Published in final edited form as:

Life Sci. 2012 November 2; 91(19-20): 921–927. doi:10.1016/j.lfs.2012.09.010.

Prenatal exposure to methanol as a dopamine system sensitization model in C57BL/6J mice

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Abstract

Aims—In this study, the effects of prenatal exposure to methanol (MeOH) on the nigrostriatal dopamine (NSDA) system were examined to determine if the interaction could sensitize this system, and serve as an underpinning for Parkinson's disease (PD) like changes that occur later in life. Methanol was studied because its toxicity resembles the symptoms of PD and the symptoms are relieved by L-dopa meaning that MeOH targets the NSDA system. Since fermentation and wood combustion are major sources for MeOH, the incidence of human encounters with MeOH is high. As a superior solvent and the precursor for formaldehyde, MeOH has a powerful and sometimes, irreversible impact on chemical processes, such as cross-linking proteins and nucleic acids. It may cause subthreshold changes that sensitizes the NSDA system to PD, that occur during aging.

Main methods—To study the prenatal effects of MeOH, pregnant C57BL/6J mice were administered 40 mg/kg MeOH by oral gavage during gestation days 8–12, twice daily. Twelve weeks after birth, behavior impairments were recorded. The striatum was dissected for the determination of tyrosine hydroxylase (TH), L-aromatic amino acid decarboxylase (LAAD), α -synuclein and levels of dopamine (DA) and its metabolites.

Key findings—MeOH reduced striatal TH and LAAD protein by 47% and 57% respectively and DA by 32%.

Significance—The results mean that in utero exposure to toxins similar to MeOH could sensitize the striatal system to changes that cause PD. This study may help identify strategies to block this type of in utero toxicity.

Keywords

L-aromatic amino acid decarboxylase; Dopamine; Methanol; Nigrostriatal neurons; Neurotoxins; Parkinson's disease; α -Synuclein; Tyrosine hydroxylase

Introduction

Parkinson's disease (PD) is characterized by degeneration of nigrostriatal dopaminergic neurons and the depletion of striatal dopamine and tyrosine hydroxylase (Hornykiewicz, 1966). Idiopathic PD represents 90–95% of all PD cases (Tanner and Ben-Shlomo, 1999).

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Conflict of interest statement

None.

PD may be initiated by exposure to neurotoxicants that result in changes prior to the classical symptoms. For instance, neurotoxicants, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Muthian et al., 2010), pesticides (Richardson et al., 2008; Shepherd et al., 2006), lipo-polysaccharide (Kirsten et al., 2010) and organic solvents, can produce subthreshold alterations in the dopamine system that may pair with the natural age-related decline in basal ganglia function leading to PD later in life (Charlton and Mack, 1994).

Methanol (MeOH) causes CNS depression, alterations in brain monoamines, and metabolic acidosis (Jeganathan and Namasivayam, 1987; Murray et al., 1991; Andrews et al., 1993; Degitz et al., 2004). MeOH has been recognized as an important neurotoxicant in humans in which its toxicity is manifested at lower doses than ethanol (Sandhir and Kaur, 2006). MeOH is widely used in manufacturing, biological research, household and cleaning business and undergoes one of the highest releases to the environment based on its mixability with water and organic fluid (Mellerick and Liu, 2004; Parthasarathy et al., 2007). Methanol is biologically produced during the hydrolysis of methyl esters, fermentation and the combustion of green plants; it generates very reactive formaldehyde and formic acid that are well known as *in vitro* and *in vivo* toxicants (Seme et al., 1999; Treichel et al., 2003).

It has been shown that the exposure to MeOH during gestation decreased striatal dopamine in the offspring (Aziz et al., 2002), highlighting the toxic effects of MeOH to the developing brain as well as the fact that the neurotoxicant causes detrimental effects that persist. MeOH caused striatal necrosis, which has been shown to be correlated with parkinsonism (Finkelstein and Vardi, 2002; Reddy et al., 2007; Paasma et al., 2009). As a metabolite of MeOH, formaldehyde cross-links proteins and DNA and increases reactive oxygen species leading to cell death (Huang et al., 2001; Lee et al., 2008). The MeOH secondary metabolite, formic acid, also increases reactive oxygen species and depletes antioxidant capacity within the brain, which may be secondary to impairing cytochrome c oxidase (Murray et al., 1991; Sakanshi et al., 1996; Mellerick and Liu, 2004).

PD is a disease of the aging but not all aging individuals develop PD (Charlton and Mack, 1994). So, there ought to be an underpinning for PD that pairs with aging, and makes aging the key risk factor for PD. The objective of this study was to determine if prenatal exposure to MeOH will impair the nigrostriatal (NS) dopamine (DA) system, making it vulnerable to changes that cause PD-like symptoms later in life. Using a mouse model, we targeted behavior, DA, key enzymes within the DA metabolic pathway and evaluated brain sections for TH-immunoreactivity. The results showed that gestational exposure to MeOH modified the dopaminergic system, leaving it apparently vulnerable to changes that may cause PD-like impairments later in life.

Materials and methods

Animals and treatment

Timed-pregnant C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and used for this study. The dams were housed two per cage, under a 12 h light 12 h dark cycle in a 22 °C temperature controlled room. The pregnant dams and the offspring had full access to standard chow and water. The offspring were born and housed under the same conditions. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health) and were approved by the Institutional Animal Care and Use Committee at Meharry Medical College. Pregnant dams were administered 40 mg/kg methanol (MeOH) by oral gavage twice daily (8 h apart) starting at gestational day (GD) 8 and ending on GD (G8–12). Control dams (n=6) received an

equivalent amount of phosphate buffered saline (PBS) during the indicated time periods. This dose was administered because it does not cause any visible toxicity to the dams. The oral exposures were chosen since the primary route of exposure to MeOH is through ingestion. Following the birth of the pups they were weighed weekly. The offspring were separated by sex after weaning and were studied at 12 weeks of age. A total of 6 male offspring from each dam were used per data group from 6 different exposed dams (controls or MeOH). The brains of 3 animals were used for immunohistochemistry.

Measurements of locomotor activity

Twelve weeks after exposure to 40 mg/kg MeOH in utero, locomotor activity was assessed in the offspring. The activity monitor system consists of plexiglass cages (one animal per cage) with infrared detection on all sides (AccuScan Instruments, Inc. Columbus, OH, USA). Locomotion was evaluated for 30 min in an isolated room. The parameters of locomotor activity were recorded as total distance traveled (TD), total number of movements (NM) and movement time (MT) using Versamax 5.0 software (AccuScan Instruments, Inc. Columbus, OH, USA).

Beam walking apparatus

Balance and motor coordination were determined in prenatally exposed MeOH offspring and PBS controls using a narrow balance beam, based on modification of the methods of Ferguson et al. (2010). The design of the apparatus consisted of a 1 cm square stainless steel beam of 105 cm in length. The walking beam was suspended 49 cm above the floor of the test chamber. The mice were acclimatized to the beam and goal box, with some modifications as follows. First, the mice were placed 10 cm from the goal box and allowed to traverse the beam to the goal box. If the mice were able to traverse the beam to the goal box at 10 cm, they were then placed at increasing distances of 30, 50, and 80 cm and trained on 2 consecutive days to completely traverse the beam. On the 3rd day, the MeOH and PBS-prenatal mice were videotaped and the mean number of hindlimb footslips during three trials was recorded.

Western blot analysis of TH, LAAD and α -synuclein

Western blots were used to quantify the level of tyrosine hydroxylase (TH), L-aromatic amino acid decarboxylase (LAAD), α -synuclein (α -syn) and α -tubulin protein present in the brains of MeOH-exposed and control offspring. At 12 weeks age, the mice were sacrificed by decapitation and the brain was removed and the striatum dissected. The striatum was weighed and homogenized in 500 μ l of 320 mM sucrose and 5 mM HEPES (Fisher Scientific, New Hampshire, CT, USA) containing 1 μ l/ml of protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA). The homogenate was centrifuged for 5 min at 3500 g (4 °C) in a Sorvall Legend Micro 17R centrifuge (Fisher Scientific, Pittsburg, PA, USA). The supernatant was collected and re-centrifuged at 14 000 g using a Marathon 26 KMR, (Fisher Scientific, Pittsburg, PA, USA) for 40 min. The supernatant was stored at -80 °C until further analysis.

An aliquot was used for the determination of the protein concentration using the Lowry method (Lowry et al., 1951). The preparations were subjected to polyacrylamide gel electrophoresis (SDS-PAGE) on a 10–12% Precast gel (Bio-Rad, Hercules, CA, USA). The samples were then transferred to a polyvinylidene difluoride membrane (PVDF, Bio-Rad, Hercules, CA, USA) and subsequently blocked in 5% non-fat dry milk diluted in Tris-buffered saline, for 1 h. All of the samples were controlled to ensure an equal amount of protein loading. The membranes were stripped and reprobed for TH, LAAD, α -syn and α -tubulin using primary 1:1000 rabbit anti-TH (Chemicon, Temecula, CA, USA), 1:1000 rabbit anti-LAAD (Chemicon, Temecula, CA, USA), 1:3000 mouse anti- α -synuclein (BD

Bioscience, San Jose, CA, USA) and 1:10 000 mouse anti- α -tubulin (Sigma Aldrich, St. Louis, MO, USA). Secondary antibodies, 1:5000 horseradish peroxidase-conjugated goat anti-rabbit (GE Healthcare, Pittsburgh, PA, USA) and 1:10 000 goat anti-mouse (BioRad, Hercules, CA, USA), were used to detect primary antibody binding to respective protein. Immunoreactive proteins were visualized and developed on film (ISC Bioexpress, Kaysville, UT, USA) using ECL Western blotting detection reagents. Densitometric analyses of the bands were measured by UN-SCAN-IT Gel Automated Digitizing System Version 6.1 (Silk Scientific Inc., Orem, Utah).

Determination of dopamine and its metabolites in the striatum and cortex by HPLC-EC

Dopamine (DA) levels in the striatum and cortex were determined using high performance liquid chromatography (HPLC) with electro-chemical (EC) detection. Samples were analyzed by the HPLC system located in the Neurochemistry Core Lab at Vanderbilt University, Nashville, TN. The mice were sacrificed by decapitation 12 weeks after in utero exposure to MeOH. Based on methods modified from Richardson et al. (2006), the brains were quickly dissected to obtain the striatum and cortex, snap frozen in liquid nitrogen and stored at -80°C . Samples were homogenized with 0.1 M trichloroacetic acid containing 10 mM sodium acetate, 1 mM EDTA, 1 μM isoproterenol (internal standard) and 10.5% methanol. The samples were centrifuged at $10\,000\times g$ for 20 min and the supernatant was used for HPLC-EC analysis. Twenty microliters of the supernatant was injected and DA and its metabolites were separated with a C18 HPLC column (150 $\times$ 4.60 mm; Phenomenex Nucleosol). Biogenic amines, dihydroxyphenylacetic acid (DOPAC), dopamine (DA), homovanillic acid (HVA), and 3-methoxytyramine (3-MT) were detected and analyzed using an Antec Decade 11 EC detector. The amines were eluted using homogenizing buffer as the mobile phase at 0.8 ml/min. Concentrations of DA, DOPAC, HVA and 3-MT were determined by assaying standards of known amounts to generate calibration curves and extrapolating from that curve.

Tyrosine hydroxylase (TH) immunohistochemistry

Twelve weeks after prenatal exposure to MeOH, TH immunohistochemistry (IHC) was performed on brain slices containing the substantia nigra of the MeOH and PBS exposed offspring. The offspring were anesthetized with 400 mg/kg chloral hydrate and transcardially perfused with cold phosphate buffered saline (PBS) and 4% paraformaldehyde prepared in PBS (Shepherd et al., 2006). The brains were removed and placed in cold 15% sucrose prepared in 4% paraformaldehyde for 24 h at 4°C . The brains were frozen in powdered dry ice and stored at -80°C for 24 h. Then the brains were sectioned at thickness of 25 μm in a cryostat (Triangle Biomedical Science, Durham, NC, USA) and mounted on gelatin coated slides and stored at -80°C prior to staining.

The slide mounted sections were stained for TH-positive cell bodies according to the methods of Charlton and Mack (1994). Briefly, the slides were kept at 4°C for 20 min; then, they were incubated in 0.3% TritonX-100 in PBS, pH 7.4 for three 5 min periods. Next, the slides were incubated with a primary antibody solution containing 1:500 dilution of rabbit anti-tyrosine hydroxylase (Chemicon, Temecula, CA, USA) prepared with the same buffer, for 24 h at 4°C . The slides were washed in 0.2% Triton-X for three 5 min periods. The slides were incubated for 1 h at room temperature in reduced light, with an Alexa Fluor 488 conjugated chicken anti-rabbit secondary antibody solution containing a 1:400 dilution (Invitrogen, Grand Island, NY, USA). Then the slides were washed for one 5-min period in 0.2% Triton-X buffer and two 5 min period in PBS. The slides were drained and air dried for 10–20 min; they were coverslipped using SlowFade® Gold antifade reagent (Invitrogen, Grand Island, NY, USA). TH-positive cells were visualized on a Nikon TE 2000E

epifluorescent microscope (Nikon Instrument Inc., Melville, NY, USA) at the Morphology Core Lab at Meharry Medical College.

Data analysis

Statistical analysis of all data was performed using a Student's t-test. GraphPad Prism® 5.0 (GraphPad Software Inc., San Diego, CA) was used to analyze the data sets. Values are expressed as the mean±SEM of 6 mice; p values less than or equal to 0.05 were considered as significant. Three animals were used for IHC.

Results

The prenatal exposure to 4 mg/kg methanol on the weight of the offspring

Methanol (40 mg/kg) was administered to pregnant dams during GD 8–12. We assessed the global effects of MeOH using changes that may occur in the weights of the offspring as a measure. The weights were recorded weekly after birth. When compared to the PBS (control) group, the MeOH offspring showed no difference in weight over a 4 week period (Fig. 1). The level of MeOH administration to C57BL/6J dams did not show overt signs of toxicity in the offspring throughout maturation and did not affect the weight of the offspring. We found that the weights appeared to reflect the natural growth pattern through-out lactation.

The prenatal exposure to MeOH reduced locomotor activity

Total distanced traveled (TD), total number of movements (NM) and movement time (MT) were the parameters used to measure spontaneous motor activities in prenatal MeOH exposed mice and PBS controls (Fig. 2). The results show that the TD was decreased by 80% in MeOH offspring (Fig. 2A) while NM was decreased by 26% when compared to the PBS control group (Fig. 2B). No change in MT occurred (Fig. 2C). The calculation of the movement rate was determined by the ratio of the TD/MT (Charlton and Crowell, 2000). The values show that mice exposed to prenatal MeOH covered about 2.31 cm/s as compared to the PBS group of 11.09 cm/s. Thus, MeOH significantly reduced the rate of movement by 79% (Fig. 2D).

Effect of prenatal MeOH on the footslip phenomenon in mice

Prenatal MeOH exposed offspring showed a significant effect on the number of footslips on the beam (Fig. 3A) at 12 weeks of age. The number of footslips in these mice increased by 48%, highlighting the poor motor performance in offspring exposed to prenatal MeOH when compared to the PBS group. The latency to traverse the beam to the goal box was also increased in the MeOH mice, but it was not significant at the 5% level of probability (Fig. 3B).

Effects of prenatal MeOH on striatal tyrosine hydroxylase (TH), L-aromatic amino acid decarboxylase (LAAD) and α -synuclein (α -syn) in the offspring

The expressions of TH, LAAD and α -syn in the striatum were determined using Western blot analysis. The striatum is the site of dopamine synthesis; TH is the rate limiting enzyme step in the synthesis of DA and is considered to be the key markers for the loss of dopamine neurons. In addition, LAAD decarboxylates L-dopa to DA and it is a key enzyme but it decarboxylates other biogenic amines. The immunoblots showed that 4 mg/kg of MeOH administered during GD 8 to 12 resulted in a 47% reduction in TH protein levels when compared to the PBS control group (Fig. 4A) and as shown in the densitometric analysis (Fig. 4B). Fig. 4C shows a 57% reduction in the immunoblot for LAAD and the comparative densitometric determination (Fig. 4D) in the MeOH exposed offspring. Prenatal MeOH

insignificantly increased α -syn by 48% (Fig. 4E; F) as the robust increase in α -syn did not occur in all animals exposed to prenatal MeOH.

The prenatal MeOH exposure on striatal DA, DOPAC, HVA and 3-MT

It was also determined whether prenatal exposure to MeOH had an effect on DA and its metabolites within the NSDA system. So, the concentrations of DA, DOPAC, HVA and 3-MT were measured in the striatum of 12 week old offspring exposed prenatally to 4 mg/kg MeOH. MeOH reduced striatal dopamine levels by 31% when compared to the PBS (control) groups (Table 1). Although, there is a noted decrease in DOPAC and HVA by 14% and 15% respectively, these changes failed to reach statistical significance. Striatal 3-MT levels also remained statistically unchanged (Table 1). The overall striatal DA turnover rate was determined (Table 1) by calculating the ratio of the metabolites to the amount of available DA. Here, we report that there was a 38% increase in striatal DOPAC+HVA +3MT/DA, $**p<0.01$; indicating that the release of DA was increased in the MeOH exposed offspring.

Dopamine and metabolite levels in the cerebral cortex of 12 week old offspring pre-exposed to in utero MeOH

DA and its metabolites were also examined in the cerebral cortex to assess whether prenatal MeOH had an effect on other brain regions outside the nigrostriatum. This report shows that cortical DA, DOPAC and 3MT levels were decreased by 84%, 66% and 60% respectively; $*p<0.05$ and $**p<0.01$ (Table 2). There was also a 27% reduction in HVA levels. The cumulative cortical turnover of DA, based on the sum of the individual metabolites showed an increase of 193% (Table 2).

Immunohistochemistry for tyrosine hydroxylase 12 weeks after prenatal MeOH exposure

It was also of interest to examine the integrity of the substantia nigra (SN) tyrosine hydroxylase immunoreactive neurons in the MeOH exposed mice, since the key feature of parkinsonian pathology is a loss of nigrostriatal neurons and TH. In the PBS mice, the SN shows high level of TH-immunoreactivity (Fig. 5, left). However, the quantity of TH-immunoreactivity was remarkably reduced in offspring prenatally exposed to MeOH, when compared to the PBS (control) groups due mainly to the reduction in the number of immunoreactive cells (Fig. 5). Some neurons in the MeOH section (Fig. 5, right) show TH-immunoreactivity extending into the proximal portion of dendritic and axonal processes (arrows), giving the appearance of a bipolar formation (black arrows). The double stripe appearance of these fibers may highlight labeling of the membranes and indicative of hypertrophied axonal processes, a likely compensation to the cellular loss.

Discussion

The brain undergoes growth and differentiation during prenatal and postnatal periods and this system is particularly susceptible to insults induced by drugs and toxicants (Ling et al., 2002; McCormack et al., 2002; Mellerick and Liu, 2004; Richardson et al., 2006; Muthian et al., 2010). In this study, methanol (MeOH) was administered at exposure periods in which mesencephalic TH cells are undergoing growth and differentiation in mouse embryos (DiPorzio et al., 1990). Previous studies have showed that methanol neurotoxicity resulted in apoptosis (Mellerick and Liu, 2004) and enhanced oxidative stress (Sandhir and Kaur, 2006). The aim of the present study was to determine whether prenatal exposure of mice to MeOH during the neurogenesis of the nigrostriatal (NS) dopamine (DA) neurons produced changes in NS markers that can be detected at 12 weeks in the adult offspring.

The first outcome shows that the weight of the 12 week old offspring was not affected following prenatal exposure to MeOH (Fig. 1). However, the mice that were prenatally exposed to MeOH demonstrated a slowness in movement since the total distance traveled and total number of movements were reduced (Fig. 2A; B). This could be due to slowness in executing the movement task since the time spent moving was the same as the control. Also, the deficit in movement was evident in the movement rate in the MeOH exposed offspring (Fig. 2C; D). It was also of interest that the 12 week old offspring also showed a reduction in balance and motor coordination by exhibiting a significantly higher number of footslips while traversing the beam (Fig. 3A). This latency in crossing the beam further points to a deficiency in movement (Fig. 3B). Several investigators corroborated our results by showing that MeOH and its metabolites can cause alterations in neurobehavioral functions (Stern et al., 1996; Aziz et al., 2002; Mellerick and Liu, 2003; Degitz et al., 2004). For instance, MeOH exposure resulted in a reduction of rota-rod performance and locomotor activity in mice (Sandhir and Kaur, 2006).

Since it is well known that behavioral deficits are correlated with NSDA damage in PD individuals, Western blot analysis of key enzymes was performed and showed that there was a reduction in both striatal tyrosine hydroxylase (TH) and L-aromatic amino acid decarboxylase (LAAD) (Fig. 4A; B). Tyrosine hydroxylase is the rate limiting enzyme step in the biosynthesis of dopamine (Nagatsu et al., 1964; Levitt et al., 1965) and impairments to nigrostriatal dopaminergic neurons have been correlated with decreases in both TH protein and dopamine levels (Ricarte et al., 1987; Charlton, 1997; Langston et al., 1999) and play a role in PD pathogenesis. Prenatal MeOH also increased α -synuclein levels in the striatum (Fig. 4F) but the findings did not reach statistical significance. Nevertheless this observation is important, primarily because the pathological hallmark of PD is the presence of α -synuclein-containing Lewy bodies, which are marked by fibrillar cytoplasmic inclusions that also contains ubiquitin and lipids (Spillantini et al., 1997, 1998; Gai et al., 2000; Perez et al., 2002; Quilty et al., 2006). Here, we show that prenatal MeOH exposure tended to produce an apparent α -synuclein elevation in some of the 12 week old offspring exposed to prenatal MeOH (Fig. 4E; F).

Dopamine and its metabolites were also measured in offspring prenatally exposed to MeOH or PBS (Table 1). The results show that DA levels were reduced in mice offspring prenatally exposed to MeOH while the metabolite levels remained unchanged (Table 1). Neurotransmission function is reflected by its turnover rate (Spencer et al., 1998). Accordingly, the increase in DA turnover suggests that an increase in DA neurotransmission occurred and may be compensatory for the reduction in DA and TH. The significant increase in 3-methoxytyramine (3-MT) is indicative of an increase in DA methylation and therefore utilization. Interestingly, as previously reported by our laboratory and others, the role of DA methylation in PD has been presented (Charlton and Way, 1978; Charlton and Mack, 1994; Shepherd et al., 2006). Whether the extent of TH and DA loss in the offspring is caused by NSDA cell damage is not known but is highly suspected since TH-immunoreactive cells were apparently reduced by MeOH (Fig. 5). Since, prenatal MeOH decreased TH and LAAD levels, it can be interpreted also as the reason for the observed loss of striatal DA. The studies of Aziz et al. (2002) showed that MeOH exposure during gestation and lactation significantly decreased DA level by 32% on post-natal day 45. Although a different dosage regime was utilized, this finding was consistent with our observation of a decrease in striatal DA levels in 12 week old offspring (Table 1). Furthermore, Jeganathan and Namasivayam (1987) found a 40–50% decrease in DA levels after administering a single dose of MeOH. Similarly, prenatal exposure to 10 mg/kg 1-methy-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP), a basal ganglia toxin, decreased striatal DA levels by 13% at 12 weeks, further substantiating the hypothesis that the early in life interaction of the NSDA with toxins or

other harmful interventions have the potential of sensitizing the affected individual to developing a PD-like phenotype that shows symptoms later in life (Muthian et al., 2010).

The cortex plays a critical role in executing motor functions and studies have shown the correlation between PD and motor cortex activity (Viaro et al., 2011). Previous studies have also showed that MeOH exposure in rodents can decrease cortical acetylcholinesterase (AChE), impair cognition and increase oxidative stress (Harris et al., 2004; Sandhir and Kaur, 2006). These results are in correlation with our study showing that 40 mg/kg MeOH decreased cortical DA level (Table 2). Similarly, DOPAC, HVA, and 3-MT levels were decreased. Studies have reported that MeOH and its metabolites can have an effect on the cortex by increasing oxidative stress thus increasing lipid peroxidation and free radical formation (Sandhir and Kaur, 2006). Taken together, the prenatal exposure to MeOH affects both the basal ganglia and cortical DA system, which could be seen as “primary measures” of the sensitization of the brain to further insults that may produce depression or dementia in a PD phenotype.

Also, the depletion of TH protein and DA levels was paralleled with a reduction in TH-immunoreactivity in the prenatal MeOH exposed offspring (Fig. 5). Our finding was similar to Shepherd et al. (2006), in which they reported a reduction in TH-immunoreactivity after paraquat administration in mice. So, MeOH can alter the biochemistry and neuronal integrity of the nigrostriatal dopamine neurons during development.

Primarily, MeOH is a protoxin whereby its effects are mediated through its metabolites formaldehyde and formic acid (Finkelstein and Vardi, 2002; Reddy et al., 2007). The toxicities of the aforementioned metabolites are well documented; they can be delivered to the deep tissues of the brain because of the unique solubility of MeOH, hence, the notable neurotoxic effects (Harris et al., 2004; Mellerick and Liu, 2004). Also, MeOH is endogenously produced through the carboxymethylation of proteins and the hydrolysis of the protein carboxyl methyl esters. The reaction is increased during the aging process (Lee et al., 2008). Formaldehyde, a MeOH metabolite, has been shown to be incorporated into DNA (Huang et al., 2001) and caused DNA damage, which can lead to cytotoxicity. Toxicity from formate has resulted in reduced ATP levels due to inhibition of mitochondrial electron transport which subjects the cells to oxidative stress (Eells et al., 1996, 2003; Harris et al., 2004). Overall, nigrostriatal DA neurons are particularly vulnerable to oxidative stress (Jones et al., 2000; Rabinovic et al., 2000); consequently, the MeOH exposed offspring with a NSDA impairment may be at risk for developing PD-like symptoms later in life.

The early exposure to an environmental toxicant can cause marked damage or even be fatal. So, the exposure of the pregnant individual to MeOH may result in harm to the brain that is determined by the vulnerability of a specific neuronal set. Permanent changes to differentiating NSDA neurons will occur if those neurons are the most vulnerable and the deficiencies will persist throughout development, causing these specific neurons to selectively succumb later in life. Consequently, this could lead to the development of PD later in life as aging induces physiological fragilities. If other neuronal groups are impaired during specific fetal exposure periods, they also will be affected, which will help to explain the pairing of other neurological degenerative disorders, such as Alzheimer's disease-type dementia or depression with PD.

Conclusion

The aim of this study was to determine if prenatal exposure to MeOH will cause subtle changes to the neuronal DA system that will render it vulnerable, producing a PD-like phenotype. Methanol was administered to pregnant mice during the period of neurogenesis

of NSDA neurons. The results showed that MeOH produced impairments to the NSDA system. Since MeOH affects the basal ganglia dopaminergic system that is impaired in PD, prenatal MeOH exposure may cause sub-threshold deficiencies that contribute to PD. Also, MeOH has been in the environment for millennia and its toxicity could be linked to PD, which was discovered in 1817 by James Parkinson. Therefore, in utero protective measure for the NSDA neurons may help to protect the brain from PD.

Acknowledgments

We would like to thank the Morphology Core Lab at Meharry Medical College which is supported in part by NIH grants U01NS041071, U54RR026140, U54CA091408 and S10RR0254970, for assistance with the immunohistochemical analysis.

This work was supported by NIH RO1NS041674, NIH R21NS049623, NIH 5U01NS041071, and NIH 5R25GMO059994.

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Fig. 1. Weights of offspring during lactation and after MeOH exposure during GD 8–12. The weights of the offspring were recorded weekly starting at postnatal day 3. The data show that there was no significant change in the weights of mice exposed to 40 mg/kg MeOH (open columns) during GD 8–12, when compared to controls (PBS; blocked columns). Student's t-test was used for statistical comparisons. The results are expressed as mean $\pm$ SEM. n=6 mice per group.

Fig. 2. Effects of prenatal exposure to 40 mg/kg MeOH on locomotor activity in mouse offspring. The total distance traveled (A), total number of movements (B) and movement time (C) were measured 12 weeks after prenatal MeOH exposure. The movement rate (D) was calculated as the ratio of total distance traveled to the movement time (TD/MT). Values are expressed as mean±SEM. of n=6 mice per group. Statistical analysis was done by using a Student's t-test with *p<0.05 and **p<0.01.

Fig. 3. Balance and motor performance in prenatal PBS and 40 mg/kg MeOH exposed offspring in a beam walking apparatus. Data show that MeOH increased the number of footslips in the offspring (A). The latency was also increased in the MeOH exposed offspring (B) but the finding did not reach statistical significance. Data are presented as mean±SEM of n=6 mice and analyzed using Student's t-test; **p<0.01.

Fig. 4. Tyrosine hydroxylase (TH), L-aromatic amino acid decarboxylase (LAAD) and α-synuclein (α-syn) protein expression in the striatum of adult male offspring exposed to PBS or methanol in utero. Prenatal MeOH exposure decreased striatal TH (A; B) and LAAD (C; D) levels by 47% and 57% respectively. There was a 48% increase in α-syn levels (E; F). Striatal levels of these proteins were determined by Western immunoblot at 12 weeks of age. Alpha tubulin was used to normalize both proteins respectively. Densitometric analyses are expressed as a ratio with reference to tubulin. Data represents mean ± SEM with n=6 offspring from different litters. Statistical significance is reported for the *p<0.05 and **p<0.01 levels compared to the PBS (control) group as determined by Student's *t*-test.

Fig. 5.

The figure shows the effects of PBS and MeOH exposure on tyrosine hydroxylase (TH) immunoreactivity (I) in the substantia nigra (SN) of 12 week old offspring exposed during gestation 8–12 to PBS or 40 mg/kg MeOH, by oral gavage to the dam. The population of TH-positive neurons was reduced in the MeOH pre-exposed offspring (left) as compared to the PBS pre-exposed offspring (right). Some neurons in the MeOH section (left) show TH-I extended into axonal processes (arrows) and with bipolar appearance (broken arrows). Representative of $n=3$ studies in which the images had a magnification of $100\times$.

Table 1

Effects of prenatal MeOH on striatal DA, DOPAC, HVA and 3MT levels (pmol/mg tissue) and turnover rate in 12 week old offspring^a.

Dopamine and its metabolites (pmol/mg)					
Treatment group	DA	DOPAC	HVA	3MT	DOPAC+HVA+3MT/DA
PBS (control)	71.03±5.30	8.70±1.65	6.85±0.60	5.00±0.40	0.29±0.01
40 mg/kg MeOH	48.63±10.50 *	7.45±2.33	5.83±0.94	6.40±1.04	0.40±0.00 **
Percent change	-31.55	-14.36	-14.89	+28.00	+37.93

^aTimed pregnant dams were administered either 40 mg/kg methanol (MeOH) or PBS during GD 8–12. At 12 weeks of age, male offspring were sacrificed and striata were isolated. Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3MT) levels were determined by HPLC with electrochemical detection. The percent changes are seen in third row. The exposure to prenatal methanol reduced striatal DA and increased its turnover. Data are presented as mean±SEM of n=6 mice.

Statistical significance is reported for the * p<0.05 and **p<0.01 levels when compared to the PBS (control) group as determined by Student's *t*-test.

Table 2

DA, DOPAC, HVA and 3MT levels (pmol/mg tissue) and DA turnover in the cerebral cortex 12 weeks after prenatal exposure to 40 mg/kg MeOH^a.

Dopamine and its metabolites (pmol/mg)					
Treatment group	DA	DOPAC	HVA	3MT	DOPAC+HVA+3MT/DA
PBS (control)	45.48±11.75	36.73±5.19	17.64±1.59	8.11±1.73	1.34±0.72
40 mg/kg MeOH	7.00±2.16 [*]	12.45±2.58 ^{**}	11.17±1.49	3.24±1.18 ^{**}	3.93±0.75 ^{**}
Percent change	−84.61	−66.10	−36.67	−60.05	+193.28

^aTimed pregnant dams were administered 40 mg/kg methanol during GD (8–12); at 12 weeks of age, male offspring was sacrificed and the cortex was isolated. Dopamine, DOPAC, HVA and 3MT levels were determined by HPLC with electrochemical detection. The percent changes are shown. Prenatal exposure to MeOH markedly reduced DA, DOPAC, HVA, and 3-MT and increased turnover of DA. Data are presented as mean ±SEM of n=6 mice from different litters.

Statistical significance is reported for the *P<0.05 and **P<0.01 levels when compared to the PBS (control) group as determined by Student's t-test.