madani et al., 1999). Long-term depression is absent in the striatum of TPA KO mice and has been coupled with decreased rearing activity and object exploration, as well as with poorer performance in a two-way active avoidance task (Calabresi et al., 2000). In 3 month-old Fischer rats, increased TPA mRNA expression is detected in Purkinje cerebellar neurons following 1 h of complex motor task learning in rats (Seeds et al., 1995). Ocular dominance plasticity in the visual cortex was also shown to be related to TPA and plasmin activities (Müller and Griesinger, 1998; Mataga et al., 2004). The authors have also suggested that a cascade of plasmin generated by TPA may selectively mediate cortical plasticity, perhaps via structural remodeling of axons (Müller and Griesinger, 1998; Mataga et al., 2004). Evidence for a function of TPA and the brain-specific protease inhibitor neuroserpin in regulating axonal

growth has come from studies of cultured cells (for a recent review see Lee et al., 2015). Hashimoto and colleagues found evidence supporting TPA involvement in long-lasting cortical plasticity following psychotomimetic administration in the rat by observing increased mRNA expression in prefrontal cortex neurons projecting to the medial striatum (Hashimoto et al., 1998).

Finally, ischemic damage is suggested to lead to excess endogenous TPA activity in the brain and contribute to neurodegeneration via extracellular matrix degradation, microglia activation, and blood brain barrier leakage (Lee et al., 2015). Neuroserpin-knockout mice have worse ischemic damage and neurological outcomes than controls, with the effects attributed to TPA-mediated activation of microglia (Gelderblom et al., 2013). Experimental intravenous (exogenous) TPA administration was shown to increase cerebrovascular permeability and decrease cerebrovascular resistance (Tsirka et al., 1995; Yepes et al., 2003; Nassar et al., 2004).

TREATMENT WITH RECOMBINANT TPA IN PREGNANT WOMEN

About 85% of all strokes are ischemic and the remaining are hemorrhagic. Spontaneous reperfusion may occur through endogenous release of plasminogen activator, which stimulates plasmin formation from plasminogen. For larger occlusions this release seems insufficient to induce reperfusion in time to avoid a cerebral lesion. Administration of alteplase, a recombinant tissue plasminogen activator (rTPA) as an injectable drug, which is commonly used to treat myocardial infarction, stroke and thrombosis, is thus one method to enhance this endogenous procedure (for a recent review see Prabhakaran et al., 2015). Acute ischemic stroke in pregnant women occurs most commonly in the third trimester and is potentiated by an increased pro-coagulant state during pregnancy, higher risk for cervical and intracranial artery dissection peri-partum, as well as by persistent foramen ovale and other underlying cardiac conditions.

The thrombolytic effect of rTPA varies among species. Humans are proposed to have a more sensitive fibrinolytic system to the effects of rTPA (Korninger and Collen, 1981). Thus, in humans the effective and safe dose for acute stroke treatment is 0.9 mg/kg. In rats, a dose of 1.8 mg/kg up to 10 mg/kg induced recanalization of carotid artery occlusion in 17–71%, whereas in humans this is only achieved in 10–30% of cases. The 1.8 mg/kg dose in the rat is proposed to be equivalent to the human dose of 0.9 mg/kg in terms of efficacy (Tomkins et al., 2015). In rabbits, a dose of 5 mg/kg—but not of 3 or 10 mg/kg—is capable of dissolving an intracerebral clot embolized from the carotid artery (Bednar et al., 1993). Until now, randomized controlled trials have excluded pregnant women and patients with increased hemorrhage risk from participation in studies regarding thrombolysis treatment. In animals rTPA does not cross the placenta and there has been no evidence of teratogenicity (Chan et al., 2000; Leonhardt et al., 2006; De Keyser et al., 2007). To date there are no reports on rTPA being able to cross the human placenta. In 2006, Leonhardt et al. had reviewed 18 cases of pregnant women treated with rTPA for other thromboembolic conditions, mainly pulmonary embolism, deep vein thrombosis and thrombosed cardiac valve prosthesis and 10 cases of pregnant women treated with rTPA for acute stroke, including an own stroke case (Leonhardt et al., 2006). Good maternal neurological outcome was reported for all but two mothers who died (one with stroke, the other with mitral valve thrombosis) and one who developed cerebral infarction. Ineffective thrombolysis or partial arterial recanalization was reported in four mothers. Twenty children were born with good outcome, however, there were two spontaneous abortions, three pregnancy interruptions owing to maternal cause and one infant died at 2 weeks' post-partum. Thus, there was a similar rate of complications in pregnant women compared to non-pregnant women, with mortality at about 7% for the mother and about 23% for the child (half of the child losses occurred in three stroke cases; the other half in two pulmonary embolisms and one valve thrombosis). Possible explanations for child loss not addressed by this review may include the severity of the underlying maternal medical condition rather than a direct effect of rTPA treatment alone. Interruptions of pregnancy may also have been carried following medical decision. Later in 2006, Wiese et al. reported use of intravenous rTPA thrombolysis in a pregnant woman with acute cardioembolic stroke. The patient improved clinically, did not develop complications after receiving rTPA, and at 37 weeks' gestation, delivered a healthy infant (Wiese et al., 2006). Yamaguchi et al. reported a 36 year-old woman, who was 18 weeks pregnant and developed a sudden onset of motor aphasia and hemiparesis on the right side. The NIH stroke scale was 6, and the brain MRI indicated occlusion of the left middle cerebral artery branches. She was treated with intravenous rTPA with subsequent recanalization of the occluded left middle cerebral artery branches. The symptoms disappeared within a few hours after treatment. She delivered a healthy infant without any apparent complications (Yamaguchi et al., 2010). There are further cases of successful use of rTPA in pregnant women with acute stroke, the majority in the third trimester of pregnancy (Dapprich and Boessenecker, 2002; Elford et al., 2002; Johnson et al., 2005; Murugappan et al., 2006). In 2012, Li et al. reported one own stroke case and reviewed 10 previously published stroke cases. They reported good to complete recovery in 10 mothers and one death during endovascular treatment, resulting in the delivery of eight healthy infants, two medical terminations of pregnancy, and one fetus death (Li et al., 2012). In 2013 and 2014, two additional stroke cases with good outcome for the mothers and the fetuses were reported by Tassi and Ritter, respectively (Tassi et al., 2013; Mantoan Ritter et al., 2014). The most recent case of successful rTPA treatment in a pregnant woman at 39 weeks of gestation with normal delivery was reported in 2015 (Ritchie et al., 2015). So far, only one mother treated with rTPA for acute stroke suffered a significant uterine bleeding complication (Demchuk, 2013), however, caution about bias publication should be taken into account when reviewing case reports. Intravenously administered rTPA has a high affinity for fibrin strands and a short half-life of 4–5 min via liver metabolism, with only 10% of its concentration remaining

in circulation after 20 min, which may explain its safety at therapeutic doses.

In menstruating women, Wein et al. described five subjects in the active arm of the National Institute of Neurological Disorders and Stroke (NINDS) intravenous thrombolysis trial, who were coded as actively menstruating. One subject who had a 1-year history of dysfunctional uterine bleeding required urgent uterine artery ligation. The authors also reported a case of a woman requiring transfusion after intravenous thrombolysis for acute ischaemic stroke (Wein et al., 2002).

So far, no neuroendocrine side-effects have been reported in pregnant women treated with alteplase.

EFFECTS OF EXPOSURE TO rTPA IN BRAIN DEVELOPMENT

As stated above, intravenous rTPA is too large a molecule (7200 KDa) to be able to pass the placental blood barrier. From clinical reports of IVT-treated pregnant women with stroke, there have been no signs of brain development issues on the surviving fetuses. The European Medicines Agency license for alteplase includes information on embryotoxicity (in the form of embryolethality and growth retardation) in pregnant rabbits given 3 mg/kg alteplase, which is over 3 times the therapeutical dose (0.9 mg/kg). However, no teratogenic effects were observed in animals treated with i.v. therapeutical doses and no effects on peri- or post-natal development or fertility were observed in rats treated with doses up to 10 mg/kg (Kojima et al., 1988) In subchronic toxicity studies in rats and marmosets no unexpected adverse effects were observed. No indicative signs of mutagenesis were found (preclinical safety data included in the European license documentation) (EMEA, 2002).

ONGOING STUDIES

Uncertainty whether fertile women with potential or known pregnancy should be treated may delay or halt thrombolysis and worsen stroke outcome. Maternal hemorrhagic complications have been reported in 8% with systemic thrombolysis across the spectrum of clinical thromboembolic indications (Cronin et al., 2008). More specifically, mortality in 172 pregnant women treated with a potent thrombolytic agent, streptokinase, was reported at 1.2% (Turrentine et al., 1995), which is far lower than the 9.5% mortality owed to stroke alone in pregnant women (Ritchie et al., 2015). Thus, considering this limited risk, pregnancy should not be considered an absolute contraindication. The risk during pregnancy must be balanced against the potential of a disabled outcome without treatment (Demchuk, 2013). To explore the safety of thrombolysis in pregnant women with acute stroke, and indeed within the whole group of fertile women, the Safe Implementation of Treatments in Stroke International Stroke Thrombolysis Register (SITS-ISTR), a prospective, international, observational registry for medical centers documenting stroke treatments (Wahlgren et al., 2007) has been expanded to include specific questions for women in the age group 13–50. The aim is to systematically collect data, to contribute to knowledge about treatment safety for these women, and to explore whether treatment in pregnant women, or indeed all women in fertile age is safe and not delayed. We estimate that a number of women will be treated despite pregnancy, partly because the condition was not considered when treatment was initiated, or because the potential benefit was judged higher than the risk. The overall aim of the study, Safe Implementation of Treatments in Stroke-Fertile Women Stroke Thrombolysis Study (SITS-FW), is to determine if pregnancy and even menstruation constitutes any safety issue when treated with thrombolysis, or if these patients can be given the same opportunity for treatment as other patients.

CONCLUSIONS

Pregnancy is still considered a relative contraindication for intravenous thrombolysis with rTPA for acute ischemic stroke within 4.5 h of symptom onset. However, the present and previous reviews indicate a similar maternal safety profile compared with non-pregnant women. This should be further analyzed in future prospective studies. It is reasonable to weigh in the benefit of rTPA vs. the risk for the fetus in this patient group and offer treatment for moderate to severe disabling stroke, particularly if there is no access to endovascular treatment. With the current ongoing implementation of mechanical thrombectomy for acute ischemic stroke in routine practice (Wahlgren et al., 2016), we expect more pregnant women to benefit from acute reperfusion strategies that may or not include intravenous thrombolysis in addition to mechanical thrombectomy for large vessel occlusions.

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AS, Planned litterature review, wrote first draft with references. TM, Planned litterature review, wrote abstract, edited first draft, and wrote final version.

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Avian Test Battery for the Evaluation of Developmental Abnormalities of Neuro- and Reproductive Systems

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Most of the currently used toxicity assays for environmental chemicals use acute or chronic systemic or reproductive toxicity endpoints rather than neurobehavioral endpoints. In addition, the current standard approaches to assess reproductive toxicity are time-consuming. Therefore, with increasing numbers of chemicals being developed with potentially harmful neurobehavioral effects in higher vertebrates, including humans, more efficient means of assessing neuro- and reproductive toxicity are required. Here we discuss the use of a Galliformes-based avian test battery in which developmental toxicity is assessed by means of a combination of chemical exposure during early embryonic development using an embryo culture system followed by analyses after hatching of sociosexual behaviors such as aggression and mating and of visual memory via filial imprinting. This Galliformes-based avian test battery shows promise as a sophisticated means not only of assessing chemical toxicity in avian species but also of assessing the risks posed to higher vertebrates, including humans, which are markedly sensitive to nervous or neuroendocrine system dysfunction.

Keywords: embryo culture system, sociosexual behavior, imprinting behavior, developmental neurotoxicity, Galliformes

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INTRODUCTION

Avian experimental models are an important tool for elucidating fundamental principles in research fields such as embryology, endocrinology, genetics, neurology, and ethology (Le Douarin, 2004; Stern, 2005; Emery, 2006; Nakamori et al., 2013). Since the embryos of common Galliformes such as the chicken *(Gallus gallus domesticus*) and Japanese quail (*Coturnix japonica*) can easily be observed and directly manipulated during embryogenesis, they have become the model organisms most widely used in developmental biology (Le Douarin and Dieterlen-Lièvre, 2013; Nakamura and Funahashi, 2013; Sato, 2013; Suzuki, 2013). Recently, Japanese quail has also been used as a model organism in neurophysiological and neuroendocrine studies of sociosexual behavior (Ubuka et al., 2013; Ubuka and Tsutsui, 2014).

Recently, it has been suggested that similarities exist between mammals and birds in the sex differentiation of core sexual behaviors that is induced by gonadal hormones during embryonic

development (Maekawa et al., 2014). Furthermore, Clayton and Emery (2015) have proposed that avian experimental models for human cognition could be adapted for studying the neural basis of complex cognition; reasoning, e.g., mean flexibility, problem solving, prospection, and declarative knowledge, and understanding the evolution and neurobiology of cognition, e.g. specific cognitive functions and critical roles of the avian and mammalian brain. Thus, avian experimental models represent a potentially powerful platform for elucidating the developmental mechanisms of the nervous and reproductive systems in higher vertebrates, including humans.

The negative effects of endocrine-disrupting chemicals, such as pesticides and herbicides, on neurodevelopmental processes and reproductive functions in wildlife and humans have been known for over a quarter of a century (Colborn et al., 1993; Lewis and Ford, 2012). Recently, house dust and flame retardants have also been identified as endocrine-disrupting chemicals (Suzuki et al., 2013). It has been proposed that endocrine-disrupting chemicals exert greater toxic effects during periods when organisms are more sensitive to hormonal disruption, such as during the intrauterine, perinatal, and juvenile periods and during puberty (Frye et al., 2012).

The existing developmental neurotoxicity tests have been carried out for some chemicals such as pesticides and their effects have been studied. However, there are problems to correspond to the large number of new chemicals because of high costs, long test period and high numbers of pregnant laboratory mammals used. Moreover, for inspection of embryonic development, the mothers have to be sacrificed which are of ethical concern (Lu et al., 2014). Therefore, simpler and alternative methods are desired. Avian culture techniques using embryonic tissues may offer certain advantages over *in vivo* experiments especially when developmental toxicity is anticipated (Neubert, 1982). The avian models are advantageous at three points in comparison to the conventional mammalian model for the assessment of the developmental toxicity: (1) direct manipulation, (2) continuous observation, and (3) reduction of unnecessary sacrifices of the pregnant individuals. In conventional developmental toxicity studies, the toxic effects of test compounds are examined in fetuses by using pregnant model organisms, such as rats and mice. However, avian embryos may be a better platform than mammalian embryos because the former can be observed and manipulated directly. Moreover, innovative avian embryo culture systems (ECSs) now allow continuous quantitative observations after administration of the test compound (Perry, 1988; Kawashima et al., 2005). Avian models also have ethical advantages because mammalian toxicity tests usually require sacrifice of the pregnant animals prior to examination of embryonic development, whereas avian models do not.

To allow more detailed evaluation of the neuro- and reproductive toxicities of environmental chemicals, new neurobehavioral endpoints in avian test models, such as sex differentiation in the gonads and brain, need to be established. Here we discuss the usefulness of an avian test battery with Galliformes for the assessment of developmental toxicity by using a combination of chemical exposure during early embryonic development by using an ECS followed by analyses after hatching of sociosexual behaviors such as aggression and mating and of visual memory via filial imprinting. Although, avian model systems are already commonly used in basic research (Le Douarin and Dieterlen-Lièvre, 2013; Nakamura and Funahashi, 2013), this article presents one of most alternative solutions as the evaluation of developmental abnormalities of neuro- and reproductive systems.

AVIAN BIORESOURCES

Genetically homogeneous inbred strains of rats or mice are often used in toxicity studies to ensure reproducibility of the experimental results. However, no fully genetically inbred avian strains are currently available. It has now been more than half a century since the Japanese quail was evaluated and recommended as a laboratory animal by Padgett and Ivey (1959), and since then the Japanese quail has become of high value to researchers, especially those in the fields of embryology and physiology, because of its hardiness, ease of handling, precocity, and high egg productivity. In 1980, our group at the National Institute for Environmental Studies (NIES), Japan, began developing a closed colony of Japanese quail with the goal of establishing a new experimental model organism. This Japanese quail is maintained by means of rotational crossbreeding (**Figure 1A**) and is fixed with a yellow-brown plumage color mutation (**Figures 1B,C**). This strain is named NIES-L as mentioned below.

Recently, a draft genome sequence of the NIES-L quail was produced by means of next-generation sequencing, and 100 microsatellite markers have been developed as useful tools

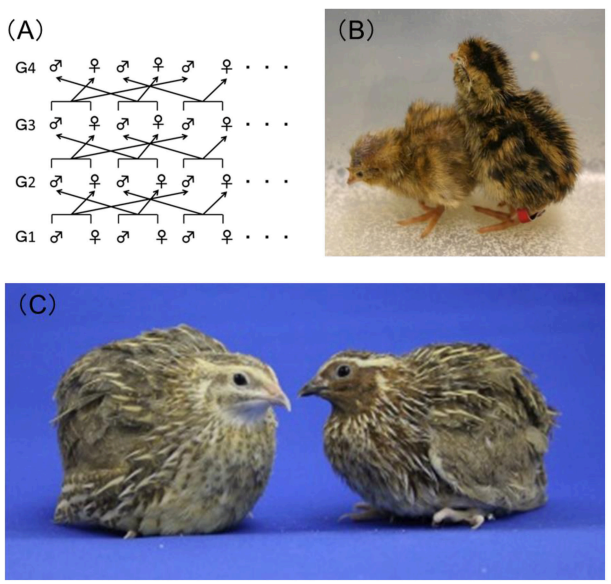

FIGURE 1 | Closed colony of Japanese quail at the National Institute for Environmental Studies, Japan. (A) Overview of the rotational crossbreeding of the NIES-L quail strain from generations 1 (G1)–4 (G4). **(B)** Newly hatched NIES-L quail chicks with the yellow-brown plumage color phenotype (left) and the wild-type phenotype (right). **(C)** Male (right) and female (left) adult NIES-L quails.

for evaluating the genetic variation within and between quail populations (Kawahara-Miki et al., 2013). Tadano et al. (2014) compared the estimated level of inbreeding within the NIES-L colony with that of a commercial random-bred colony by using polymorphic microsatellite marker analyses and confirmed that the heterozygosity in the NIES-L colony is gradually being lost over time. Our group is also using specific alleles of the microsatellite loci to monitor for genetic contamination in the colony by genetic markers developed. The Japanese quail is a potentially useful experimental organism not only for studies in the field of poultry science but also in the field of basic research and/or environmental studies, which are more necessary to analyze genetically.

About 35 years ago, we started to select and breed Japanese quails for high (H) and low (i.e., NIES-L) serum antibody titers against inactivated Newcastle disease virus (Takahashi et al., 1984), but the H quails have been extinct. It is clear that heterozygosity in the NIES-L closed colony is being gradually lost over time. However, it is difficult to establish mutant quail strains and maintain inbred strains because they are particularly susceptible to inbreeding depression (Sittmann et al., 1966). Indeed, no inbred strain of Japanese quail surviving more than 20 consecutive generations of full-sib mating has ever been developed, unlike in mouse and rat. Therefore, if we could fully establish an inbred strain of Japanese quail by continuing our breeding of NIES-L, this model organism would be useful for toxicity testing and would provide a simple and reproducible platform for the assessment of nervous and reproductive system developmental toxicity in birds and mammals.

AVIAN EMBRYO CULTURE SYSTEM

The first avian whole ECS was established in chicken by Perry (1988) and developed further by Naito et al. (1990). An ECS has also been developed for Japanese quail (Ono et al., 1994). Perry's ECS (1988) covers the period from fertilization of the ovum to hatching and comprises three culture systems (Perry, 1988). System I is used to culture embryos obtained from the oviduct in the early cleavage stages, that is, to culture embryos from fertilization to blastoderm formation (Phase I). Systems II (**Figure 2A**) and III (**Figure 2B**) use a surrogate eggshell to culture embryos obtained from newly laid eggs through the period of embryogenesis (Phase II) and from embryonic growth to hatching (Phase III).

Our group has also succeeded in cultivating newly laid chicken or quail eggs through the period of embryogenesis (Phase II) by using a clear, egg-shaped artificial culture vessel (**Figure 2C**), which allowed direct observation of the developmental stage at any time throughout the cultivation period simply by tipping the vessel (Kawashima et al., 2005). In a comparison of our ECS, which uses an artificial culture vessel, and Perry's System II culture system, which uses a surrogate eggshell culture vessel, no obvious detrimental effects were found in Phase II embryos cultured by using our shell-less culture method (Kawashima et al., 2005).

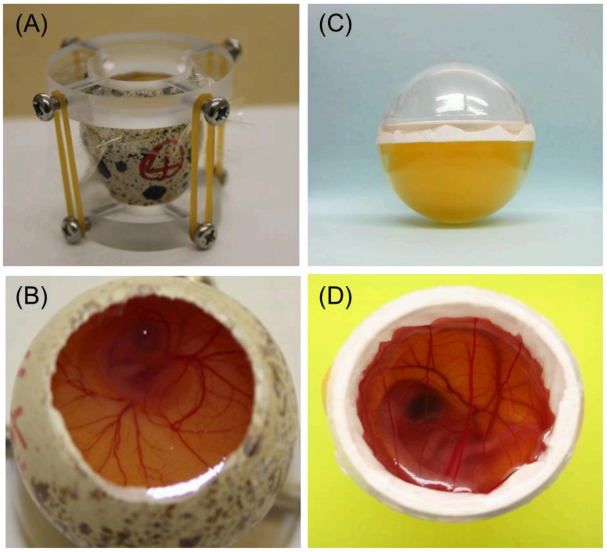

FIGURE 2 | Photographs of two quail embryo culture systems (ECSs). **(A)** Perry's ECS (System II) uses a surrogate eggshell for the period from blastoderm to embryogenesis. **(B)** Perry's ECS (System III) uses a surrogate eggshell during embryonic growth until hatching. **(C)** Alternative ECS using an artificial culture vessel for the period from blastoderm to embryogenesis. Developing quail embryos can be observed by tipping the vessel slightly, allowing easy determination of the developmental stage. The volumes of the lower halves of the plastic cases (diameter across the equatorial plane, 26 mm) were comparable with those of quail eggs. **(D)** Alternative ECS using an artificial culture vessel during embryonic growth until hatching.

Attempts to culture avian embryos *in vitro* from embryonic growth to hatching (i.e., Phase III) have also been made. Auerbach et al. (1974) cultured 3- to 4-day-old chicken embryos in Petri dishes and obtained approximately 40% viability at 14 days of total incubation with a maximum development period of 18 days. Dunn and Boone (1976) cultured 3-day-old chicken embryos in egg-shaped plastic wrap and reported that the maximum developmental stage reached was almost before hatching. Ono and Wakasugi (1983) cultured quail embryos, preincubated for 2.5 days, by using a plastic wrap technique, but the embryos did not survive beyond 14 days. Finally, Kamihira et al. (1998) succeeded in culturing 2-day-old quail embryos to hatching by using a gas-permeable Teflon membrane (Milliwrap; Nihon Millipore Co., Tokyo, Japan) with calcium supplementation and oxygen aeration (**Figure 2D**). Therefore, it is now possible to cultivate avian embryos in an artificial vessel throughout the whole embryonic growth phase.

Avian-based toxicity screening tests that are simple to conduct, rapid, and cost-effective are urgently required to obtain preliminary information on the effects of exposure to endocrine-disrupting chemicals in vertebrates (Flint et al., 2012; Oshima et al., 2012). An avian *in ovo* model called the sex reversal test has been established that uses Japanese quail embryos (Shibuya et al., 2004). Histologically, intact male Japanese quail embryos in the later embryonic stages just before hatching develop both left and right testes, whereas intact female embryos develop

only a left-side ovary. Exposure of the embryo to estrogen or to an estrogen-like chemical such as an endocrine-disrupting chemical during an early embryonic developmental stage causes transformation of the left testis into an ovotestis and persistence of the left oviduct in genetic males (Shibuya et al., 2005). Therefore, the sex reversal test using an avian ECS represents a high-throughput, quantitative approach for the evaluation of the estrogenic effects of endocrine-disrupting or other chemicals.

Avian ECSs offer ethical advantages over other *in vivo* means of toxicity screening, especially when developmental toxicity of a chemical is anticipated. Furthermore, avian ECSs offer the additional advantage of allowing direct quantitative determination of the effects of test chemicals on the fetus without any maternal influence through placental transmission. Furthermore, because avian ECSs facilitate direct observation and manipulation, it is now possible to investigate the time-specific influences of chemicals with embryonic toxicity on the embryo. Suitable ECSs should be developed depending on the research or testing goal.

FEATURES OF AVIAN NEUROBEHAVIOR

Avian experimental models may also be useful for assessing aspects of neurobehavior such as the sociosexual behaviors of aggression and mating using Japanese quail (Ubuka et al., 2013). Indeed, some avian species have already been used as models of learning and memory in studies examining the neural basis of cognition (Emery, 2006). Corvids, songbirds, and domestic fowl are currently considered the best models available to examine specific aspects of the neurobiology underlying learning and cognition (Clayton and Emery, 2015). To apply the usefulness of avian models for learning and cognition to the developmental toxicity research, we need to establish new endpoints appropriate for the evaluation of neurotoxic and neurobehavioral effects.

Japanese quails have been used as a model organism in studies of the neurophysiological and neuroendocrine bases of aggression and reproductive behavior for a long period (Selinger and Bermant, 1967; Mills et al., 1997; Ubuka et al., 2013; Ubuka and Tsutsui, 2014). Sexually mature male quail frequently fight with intense aggression and display a series of stereotypical actions. They often approach, chase, and peck their opponent (peck), grab the back of their opponent's head or neck with their beak (grab), attempt to ride on the back of their opponent (mount), or ride on the back of their opponent and lower their cloaca close to their opponent's cloaca (cloacal contact-like action). Since the behavior of each quail can be recorded and analyzed by using a digital video camera, the numbers of pecks, grabs, mounts, and cloacal contact-like actions attempted by each animal can be quantified (see in **Supplementary Movie 1**). Since the frequency of these actions represents the degree of the sociosexual behavior of individual male quails (Ubuka et al., 2013), these actions represent novel endpoints that could be used to assess developmental toxicity.

Imprinting, which occurs limitedly during the initial stage of avian chicks, may be useful to establish a behavioral index for investigating the neural plasticity involved in juvenile learning (Yamaguchi et al., 2012; Nakamori et al., 2013). Imprinting is characterized by a high learning efficiency and robust memory retention, which are features that distinguish it from general learning and memory. Maekawa et al. (2006) established an experimental procedure for imprinting to visual stimuli presented on a liquid crystal display. After training, the chicks are placed on a running wheel, and an angle sensor is used to record the number of forward and backward rotations of the wheel as the chick is shown either images they had been shown since hatching or new, previously unseen images. The effects of imprinting on neural activity in the visual wulst can also be investigated by using *in vivo* intrinsic optical imaging techniques (Maekawa et al., 2007). It may also be possible to apply a measurement system of juvenile learning and memory that utilizes avian imprinting behavior as a novel means of rapidly assessing developmental neurotoxicity in early childhood.

Imprinting in Japanese quail has also been reported. It has been found that Japanese quail will imprint on achromatic stimuli, flashing lights, and models of quail hens (Mills et al., 1997). Imprinting in chicks may be a good model of learning in human infants because human infants and chicks share a critical period during which they are sensitive to specific experiences. Therefore, uncovering the molecular mechanisms underlying the imprinting process will help clarify the juvenile learning, which has an obvious critical period (Suzuki et al., 2012). Since future studies are expected to reveal the molecular mechanisms underlying learning and memory in higher vertebrates, new developmental neurotoxicity endpoints are also likely to be established in the future.

CAN AVIAN TEST BATTERY BE ALTERNATIVE TO DEVELOPMENTAL NEUROTOXICITY TESTS FOR HUMAN?

The combination of an avian ECS followed by neurobehavioral assessment is also a promising means of assessing developmental neurotoxicity. For many chemicals, it is high prenatal exposure that induces postnatal neurobehavioral disorders (Grandjean and Landrigan, 2006; Huizink and Mulder, 2006). However, even if exposure to a certain chemical is not found to induce apparently morphological abnormalities in the fetal and infantile brain, the chemical may still cause functional disorders and affect learning and memory. By using brain functions such as behavior, memory, and learning as advanced endpoints, it may be possible to use avian-based assay systems in place of the currently used tests of developmental neurotoxicity. However, given that the morphological development of the brain may differ between humans and birds, further studies are needed to determine how best to extrapolate test results.

Most of the currently used toxicity studies for environmental chemicals use acute or chronic systemic or reproductive toxicity endpoints and use mammalian or avian laboratory animals. However, since exposure in mammalian models is usually based on how much chemical is administered to the mother, it is difficult to determine exactly how much the fetus

receives through the placenta. The Organization for Economic Cooperation and Development has established a series of standardized ecological risk assessments for chemicals such as endocrine disruptors (Buschmann, 2013). Although, these tests are accepted internationally as standard methods for evaluating the risk to human health and wildlife posed by persistent and degradable chemicals in the environment, neurobehavioral endpoints of toxicity are rarely examined. In addition, given the increasing number of new chemicals that potentially have neurotoxic effects in vertebrates, including humans, a rapid means of assessing the neurotoxic effects of these chemicals in humans is required.

Recently, it has been suggested that neonicotinoid pesticides have caused a decline in wild bird populations (Hallmann et al., 2014; Gibbons et al., 2015). Since the use of neonicotinoid pesticides is rapidly increasing throughout the world, the effects of these pesticides on human health are now a concern. Even if neonicotinoid pesticides were found not to adversely affect the morphological development of the embryonic human brain, the question of whether or not they cause postnatal neurobehavioral disorders would remain. Therefore, there is an urgent need to establish animal-based evaluation systems that can be used to assess neurotoxic effects of neonicotinoid pesticides. The avian test battery represents a potentially rapid and cost-effective way of conducting these assays.

CONCLUSION

Avian test batteries represent a potentially rapid, cost-effective, ethical alternative to the currently available means of assessing developmental toxicity using higher vertebrates. Here we have discussed using a Galliformes-based avian test battery in which developmental toxicity is assessed by means of a combination of chemical exposure during early embryonic development by using an ECS followed by analyses in later life of sociosexual behaviors such as aggression and mating and of visual memory via filial imprinting. However, to fully harness the potential of this novel means of chemical toxicity screening, it will be necessary to establish a variety of evaluation systems and experimental models before we can extrapolate the test results to humans.

AUTHOR CONTRIBUTIONS

All authors contributed to substantial contributions to the conception or design of the work. TK, WA, and KN designed this work. TK, TU, and KT revised the important intellectual content critically. TK wrote the article and all authors contributed to the editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2016.00296

Supplementary Movie 1 | Series of stereotyped aggressive and reproductive behaviors in masculine Japanese quail, namely, pecks, grabs, mounts, and cloacal contact-like actions.

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Resveratrol Ameliorates the Anxiety- and Depression-Like Behavior of Subclinical Hypothyroidism Rat: Possible Involvement of the HPT Axis, HPA Axis, and Wnt/β-Catenin Pathway

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Metabolic disease subclinical hypothyroidism (SCH) is closely associated with depression-like behavior both in human and animal studies, and our previous studies have identified the antidepressant effect of resveratrol (RES) in stressed rat model. The aim of this study was to investigate whether RES would manifest an antidepressant effect in SCH rat model and explore the possible mechanism. A SCH rat model was induced by hemi-thyroid electrocauterization, after which the model rats in the RES and LT4 groups received a daily intragastric injection of RES at the dose of 15 mg/kg or LT4 at the dose of 60 μg/kg for 16 days. The rats' plasma concentrations of thyroid hormones were measured. Behavioral performance and hypothalamic–pituitary–adrenal (HPA) activity were evaluated. The protein expression levels of the Wnt/β-catenin in the hippocampus were detected by western blot. The results showed that RES treatment downregulated the elevated plasma thyroid-stimulating hormone concentration and the hypothalamic mRNA expression of thyrotropin-releasing hormone in the SCH rats. RES-treated rats showed increased rearing frequency and distance in the open-field test, increased sucrose preference in the sucrose preference test, and decreased immobility in the forced swimming test compared with SCH rats. The ratio of the adrenal gland weight to body weight, the plasma corticosterone levels, and the hypothalamic corticotrophin-releasing hormone mRNA expression were reduced in the RES-treated rats. Moreover, RES treatment upregulated the relative ratio of phosphorylated-GSK3β (p-GSK3β)/GSK3β and protein levels of p-GSK3β, cyclin D1, and c-myc, while downregulating the relative ratio of phosphorylated-β-catenin (p-β-catenin)/β-catenin and expression of GSK3β in the hippocampus. These findings suggest that RES exerts anxiolytic- and antidepressant-like effect in SCH rats by downregulating hyperactivity of the HPA axis and regulating both the HPT axis and the Wnt/β-catenin pathway.

Keywords: depression, hypothalamus–pituitary–adrenal axis, hypothalamic–pituitary–thyroid axis, resveratrol, subclinical hypothyroidism, Wnt/β-catenin pathway

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INTRODUCTION

Imbalances in thyroid hormone homeostasis are associated with both functional and structural brain alterations, resulting in neurobehavioral alterations, including depression (1, 2). Subclinical hypothyroidism (SCH) is defined as an elevated plasma thyroid-stimulating hormone (TSH) level associated with normal total or free thyroxine (fT4) and triiodothyronine (T3) levels. Although the hypometabolism symptoms, including fatigue, weakness, and cold intolerance, are dormant and non-specific in SCH patients, increasing evidence suggests that SCH is associated with neuropsychiatric disorders such as cognitive dysfunction (2) and depression (3, 4). Depression is observed more frequently among individuals with SCH than those with overt hypothyroidism (3), and SCH patients exhibit a twofold higher prevalence of depressive-like symptoms than healthy individuals (5). In our previous study, SCH induced depression-like behavior in rats accompanied by subtle hyperactivity of the hypothalamus–pituitary–adrenal (HPA) axis (6). Clinical studies have demonstrated that treatment with levothyroxine (LT4) improves mood and normalizes the elevated relative cerebral glucose metabolism in several brain areas of depression patients (7, 8). However, the correct dosage of LT4 remains elusive. Moreover, the unpredictable clinical effects of the currently available antidepressants, including poor efficacy and adverse reactions, make the development of new drugs to alleviate depression in SCH patients an urgent clinical need.

Resveratrol (*trans*-3,5,4′-trihydroxy-*trans*-stilbene, RES), a polyphenol component found mainly in grape and *Polygonum cuspidatum*, possesses multiple biological and pharmacological activities, including metabolism regulation (9) and antioxidant effects (10). Recently, our results (11), together with findings from other studies (12–15), have demonstrated that RES alleviates depression-like behavior in a rat model of chronic unpredicted mild stress (CUMS) through its antioxidant effects and by ameliorating hyperactivity of the HPA axis. In addition, the monoaminergic system and the molecular markers related to depression were also altered by RES treatment (16). However, it remains unknown whether RES can alleviate the depression-like symptoms in SCH, which is complicated with the balance of both the hypothalamic–pituitary–thyroid (HPT) axis and the HPA axis. Clinic studies showed that RES was well tolerated with the dose ranging from 200 mg (17) to 1000 mg (18) daily. According to the formula for dose translation based on body surface area (BSA), the corresponding dose in rats should range from 18 to 90 mg/kg. With regard to animal research, it has been reported that RES (20, 40, and 80 mg/kg) could significantly decreased the immobility time of mice in the despair tests (19). Consistently, results of our previous study also demonstrated that RES (15 mg/kg/day × 7 day) could significantly alleviate the depression-like behavior of CUMS rat (11). Thus, the dose of 15 mg/kg/day was chosen in this study.

Multiple approaches have been adopted to evaluate the antidepressant potential of compounds in animal models, including behavioral tests and biochemical/neurochemical assays. In rodents, spontaneous motor activity and anxiety are often analyzed in terms of exploratory behavior, especially during exposure to an open field (20). And the clinical symptoms/signs of depression such as anhedonia (incapability to perform rewarded behaviors) and helplessness are usually measured using the sucrose preference test (SPT) and the forced swimming test (FST), respectively (13). In this study, these behavior tasks were used, and the activity of the HPA axis was also detected.

Several studies in recent years have implicated the canonical Wnt signaling pathway in mood disorders such as bipolar disorder (21) and major depression (22). Activation of the canonical Wnt pathway leads to the inhibition of GSK-3β, allowing β-catenin to be stabilized in the cytosol and translocated to the nucleus, where it activates the transcription of target genes (23). Mutant mice with a heterozygous GSK-3β deletion showed decreased evidence of depression in the FST (23), and infusion of L803-mts, a selective GSK-3 inhibitor, also decreased immobility in the same test (24). Additionally, upregulated β-catenin has been used as a marker for antidepressive-like effects (24). Furthermore, thyroid hormone exerts a negative effect on the canonical Wnt signaling pathway, as demonstrated in a previous study (25). Thyroid hormone stimulates cell proliferation, represses the expression of key members of the Wnt signaling pathway, and suppresses β-catenin levels (25). Therefore, the Wnt/β-catenin pathway may participate in SCH-associated depression.

In this study, in order to explore the potential antidepressant-like effect of RES in the SCH rats and the possible mechanisms, behavior performance were evaluated using a series of behavioral tasks [open-field test (OFT), SPT, and FST]. Moreover, the activities of the HPA axis, the HPT axis, and the canonical Wnt pathway were assessed biochemically.

EXPERIMENTAL PROCEDURES

Drugs

Resveratrol was purchased from Sigma Chemical Co. (St. Louis, MO, USA). LT4 was purchased from Berlin-Chemie AG (Berlin, Germany). Both drugs were dissolved in an aqueous solution of 0.5% sodium carboxymethyl cellulose to be a mixed suspension. Control and untreated model rats received a daily intragastric injection of 0.5% sodium carboxymethyl cellulose.

Animals

Male, 2-month-old Sprague-Dawley rats were purchased from the Anhui Experimental Animal Center of China. They were housed three to four per cage (43 cm length × 31 cm width × 19 cm height) with access to food and water *ad libitum* and were maintained under a 12:12-h light/dark cycle. The light onset is at 8 o'clock. The ambient temperature was maintained at 21–22°C with 50–60% relative humidity. The rats were handled for 5 min daily over 7 days before drug administration. All experimental procedures in this study were approved by the Animal Care and Use Committee at the University of Science and Technology of China, which complies with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985).

Animal Model of SCH

The SCH rat model was established *via* hemi-electrocauterization, according to the procedures in our previous study (6). In brief, 33 rats underwent hemi-thyroid electrocauterization to establish the SCH model, and 8 sham rats underwent the same operation, but the thyroid tissues were exposed without electrocauterization. The SCH model was evaluated 2 weeks later, and the success rate of SCH modeling was 75.8% (25/33), according to the criterion that the plasma TSH concentration was higher than the 97.5 percentile of the sham group accompanied by a plasma fT4 level between the 2.5 and 97.5 percentile of the sham group. Consequently, 24 successful SCH rats were randomly divided into 3 groups with 8 rats in each group: an untreated model group, a RES treatment group (15 mg/kg/day + model) and an LT4 treatment group (60 μg/kg/day + model). The rats in the RES and LT4 groups received a daily intragastric injection of RES and LT4 at the corresponding dose for 16 days, respectively, and the rats in the sham and untreated model groups simultaneously received the same injection with 0.5% sodium carboxymethyl cellulose. To prevent hypocalcemia resulting from destruction of the parathyroid glands by electrocauterization, the rats were provided with 0.1% (w/v) calcium lactate in their drinking water after surgery.

Behavioral Tests

Behavioral tests were performed in a soundproof room with a neutral environment in the order listed in **Figure 1**. Briefly, SPT was carried out on day (D) 34, OFT on D 35, and FST on D 36 and D 37. All of the tests were carried out between 0900 and 1430 hours, with matching between the groups. The observers were blind to the treatment. The behavioral performance was monitored and recorded by a digital camera above the apparatus interfaced to a computer running the ANY-maze video imaging software (Stoelting Co., Wood Dale, IL, USA).

Sucrose Preference Test

After a 12-h period of food and water deprivation, the animals were individually housed in a cage (28 cm length × 17 cm width × 14.5 cm height) during the test and given two bottles (capacity: 250 ml) containing either water or a 2% sucrose solution. Six hours later, the volumes of water and sucrose consumed were measured. The percentage of the total liquid ingested was sucrose solution that was used as a measure of the sensitivity to reward.

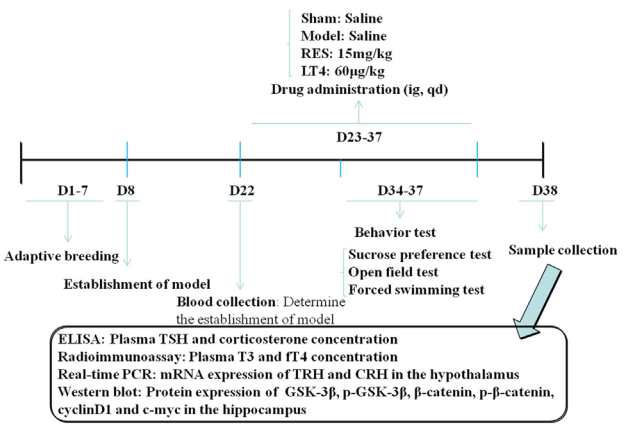

FIGURE 1 | Schedule of the experimental design. RES, resveratrol; LT4, levothyroxine; T3, triiodothyronine; fT4, free thyroxine; CRH, corticotrophin-releasing hormone; TRH, thyrotropin-releasing hormone.

Open-Field Test

The open-field apparatus consisted of a black square arena (100 cm × 100 cm), with a 30-cm-high wall. The floor was marked with a grid dividing it into 16 equally sized squares. During a 5-min observation period, the rat was placed at one corner of the apparatus facing the wall. After the 5-min test, rats were returned to their home cages, and the open field was cleaned with 75% ethyl alcohol and permitted to dry between tests. The total distance; average velocity; the distance, frequency, and duration in the center; and the frequencies of rearing, grooming, and defecation were recorded.

Forced Swimming Test

The behavioral cylinder was 60 cm high and 25 cm in diameter, maintained at 24–25°C, and filled with 30 cm of water, so that rats could not support themselves by touching the bottom with their paws. The FST paradigm includes two sections: an initial 15-min pretest followed by a 5-min test 24 h later. Rats were considered immobile when they did not make any active movements. Struggling was indicated when the rats made active movements with their forepaws in and out of the water along the side of the swim chamber. Swimming was indicated when the rats made active swimming or circular movements.

Measurement of Plasma Concentrations of Thyroid Hormones and Corticosterone

Two weeks after the operation, blood samples (approximately 1 ml) were collected from the angular vein to test whether the SCH rat model was successfully established. Twenty-four hours after the last behavioral test, the rats were deeply anesthetized with chloral hydrate, and blood was taken from the abdominal aorta. Plasma concentrations of TSH and corticosterone were measured using ELISA kits (TSH: Cusabio Biotech. Co., Ltd., Wuhan, Hubei, China; corticosterone: Enzo Life Sciences, Inc., USA), according to the manufacturer's instructions, and fT4 and T3 were measured with radioimmunoassay kits (North Institute of Biological Technology, Beijing, China), with the apparatus used in the assay that came from University of Science and Technology of China Zonkia (AnHui Ustc ZonKia Scientific Instruments Co., Ltd., Anhui China).

The Ratio of the Adrenal Gland Weight to Body Weight

The adrenal glands of each side were removed and weighed immediately postmortem. The ratio of the average of both glands weight to body weight was measured.

RNA Isolation and Real Time PCR

After blood collection, eight rats in each group were sacrificed by decapitation to collect the hypothalamus. The hypothalamus

was rapidly dissected and frozen quickly in liquid nitrogen before storage at −80°C. Total RNA was extracted using the TRIzol (Invitrogen, Carlsbad, CA, USA) method. cDNA was synthesized using reverse transcriptase (Promega, WI, USA). Q-PCR was performed using the SYBR Green PCR Kit (Applied Biosystems, USA) and an ABI Prism 7000 Sequence Detector system in a 25-μl total reaction volume for 40 cycles (15 s at 95°C and 60 s at 62°C). The primers used in our study were as follows: rat β-actin 5′-TTGCTGACAGGATGCAGAA-3′ and 5′-ACCAATCCACACAGAGTACTT-3′; thyrotropin-releasing hormone (TRH) 5′-AGCTCAGCATCTTGGAAAGC-3′ and 5′-CCAGCAGCAACCAAGTC-3′; and corticotrophin-releasing hormone (CRH) 5′-CAGAACAACAGTGCGGGCTCA-3′ and 5′-AAGGCAGACAGGGCGACAGAG-3′. The relative amount of each target gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blot Assays

The hippocampus was homogenized in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl at pH 7.4, 0.1% SDS, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM Na_3VO_4). Before homogenization, a protease inhibitor cocktail (Roche, IN, USA) and the phosphatase inhibitor PhosSTOP (Roche, IN, USA) were added. Protein quantitation was conducted using a Lowry Protein Assay Kit (Meiji Biotech Co., Ltd., Shanghai, China). The same quantity (approximately 50 μg) of protein from each animal was loaded and separated by 15% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Amersham Biosciences, UK). The membrane was blocked with 5% skim milk for 1 h, incubated with antibodies targeting GSK3β, p-GSK3β (Ser9), β-catenin, p-β-catenin (1:1000; Cell Signaling Technology, USA), cyclin D1, c-myc (1:1000; ImmunoWay, Newark, DE, USA), or β-actin (1:1000; Bioworld Technology, Inc., USA) at 4°C overnight, and then incubated with a horseradish peroxidase-conjugated secondary antibody (1:10,000) at 37°C for 1 h. The blots were developed with the Easy Enhanced Chemiluminescence Western Blot Kit (Pierce Biotechnology, Rockford, IL, USA). The protein bands were scanned and analyzed using Image J software (NIH).

Statistical Analyses

All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) version 12.0.1 (SPSS Inc., Chicago, IL, USA). The data are expressed as the means ± SEM, and $P < 0.05$ was considered statistically significant. The distribution of the data was determined by the Kolmogorov–Smirnov test. Between-group effects on body weight, TSH, fT4, and T3 were analyzed by repeated measures ANOVA with group and days as the factors. Statistical analyses of the between-group effects of RES on behavioral performance, the ratio of the adrenal gland weight to body weight, plasma corticosterone, the hypothalamic mRNA expression of CRH and TRH, and the hippocampal protein expression of GSK3β, p-GSK3β (Ser9), β-catenin, p-β-catenin, cyclin D1, and c-myc were carried out using ANOVA followed by LSD *post hoc* tests. Correlation analysis was performed using a Pearson correlation test.

RESULTS

RES Administration Decreased the Elevated Plasma TSH and the Hypothalamic TRH mRNA in SCH Rats

The repeated measures ANOVA revealed that both the treatment [$F(5,140) = 11.975$, $P < 0.001$] and time [$F(3,28) = 76.925$, $P < 0.001$] had a significant effect on the TSH levels, with no significant interaction [$F(5,140) = 0.583$, $P = 0.614$]. When it comes to the T3 levels, the results showed that neither the time [$F(5,140) = 0.555$, $P = 0.461$] nor the treatment [$F(3,28) = 0.673$, $P = 0.574$] had a significant effect, with no significant interaction [$F(5,140) = 0.452$, $P = 0.718$]. Moreover, repeated measures ANOVA revealed a significant interaction between the treatments and weeks in body weight [$F(15,140) = 23.989$, $P < 0.001$]. The individual factor treatment also had a significant effect [$F(5,140) = 26.113$, $P < 0.001$], but the factor time did not [$F(3,28) = 2.509$, $P = 0.122$].

As shown in **Table 1**, before the treatment, SCH rats in the untreated model, RES and LT4 groups showed elevated plasma TSH level [$F(3,28) = 13.263$, $P < 0.01$; LSD: sham vs. untreated model: $P = 0.002$, sham vs. RES: $P < 0.001$, sham vs. LT4: $P < 0.001$] with normal plasma fT4 [$F(3,28) = 0.737$, $P = 0.539$; LSD: sham vs. untreated model: $P = 0.378$, sham vs. RES: $P = 0.689$, sham vs. LT4: $P = 0.876$] and T3 [$F(3,28) = 1.505$, $P = 0.235$; LSD: sham vs. untreated model: $P = 0.778$, sham vs. RES: $P = 0.701$, sham vs. LT4: $P = 0.423$] concentrations compared with the sham ones. After 16 days of treatment, both RES

TABLE 1 | Concentrations of plasma total triiodothyronine (T3), free thyroxine (fT4), thyroid-stimulating hormone (TSH) in sham ($n = 8$), model ($n = 8$), RES ($n = 8$), and LT4 ($n = 8$) before and after treatment.

Group	*n*	T3 (nmol/L)		fT4 (ρmol/L)		TSH (mIU/L)	
		Before	After	Before	After	Before	After
Sham	8	0.78 ± 0.03	0.72 ± 0.04	9.45 ± 0.34	5.78 ± 0.51	0.45 ± 0.06	0.46 ± 0.05
Model	8	0.75 ± 0.04	0.74 ± 0.05	9.44 ± 0.41	6.36 ± 0.40	2.28 ± 0.34**	1.31 ± 0.03**
RES	8	0.78 ± 0.05	0.66 ± 0.16	9.36 ± 0.49	6.84 ± 0.53	2.58 ± 0.29**	0.55 ± 0.09#
LT4	8	0.79 ± 0.02	0.81 ± 0.05	8.91 ± 0.25	15.67 ± 0.85**,##	2.68 ± 0.28**	0.09 ± 0.01##

The data were presented as mean ± SEM.

***P < 0.01 compared with sham group.*

#P < 0.05 and ##P < 0.01 compared with model group.

RES, resveratrol; LT4, levothyroxine.

and LT4 decreased the elevated TSH level of untreated model rats [$F(3,28) = 11.269$, $P < 0.01$; LSD: RES vs. untreated model: $P = 0.028$, LT4 vs. untreated model: $P < 0.01$]. No significant difference was found between sham rats and RES- or LT4-treated rats (LSD: RES vs. sham: $P = 0.213$, LT4 vs. untreated model: $P = 0.711$). Consistent with this result, the hypothalamic mRNA expression of TRH was inhibited by treatment with RES or LT4 [**Figure 2**, $F(3,16) = 6.668$, $P < 0.001$; LSD: RES vs. untreated model: $P = 0.050$, LT4 vs. untreated model: $P = 0.01$, RES vs. sham: $P = 0.221$, LT4 vs. untreated model: $P = 0.611$]. No significant difference was observed between the plasma T3 concentrations of the groups [**Table 1**, $F(3,28) = 2.563$, $P = 0.458$]. LT4, but not RES, increased the plasma fT4 concentrations compared to sham or untreated model rats [**Table 1**, $F(3, 28) = 60.583$, $P < 0.01$; LSD: RES vs. sham: $P = 0.202$; LT4 vs. sham: $P < 0.001$; RES vs. untreated model: $P = 0.340$, LT4 vs. untreated model: $P < 0.001$].

RES Administration Did Not Reverse the Decreased Bodyweight in the SCH Rats

Figure 3A shows the effect of RES on the body weight and behaviors of the SCH rats. Repeated measures ANOVA revealed a significant interaction between the treatments and weeks in body weight [$F(15,140) = 3.707$, $P < 0.01$]. The individual factor weeks also had a significant effect [$F(5,140) = 165.550$, $P < 0.01$], but the factor treatments did not [$F(3,28) = 1.633$, $P = 0.198$]. A significant difference in body weight was noticed in week 5 in the sham rats compared to the untreated model rats [$F(3,28) = 3.179$, $P = 0.035$; LSD: sham vs. untreated model: $P = 0.027$, RES vs. untreated model: $P = 0.785$, LT4 vs. untreated model: $P = 0.745$, RES vs. sham: $P = 0.015$, LT4 vs. untreated model: $P = 0.052$]. However, no change in body weight was observed during the experimental period in the untreated model rats compared to the RES- or LT4-treated rats, indicating that neither drug had an effect on the body weights of untreated model rats.

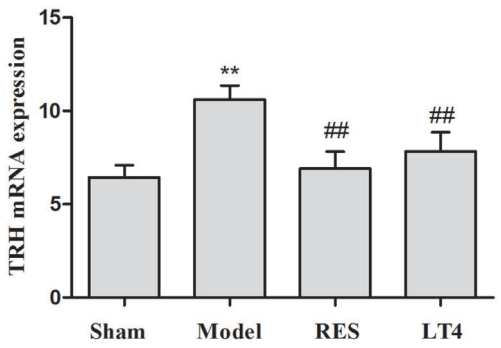

FIGURE 2 | Effect of RES on hypothalamic TRH mRNA expression in the SCH rats. The mRNA expression of TRH in the hypothalamus is illustrated. The data are presented as the means ± SEM, with $n = 8$ for each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the sham group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to the untreated model group. RES, resveratrol; TRH, thyrotropin-releasing hormone; SCH, subclinical hypothyroidism.

RES Administration Alleviated the Anxiety- and Depressive-Like Behavior in the SCH Rats

The sucrose preference of the untreated model rats was remarkably lower than that of the sham group. Both the RES and LT4 groups showed an elevated sucrose preference index compared to the untreated model rats [**Figure 3B**, $F(3,28) = 6.387$, $P = 0.001$; LSD: RES vs. untreated model: $P = 0.002$, LT4 vs. untreated model: $P = 0.035$, RES vs. sham: $P = 0.849$, LT4 vs. untreated model: $P = 0.224$], indicating an anti-anhedonia effect of RES and LT4.

In the OFT (**Figures 3C–G**), the untreated model group traveled over a shorter total distance [$F(3,28) = 9.983$, $P < 0.01$; LSD: RES vs. untreated model: $P = 0.002$, LT4 vs. untreated model: $P = 0.018$, RES vs. sham: $P = 0.022$, LT4 vs. untreated model: $P = 0.004$] and at a lower velocity [$F(3,28) = 9.987$, $P < 0.01$; LSD: RES vs. untreated model: $P = 0.002$, LT4 vs. untreated model: $P = 0.017$, RES vs. sham: $P = 0.023$, LT4 vs. untreated model: $P = 0.004$] than the sham group, and these measures were ameliorated by the RES and LT4 treatments. These results indicate that both drug treatments improved the SCH rats' locomotor activity. Moreover, a decreased number of rearing was observed in the untreated model rats, and this behavior was increased in the SCH rats treated with RES or LT4 [$F(3,28) = 13.560$, $P < 0.01$; LSD: RES vs. untreated model: $P = 0.001$, LT4 vs. untreated model: $P = 0.008$, RES vs. sham: $P = 0.006$, LT4 vs. untreated model: $P = 0.001$], indicating that both drug treatments improved exploratory behavior. As the total distance and frequency of rearing are also used as measures of anxiety (26), these data revealed that the drug treatments may have decreased the high anxiety levels in the untreated model rats. However, no differences in the number of grooming behaviors and defecations were observed between the SCH rats with and without RES treatment.

In the FST (**Figures 3H–J**), the untreated model rats spent a longer time immobile [$F(3,28) = 4.792$, $P < 0.01$; LSD: RES vs. untreated model: $P = 0.014$, LT4 vs. untreated model: $P = 0.004$, RES vs. sham: $P = 0.351$, LT4 vs. untreated model: $P = 0.646$] and less time swimming [$F(3,28) = 3.557$, $P = 0.023$; LSD: RES vs. untreated model: $P = 0.014$, LT4 vs. untreated model: $P = 0.004$, RES vs. sham: $P = 0.351$, LT4 vs. untreated model: $P = 0.646$], and these changes were reversed by RES or LT4 treatment, indicating that RES alleviated the despairing behavior in the SCH rats.

RES Administration Decreased the Ratio of the Adrenal Gland Weight to Body Weight, Plasma Corticosterone, and Hypothalamic CRH mRNA in SCH Rats

Although the ratio of the adrenal gland weight to body weight was significantly increased in the untreated model rats (**Figure 4A**), both RES and LT4 significantly decreased the ratio compared to the sham group [$F(3,28) = 22.777$, $P < 0.001$; LSD: sham vs. untreated model: $P < 0.001$, RES vs. untreated model: $P < 0.001$, LT4 vs. untreated model: $P < 0.001$, RES vs. sham: $P = 0.011$, LT4 vs. untreated model: $P = 0.604$]. After the hemi-thyroid electrocauterization, plasma corticosterone in the

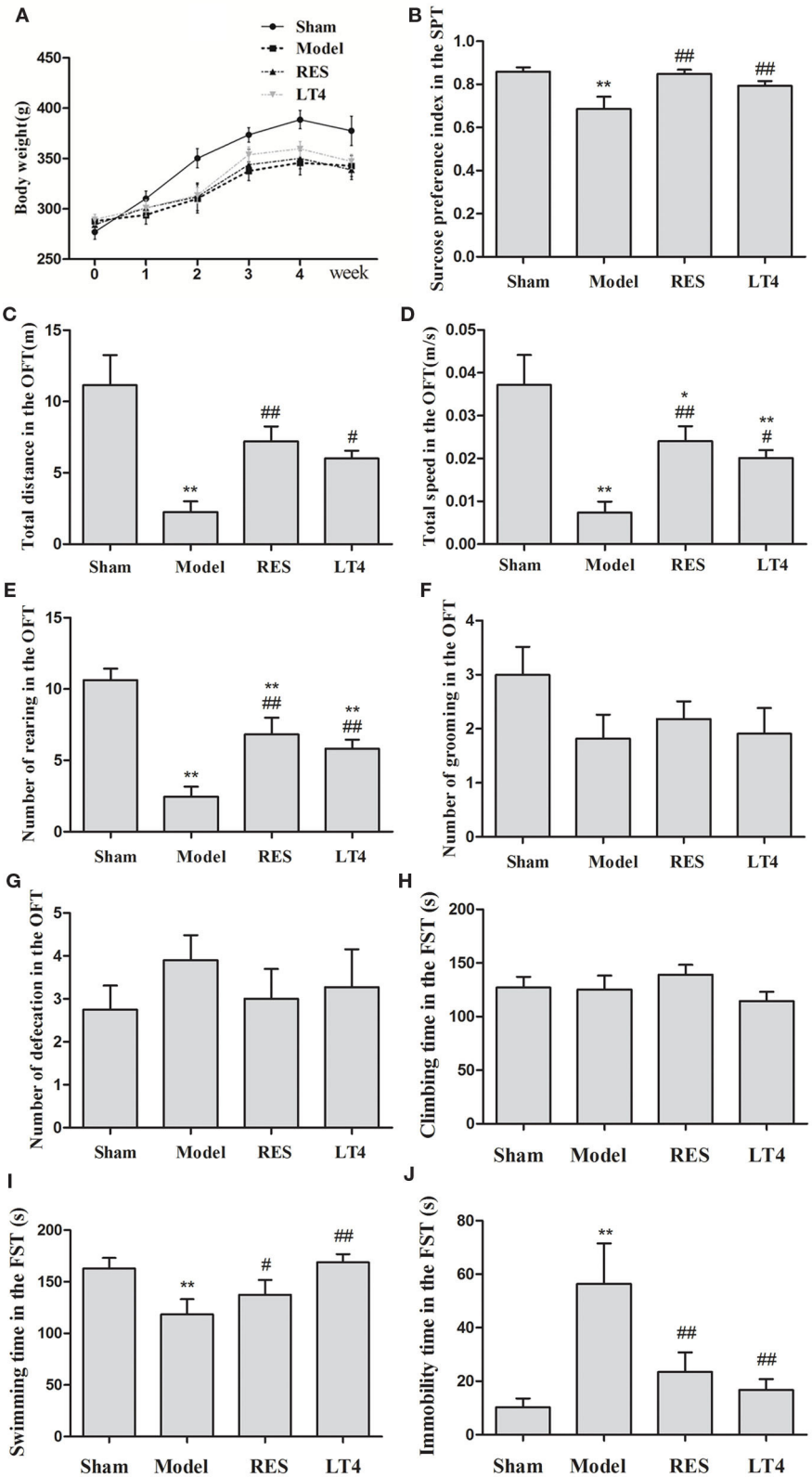

FIGURE 3 | **Effect of RES on the body weight and behaviors of the SCH rats**. The body weight **(A)**, sucrose preference **(B)**, and performance in the OFT **(C–G)** and FST **(H–J)** were observed. The data are presented as the means ± SEM, with $n = 8$ for each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the sham group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to the untreated model group. RES, resveratrol; SCH, subclinical hypothyroidism; OFT, open-field test; FST, forced swimming test.

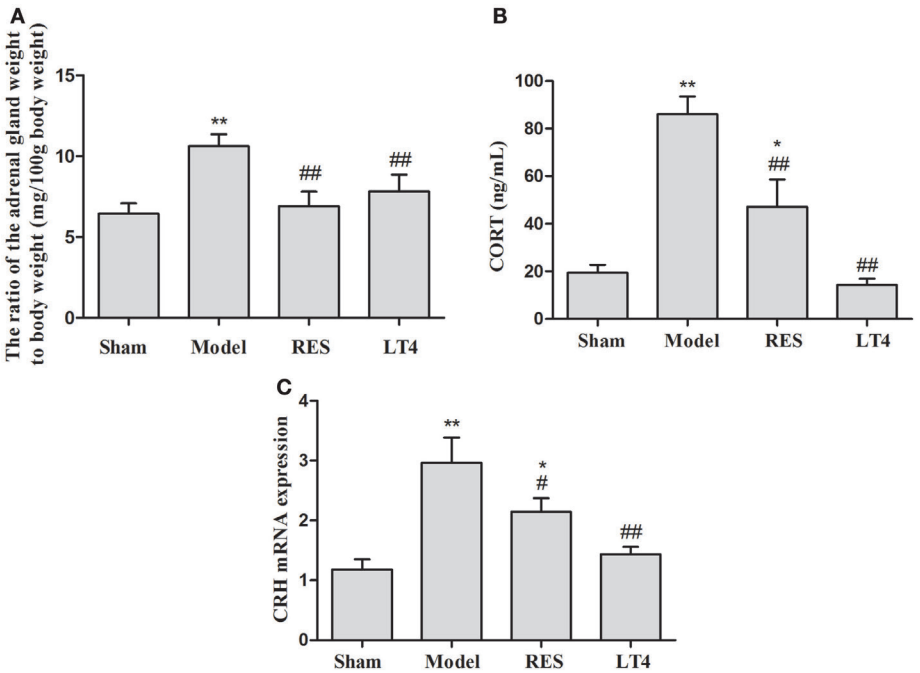

FIGURE 4 | Effects of RES on the ratio of the adrenal gland weight to body weight, the plasma corticosterone, and the expression of CRH mRNA in the hypothalamus in SCH rats. The ratio of the adrenal gland weight to body weight **(A)**, the plasma corticosterone **(B)**, and the expression of CRH mRNA in the hypothalamus **(C)** are shown. The data are presented as the means ± SEM, with $n = 8$ for each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the sham group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to the untreated model group. RES = resveratrol, SCH = subclinical hypothyroidism, CRH = corticotrophin-releasing hormone.

untreated model rats increased significantly, while treatment with either RES or LT4 decreased the elevated corticosterone levels [**Figure 4B**, $F(3,28) = 19.066$, $P < 0.01$]. In line with this result, the elevated hypothalamic CRH mRNA expression noted in the untreated model rats was decreased by treatment with either RES or LT4 [**Figure 4C**, $F(3,28) = 9.468$, $P < 0.01$].

RES Administration Decreased Activation of the Canonical Wnt Pathway in the Hippocampus of SCH Rats

Figure 5 shows the protein expression levels of GSK-3β, p-GSK-3β (Ser9), β-catenin, p-β-catenin, cyclin D1, and c-myc in rat hippocampi. Compared with those of the sham group, a lower protein expression of p-GSK-3β [**Figure 5A**, $F(3,28) = 23.969$, $P < 0.01$] and relative ratio of p-GSK-3β/GSK-3β [$F(3,28) = 21.902$, $P < 0.01$] and a higher protein expression of GSK-3β [$F(3,28) = 23.673$, $P < 0.01$] were observed in the hippocampus of the untreated model rats, which was reversed by the RES or LT4 treatment.

As shown in **Figure 5B**, a higher protein expression of p-β-catenin [**Figure 5B**, $F(3,28) = 11.322$, $P < 0.01$] and relative ratio of p-β-catenin/β-catenin [**Figure 5B**, $F(3,28) = 11.641$, $P < 0.01$] were observed in the hippocampus of the untreated model rats. Conversely, the protein expression of β-catenin [**Figure 5B**, $F(3,28) = 7.44$, $P = 0.002$] was lower than that of the sham group. These changes were reversed by treatment with RES or LT4.

Consistently, hippocampal protein levels of cyclin D1 [**Figure 5C**, $F(3,28) = 7.457$, $P < 0.01$] and c-myc [$F(3,28) = 8.922$, $P < 0.01$] were lower in the untreated model rats than in the sham ones, but these changes were improved by treatment with RES or LT4. Altogether, these results indicate that the canonical Wnt pathway was activated in the hippocampus of the untreated model rats and that activation was ameliorated by the RES treatment.

Results of Pearson correlation analysis showed that the expressions of p-β-catenin/β-catenin were both negatively correlated to cyclin D1 ($r = -0.566$, $P = 0.004$) or c-myc ($r = -0.565$, $P = 0.004$).

DISCUSSION

In this study, we explored the antidepressant effects of RES in SCH rats. The results showed that RES treatment could alleviate anxiety- and depression-like behavior in SCH rats, as indicated by their increased rearing frequency and moving distance in the OFT, their elevated sucrose preference index, and their decreased immobility in the FST. Moreover, RES treatment improved the imbalance of HPA and HPT axes observed in the SCH rats. Furthermore, RES treatment downregulated activation of the canonical Wnt pathway in the hippocampus of SCH rats.

Subclinical hypothyroidism is a common thyroid dysfunction that occurs in 4–20% of the adult population. The risk of SCH progressing to overt hypothyroidism is approximately 7% (27), and both overt and SCH are associated with an increase in

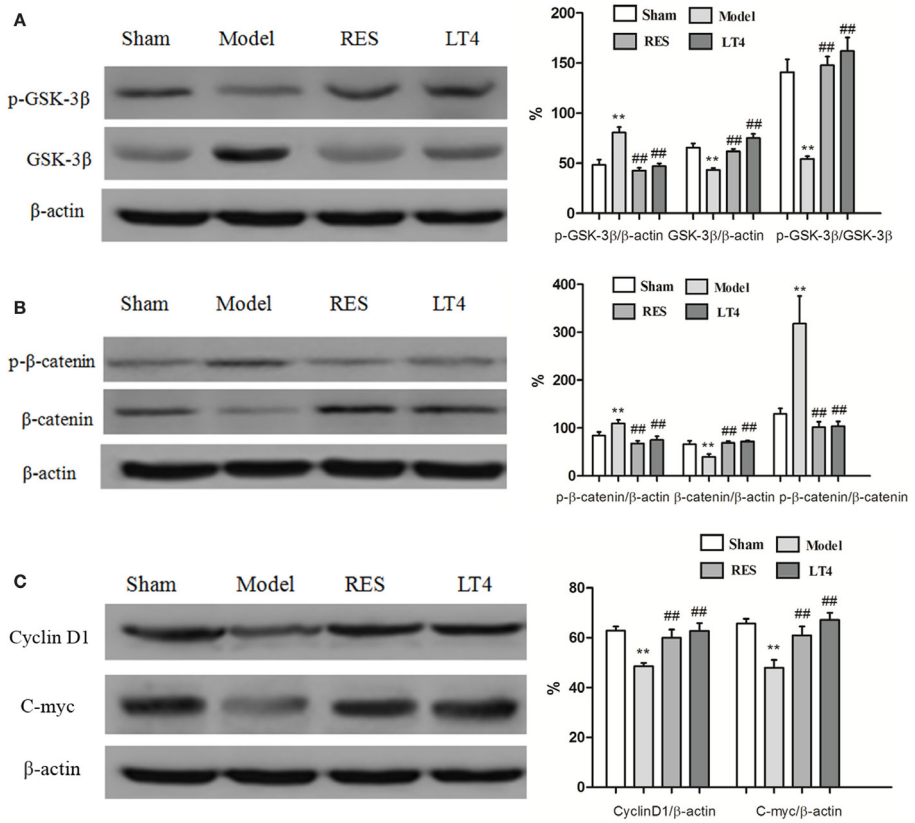

FIGURE 5 | Effects of RES on the activation of the canonical Wnt pathway in the hippocampi of SCH rats. Representative blots and the quantitative analysis of GSK3β and p-GSK3β **(A)**, β-catenin and p-β-catenin **(B)**, cyclin D1 and c-myc **(C)** are presented. The data are presented as the means ± SEM, with $n = 8$ for each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the sham group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to the untreated model group. RES, resveratrol; SCH, subclinical hypothyroidism.

the number and severity of depressive-like symptoms (28, 29). LT4 is the routine clinical treatment for SCH. In this study, our results show that the imbalance of the HPT axis in SCH rats was improved by the treatment of LT4, as indicated by the decrease of both the plasma TSH and the hypothalamic TRH mRNA expression. However, the possibility of overtreatment is one of the adverse effects of LT4 treatment, and the risk ranges from 14 to 21% (30). Consistently, with a dose parallel to the routine dose used clinically, LT4-treated SCH rats showed significantly higher plasma fT4 concentration than the sham rats in our study.

The adverse side effects of LT4, including the hyperthyroxinemia, make the development of new therapeutic drugs to treat SCH necessary. Recently, a new concept of antidepressant mechanisms of action has been proposed based on the findings that antidepressants showed immediate antioxidant effects in the treatment of major depressive disorder (31). Increasing evidence from animal studies suggests that treatment with antioxidants can reduce oxidative stress and alleviate depressive-like behaviors (32). RES is a polyphenol antioxidant that has versatile biological and pharmacological activities, including neuroprotective effects (19, 33). Our results reveal that treatment with RES decreases both the plasma TSH concentration and the hypothalamic TRH mRNA expression in SCH rats without increasing the plasma concentration of fT4. Although the specific mechanism remains unknown, this effect might be partly attributable to the capacity of RES to regulate TSH secretion by manipulating the levels of SIRT1 (34).

The OFT provides simultaneous measures of locomotion, exploration, and anxiety (26). In this study, the untreated model rats showed a decrease in the total distance traveled and the frequency of rearing in the OFT, which was increased in the SCH rats treated with RES, indicating that RES treatment improved the decreased locomotor activity and exploration in the untreated model rats. Moreover, the decreased total distance traveled and frequency of rearing indicated a higher level of anxiety in the untreated model rats, which may also have been reduced by the treatment with RES and LT4. The SPT is commonly used to assess anhedonia, which is a prominent symptom of depression in rodents. Immobility in the FST is taken as an index of despair behavior, which is another prominent symptom of depression. In this study, the SCH rats showed decreased sucrose preference index in the SPT and increased immobility in the FST, indicating a depression-like behavior in SCH rats, which was consistent with the findings in our previous study (6). Moreover, Detke et al. found

that antidepressant drugs, which inhibit norepinephrine reuptake (desipramine or reboxetine) effectively, reduced immobility and selectively increased climbing behavior without affecting swimming, whereas the selective serotonin reuptake inhibitor (SSRI), which works through the serotonin system, reduced immobility and selectively increased swimming, without affecting climbing. In this study, our results showed that both RES and LT4 reduced immobility behaviors in the model rats (35). Thus, it is possible that RES might have an analogous therapeutic effect with the SSRIs in certain types of depression. Further studies are in need to verify the reliability of this hypothesis.

Treatment of thyroid dysfunction could reduce the psychiatric symptoms of depression in general (36), and clinical studies have shown an improvement in depressive-like symptoms after treatment with LT4 in SCH patients (37, 38). Thus, LT4 was selected as a positive control in this study. The results showed the expected antidepressant effect of LT4 on the SCH rats. Similarly, RES-treated rats showed an increased sucrose preference index and decreased immobility in the FST. These results indicate that treatment with RES also alleviated the depression-like behavior of the SCH rats.

Hyperactivity of the HPA axis is one of the most potent factors that trigger depression episodes (39), and abnormalities in HPA axis function are also well documented in rats with hypothyroidism (6). Similarly, the SCH rats displayed elevated adrenal mass, plasma corticosterone, and hypothalamic CRH mRNA expression. However, these changes were ameliorated by treatment with RES. Although RES reportedly stimulated cortisol biosynthesis and secretion in H295R adrenocortical cells *in vitro* (40), our results and other reports indicate that RES reduced the serum corticosterone concentration in stressed rats (11, 14) and unstressed mice (41). This discrepancy about the effect of RES on the HPA axis may be partly ascribed to the different experimental techniques and methods in the different studies.

Studies on human brains have evaluated the levels or activity of total GSK-3β protein in the prefrontal cortex in mood disorders, including depression (22, 42), and increasing evidence suggests that inhibition of GSK-3β might contribute to antidepressant activity (43). Consistent with the report that the enzymatic activity of GSK-3β was increased in depressed suicide victims (42), lower pGSK-3β and pGSK-3β/GSK-3β levels in the hippocampus were found in the SCH rats, which could induce depression-like behavior in this study (6). However, the increased GSK-3β levels were improved by the treatment with RES. L803-mts, a known GSK-3β inhibitor (24), induced an anti-immobility effect in the FST. Moreover, a lower GSK-3β level expressed in heterozygotic GSK-3$\beta^{+/-}$ mice was associated with reduced immobility time in the FST (44). These results suggest a role for GSK-3β in the antidepressive-like effects of RES and highlight GSK-3β as a potential target in the treatment of SCH-associated depression.

β-catenin, a substrate of GSK-3β (45), has been implicated in brain development, cognitive activity, and dendritic growth (46). Phosphorylation of β-catenin by GSK-3β enhances the degradation of the protein, whereas phosphorylation of GSK-3β stabilizes β-catenin and promotes its accumulation in the cell cytoplasm. The unphosphorylated β-catenin can then migrate into the nucleus, where it associates with transcription factors to stimulate gene expression (47). β-catenin protein levels were lower in the postmortem prefrontal cortices of depressed subjects compared to non-depressed controls (22), and β-catenin levels in the hippocampus can serve as a marker for antidepressant behavior (48). Consistent with these results, the protein levels of p-β-catenin and p-β-catenin/β-catenin were upregulated in the hippocampus of the SCH rats, indicating β-catenin degradation. Together with these reports, the findings from this study reinforce the clinical observation that depressed subjects display a high GSK-3β activation state and low β-catenin levels (22).

Recent studies have demonstrated that cyclin D1 and c-myc, critical genes involved in cell proliferation and differentiation, were important target genes of the Wnt signaling pathway. Overexpression of cyclin D1 and c-myc is highly associated with the accumulation of β-catenin and mutational defects of the Wnt signaling pathway (49). In this study, protein levels of cyclin D1 and c-myc were decreased due to the increased β-catenin protein levels in the SCH rats, which was improved by RES treatment, further confirming the therapeutic effect of RES. Several recent studies have focused on the relationship between β-catenin and cyclin D1 or c-myc. For instance, a strong correlation was reported between β-catenin deregulation and cyclin D1 expression in primary colorectal tumors (50). Brabletz et al. (51) also reported a tight correlation between nuclear β-catenin accumulation and c-myc expression in colorectal adenomas. In this study, the results of Pearson's correlation test suggested a significant positive association between the expression of p-β-catenin/β-catenin and cyclin D1 or c-myc. Based on our findings, the abnormal expression of β-catenin and its associated dysfunction of cyclin D1 and c-myc may play a key role in SCH-associated depression.

In this study, we first observed that RES alleviates depression-like behavior in SCH rats, which may be due to the regulation of HPA and HPT axes and the activity of the Wnt/β-catenin pathway in the hippocampus. Consistent with reports that RES does not cause adverse effects (15, 52), our results confirm that RES does not cause hyperthyroxinemia, which is a potential therapeutic advantage over LT4 treatment. However, this study presented several limitations. First, RES was administered only in a single dose. Thus, its dose relationship and long-term side-effects should be determined using different doses in future studies. Second, considering our previous finding that RES could alleviate the depression-like behavior of CUMS rats, together with the reports that RES treatment does not cause adverse effects (15, 52), we did not administer RES to the sham rats, which might make it subtly difficult to interpret many of the results. Third, although LT4 treatment was selected as a positive control in this study, the effect of RES on the depression-like behavior of the SCH rats was not compared with that of conventional antidepressant medications, such as fluoxetine.

In conclusion, our results demonstrate that RES improved anxiety- and depression-like behavior in SCH rats. This effect may be due, at least in part, to regulation of the HPA and HPT axes and the Wnt/β-catenin pathway in the hippocampus. Compared with the possible adverse effects of LT4 treatment,

including cardiovascular events and symptoms associated with excess thyroid hormone, such as nervousness and palpitations (53), the credible efficacy with high safety margins (54, 55) of RES make it a promising candidate for the treatment of SCH-associated depression.

AUTHOR CONTRIBUTIONS

Associate Prof. Jin-Fang Ge and Prof. Fei-Hu Chen designed the study, and wrote the protocol and the first draft of the manuscript. Prof. Jin-Fang Ge and Dr. Ya-Yun Xu managed the literature searches and the statistical analyses. Ya-Yun Xu, Gan Qin, and Jiang-Qun Cheng performed animal model experiments. All authors contributed to and have approved the final manuscript.

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Murine Depression Model and its Potential Applications for Discovering Foods and Farm Products with Antidepressant-Like Effects

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Advanced societies face increased health problems related to various stresses. Chronic psychological stress is a major risk factor for psychiatric disorders such as depression. Although therapeutic agents reduce several symptoms of depression, most have side effects in a broad range of the population. Furthermore, some victims of depression do not show significant improvement with any drugs, so alternative approaches are needed. Good dietary habits may potentially reduce depressive symptoms, but there is little scientific evidence thus far. Murine depression models are useful to test nutritional approaches *in vivo*. Our model mice subjected to a subchronic mild social defeat stress (sCSDS) paradigm show several alterations in physiological parameters and social behavior. These stress-induced symptoms in sCSDS mice can be used as cues to identify antidepressant-like natural resources including foods and farm products. We previously discovered that sCSDS mice show more vulnerability to social stress by changing dietary condition. In addition, we developed a more objective system for analyzing mouse behavior using a 3D depth-sensing camera to understand relationships between diet and behavior. The combination of sCSDS mice with 3D behavioral analysis is a powerful method for screening ingredients in foods and farm products for antidepressant-like effects.

Keywords: depression, food, behavior, metabolomics, social defeat stress, depth camera

INTRODUCTION

Advanced societies face increased health problems related to various stresses; chronic psychological stress in particular is a major risk factor for precipitating psychiatric disorders such as depression. A Global Burden of Disease study showed depression is the most disabling disorder worldwide (Whiteford et al., 2013). Since this issue will face the next generation of developing countries, it is imperative to find some solution. Therapeutic agents reduce several symptoms of depression, but most have several side effects in a broad population (Stevens et al., 2014; Galling et al., 2015). Moreover, some victims of depression do not show significant improvement with any

drugs (treatment-resistant depression), so alternative approaches are needed (El-Hage et al., 2013). For prevention, it is more important to change dietary habits rather than resort to conventional treatments. Recently, interest has increased in Kampo (Watanabe et al., 2011) and functional foods (Arai, 1996). In fact, some reports indicate that Kampo (Ito et al., 2012; Hori et al., 2015) and functional ingredients (Tomonaga et al., 2008; Iio et al., 2012a) have antidepressant effects in animal models. This study further explores functional ingredients from natural sources for prevention and attenuation of symptoms of depression.

Chronic social defeat stress (CSDS) models of rats and mice are recognized as good animal models of depression. CSDS models establish social stress using male territorial aggression (Kudryavtseva et al., 1991; Miczek et al., 2008; Hammels et al., 2015). We previously studied CSDS rats and found that social stress induces alterations in the MAP kinase cascade, hypothalamic malonyl-CoA, peripheral leptin, digestive system, and behaviors (Iio et al., 2011, 2012b, 2014; Toyoda et al., 2015). Others have analyzed both central nervous system and peripheral tissues in relation to stress resilience using CSDS mice (Russo and Nestler, 2013; Hodes et al., 2014; Pfau and Russo, 2015). In addition, we have established a milder model of depression, subchronic mild social defeat stress (sCSDS; Goto et al., 2014, Goto and Toyoda, 2015) than CSDS (Krishnan et al., 2007). Since several models are required (Bartolomucci and Leopardi, 2009), sCSDS mice may provide insights about pathogenic mechanisms and preventive measures for depression.

The 3D behavioral analysis allows evaluation of more natural, realistic animal behavior. Although animals behave in 3D space and show several postures sterically, conventional animal monitoring systems have been primarily 2D video cameras. Recently, commercially available 3D depth-sensing cameras have been developed in the computer game industry, and can thus be affordably obtained (∼$200/camera). Using 3D cameras, rats and mice have been monitored (Ou-Yang et al., 2011). Since behavioral scientists can derive depth information from animal behavior, the 3D camera enables discrimination of slight differences in 3D behavioral characteristics that have not been previously well detected.

In this article, we introduce features of our murine stress model and novel 3D monitoring system. We discuss the use of both for discovering foods and farm products providing antidepressant-like effects.

SUBCHRONIC MILD SOCIAL DEFEAT STRESS (SCSDS) MODEL MOUSE

Phenotypes of sCSDS (Figure 1A)

sCSDS mice are established by the method (Goto and Toyoda, 2015). Subject C57BL/6J mice are exposed to psychosocial stress from aggressive ICR mice for 10 consecutive days. During establishment, body weight gain, food intake, and water intake in sCSDS mice are significantly higher than those of non-stressed control mice (Goto et al., 2014). The sCSDS mice show increased body water content and social avoidance behavior after the stress. Moreover, nest-building behavior in sCSDS mice is significantly delayed compared to control mice (Otabi et al., 2016). In CSDS mice, social avoidance behavior has been widely reported (Tanaka et al., 2012; Russo and Nestler, 2013). Increased body weight gain has been reported in defeated mice (Goto et al., 2014) and lower ranking mice (Kim et al., 2015). Stress-induced polydipsia has been reported in both CSDS (Krishnan et al., 2007) and chronic mild stress (CMS) conditions (Gross and Pinhasov, 2016). Although, these features are supported by several studies, social stress-induced increases in body water content (Goto et al., 2014) and delays in nest-building behavior (Otabi et al., 2016) are, to our knowledge, unique findings.

Metabolomic Analyses of sCSDS (Figure 1A)

To find key metabolites altered by social defeat stress, we tested the blood plasma/serum, urine, and liver. Biochemical assays for blood serum components revealed that sCSDS mice showed low levels of albumin (ALB) and blood urea nitrogen (BUN) just after stress (Goto et al., 2014). Low levels of sodium were found in the urine of sCSDS mice. Metabolomics revealed that four liver metabolites, taurocyamine (GES), phosphorylcholine, D-alanyl-D-alanine (D-ala-D-ala), and 1-methylnicotinamide (MNA), were significantly upregulated in sCSDS mice vs. control mice (Goto et al., 2015a).

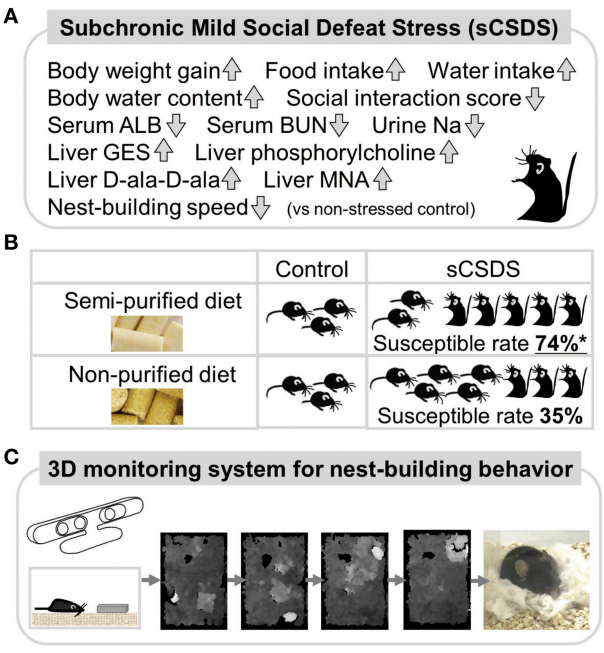

FIGURE 1 | Summary of a mouse model and 3D behavioral analysis.(A) Social stress-induced symptoms in sCSDS mice. **(B)** Dietary quality influence stress-vulnerability behavior in sCSDS mice. sCSDS mice fed semi-purified diet indicate significantly higher susceptible rate than sCSDS mice fed non-purified diet. *$P < 0.05$ (Goto et al., in press). **(C)** 3D monitoring system of nest-building behavior. Temporal changes of nesting behavior are clearly identified by depth images (higher regions are indicated by whiter color).

Food Habits Attenuate Stress-Induced Symptoms in sCSDS (Figure 1B)

We established sCSDS mice under feeding conditions with two kinds of pellet food, a semi-purified and a non-purified diet. We confirmed that the increased body weight gain, food intake, and water intake of sCSDS mice during stress were common to both diets (Goto et al., in press). Interestingly, we found that the vulnerability of mice to social defeat stress was affected by diet quality. sCSDS mice fed a semi-purified diet were more susceptible than sCSDS mice fed a non-purified diet (Goto et al., in press). This may be due to changing gut environments, as gut microbiota and their metabolic products can affect animal brain function and behavior (Cryan and Dinan, 2012). Especially, commensal microbiota can influence the hypothalamus-pituitary-adrenal reaction to stress in mice (Sudo et al., 2004). And, gut microbiota can modulate brain development and modulation of the serotonergic system, which is directly related to mood, in the limbic system (Heijtz et al., 2011). Although the microflora of sCSDS mice have not been fully studied, intestinal flora will be influenced by both stress and food. Metabolomics with the sCSDS mice fed both diets hope to find some solutions to attenuate stress-induced behavior by changing the metabolic environments of peripheral tissues.

A NOVEL 3D BEHAVIORAL TESTING METHOD FOR INNATE BEHAVIOR (FIGURE 1C)

Infrared 3D depth-sensing cameras have been developed intensely in the computer game industry. Both Microsoft Kinect (Microsoft Corp., USA) and Xtion PRO LIVE (ASUSTek Computer Inc., Taiwan) have become commercially available and affordable. The 3D depth-sensing camera has thus recently become available for use in rodent behavioral tests. The first reported behavioral analyses using a 3D camera focused on locomotion and pose in rats and mice (Ou-Yang et al., 2011). Matsumoto et al. constructed skeleton models of rats using four 3D cameras simultaneously and analyzed social and sexual interactions and novel object recognition behavior (Matsumoto et al., 2013, 2014). We monitored nest-building behavior in mice using a 3D camera (Okayama et al., 2015), and confirmed its effective utilization in a genetic study (Goto et al., 2015b). Nakamura et al. have developed a gait analysis system for mice using a 3D camera (Nakamura et al., 2015). Hong et al. succeeded in automating measurement of mouse social behaviors with machine learning algorithms from images captured simultaneously by a 3D camera and two 2D video cameras (Hong et al., 2015). Although conventional testing has been performed with 2D video cameras, future animal behavioral testing will evolve dramatically through use of 3D cameras.

Our 3D monitoring system focused on nesting behavior in mice, because the steric nest can be evaluated as a visible behavior. Deacon codified a standard method of nest-building behavior in mice using pressed cotton and rated nest quality on a scale of 1–5 (Deacon, 2006). By utilizing 3D depth-sensing cameras, we are able to conduct objective 3D evaluation of the final nest in one point evaluation (Okayama et al., 2015). In addition, we have analyzed an untapped behavioral characteristic, the construction process of the nest. Our 3D monitoring system could discriminate slight differences in temporal nesting behavior using 3D depth images and Deacon score 1-5 (Goto et al., 2015b). Since nest-building behavior is altered by social stress (Otabi et al., 2016), several stress models such as CSDS, CMS, and restraint stress should also be analyzed while nest-building in future.

STRATEGY FOR FINDING ANTIDEPRESSANTS FROM NATURAL INGREDIENTS (FIGURE 2)

Using sCSDS mice, we will investigate functional ingredients from natural sources that should attenuate stress-induced symptoms. Since sCSDS mice fed a semi-purified diet show more vulnerable behavior than mice fed a non-purified diet (Goto et al., in press), a change to a semi-purified diet as a base diet would be a good approach to identify functional ingredients enhancing stress resilience behavior. A semi-purified diet is preferred for nutritional research because the non-purified diet contains unknown raw materials and ingredients. Since the non-purified diet potentially improves stress vulnerability, it would be worthwhile to note differences between semi-purified and non-purified diets. Components including dietary fiber, resistant starch, and unavailable carbohydrates will be targets in future studies. These components are available to gut microbiota, resulting in a variety of species living in the gut. The brain-gut axis (Kelly et al., 2015) should be a main target for food-mediated approaches to finding antidepressants and preventing stress-related diseases by shifting food habits. In addition, sCSDS mice show stress-induced symptoms described above. By checking these indicators, the depression model can be useful for screening antidepressant effects of functional food and farm products.

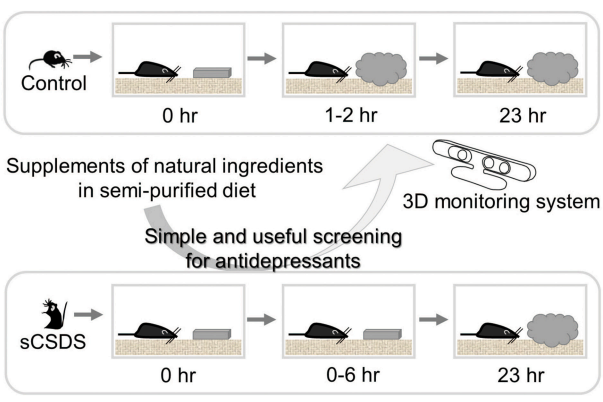

FIGURE 2 | Strategy for finding antidepressants from natural sources. The combination of sCSDS mice with 3D monitoring system is a powerful method for screening antidepressants from natural ingredients. Natural ingredients which rescue retarded nest-building can be screened.

Our novel 3D monitoring system for nest-building enabled us to detect small differences among groups in different treatments and strains (Okayama et al., 2015; Goto et al., 2015b). Furthermore, we found simple and useful nesting behavioral test (Otabi et al., 2016). By using this combined method with sCSDS mice and 3D monitoring systems, we can discern slight differences in innate behavior and screen candidate supplements quickly. In future, automated behavioral analysis for nesting behavior will make this method easy for many researchers.

Toyoda and colleagues conducted research in the interdisciplinary field of agricultural-medical science (http://iucam-ibaraki.wix.com/iucam), exploring functional food and farm products for prevention and attenuation of psychiatric symptoms. Establishing animal models of depression, searching and screening supplemental resources, and applying engineering techniques will be essential in the big data era. Now that the fundamental platform has been established, we can screen ingredients from natural sources such as dairy products, fruits, and vegetables.

CONCLUSIONS

In this article, we introduced our mouse model of depression and novel 3D evaluation system for nesting behavior. Good dietary habits should provide potential effects for reducing depressive symptoms and extending healthy life. The importance of food habits in overcoming stress can be shown by identifying antidepressant-like natural resources using sCSDS mice. The combination of sCSDS mice with 3D behavioral assays is a powerful method for screening the ingredients showing antidepressant-like effects in foods and farm products.

AUTHOR CONTRIBUTIONS

TG and AT wrote overall manuscript. ST and TO wrote a part of the manuscript about metabolome and 3D sensor, respectively. TG, ST, TO, and AT have checked the manuscript entirely and agree with submission.

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