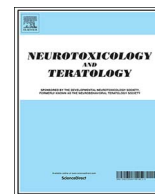




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Full length article

## Periconceptional folate deficiency leads to autism-like traits in Wistar rat offspring

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## ABSTRACT

**Background:** Folates in their role as key one carbon donors, are essential for two major pathways: the synthesis of DNA and RNA precursors and DNA methylation. A growing body of evidence from epidemiological studies indicates a possible association between nutritional and functional deficiency in folates and Autism Spectrum Disorders (ASD). However, there are no available behavioral animal studies on periconceptional one-carbon donor deficiency during gestation and the autistic phenotype.

**Objective:** The objective of this study was to determine if the periconceptional alteration of one-carbon metabolism induced with a folate deficient diet would affect the behaviour of rat offspring.

**Methods:** Female Wistar rats were divided in two groups: control (basal diet, in compliance with standards of regular laboratory diets), or exposed during one month before breeding until Gestational Day 15 to a modified diet with no added folic acid (0.2 mg/kg of food), reduced choline (750 mg/kg of food), and added 1% SST (a non-absorbable antibiotic used to inhibit folate synthesis by gut bacteria). We administered behavioral tests to offspring, i.e., open field (P20), social interactions (P25), marble burying (P30), elevated plus maze (P35), and prepulse inhibition of the acoustic startle reflex (sensorimotor gating) (P45). Blood homocysteine levels were used to confirm the deficit in one-carbon donors.

**Results:** Compared to controls, offspring with the periconceptional deficit in folate showed: (i) congenital body malformations; (ii) reduced social interactions, with a ~30% decrease in social sniffing behavior; (iii) reduced exploration of the open arm by 50% in the elevated plus maze test, indicating increased anxiety; (iv) a ~160% increased number of marbles buried, indicating repetitive behaviors; and (v) altered sensorimotor gating, with a global 50% decrease in startle inhibition.

**Conclusion:** Maternal periconceptional deficit in folate provokes alterations in the behavior of offspring relevant to the autistic-like phenotype.

## 1. Introduction

Autism Spectrum Disorders (ASD) are characterized by social and communication deficits, and by repetitive behavior and restricted interests. The prevalence of autism has exploded over the last 30 years and, according to the Center for Disease Control, it affected 1/68 children in 2016 (Christensen et al., 2016). This exponential increase in ASD suggests an interaction between unknown environmental/nutritional factors and genetic causes, because genetics alone cannot explain this increase. Two large independent studies reported a 40% reduction of autism risk in children from mothers who took periconceptional folic acid supplements above the recommended daily intake of 600 µg/day

(Schmidt et al., 2012; Surén et al., 2013), which suggests the potential role of one carbon donors and epigenetic methylation mechanisms in the development of the autistic phenotype. In the California study, this reduction of risk was 70% when both the mothers and their children were carriers of a specific but common genetic polymorphism in a key enzyme involved in first steps of folate metabolism, MTHFR (Methylenetetrahydrofolate reductase) (Schmidt et al., 2012).

Folates are vitamins, which can be of natural origin (from fruits and vegetables) or synthetic origin (folic acid). Mammals lack the ability to synthesize folates de novo and require preformed folates in the diet (Molloy, 2002). Folates in their role as one carbon donors are essential for two major pathways: (i) the synthesis of RNA and DNA precursors

**Abbreviations:** ASD, Autism Spectrum Disorders; CDC, Center for Disease Control and Prevention; G1, Gestational Day 1; MTHFR, methylenetetrahydrofolate reductase; P1, post-natal day 1; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SEM, standard error of the mean; SST, SuccinylSulfaThiazole

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and (ii) DNA methylation (Fox and Stover, 2008). DNA methylation is a major mechanism of epigenetic regulation, which is particularly important during early development (embryo and fetus) and which determines long term brain development (Geraghty et al., 2015). In human fetuses, maternal folate levels in pregnancy were highly positively correlated with mean 5mC content in fetal brain (Chang et al., 2011). Few studies report hypomethylation profiles in autistic subjects and their parents (Melnyk et al., 2012; James et al., 2010). Several small case-control studies have reported abnormalities in biochemical parameters reflecting low folate status in autistic children, such as decreased methionine, increased homocysteine, and low blood SAM:SAH (S-adenosylmethionine and S-adenosylhomocysteine) ratio (James et al., 2004; Naushad et al., 2013; Paşca et al., 2006).

Several animal studies have revealed an association between pre- and perinatal deficiencies in one-carbon donors, especially choline, and decreased cognitive performance, neurogenesis and neural plasticity in adulthood (Blusztajn and Mellott, 2012; El Hajj et al., 2014). To date there is no research on the development of an autism-like phenotype in animals in relation to periconceptional one-carbon donor deficiency. To our knowledge, two models of folate deficiency have been used for studies of cognitive performance. One pilot study with 1 dam per group with injections of folate Receptor Alpha Antibodies during Gestation (GD8) or post natal or weaning days (Sequeira et al., 2016) showed severe learning deficit and an excessive self-grooming behavior in exposed pups, but did not specifically test autistic traits. Another model of dietary induced folate deficiency during weaning also showed irreversibly decreased learning and memory, as well as increased self-grooming behavior, which is akin to a behavioral stereotypy (Berrocal-Zaragoza et al., 2014).

The objective of this study was to determine if periconceptional alteration of one-carbon metabolism induced with a folate deprived diet can affect the behaviour of rat offspring. Given that high homocysteine levels indicate a deficit of one-carbon donors needed for the transformation of homocysteine to methionine (Finkelstein, 1974), we measured homocysteine in the blood from dams after one month of a specific diet and before conception to control for folate status. Our hypothesis is that periconceptional folate deficiency in the dams provokes neurobehavioral alterations similar to autism-like phenotypes in the offspring. We therefore used behavioral tests which focus on ASD core symptoms and/or comorbidities: sociability and communication, locomotor activity and stereotypies, anxiety, and sensorimotor gating.

## 2. Methods

### 2.1. Animals husbandry and food

We obtained 12 female Wistar rats (250–290 g) from Charles River Laboratories (St. Constant, Québec, Canada) and 8 male Wistar rats (310–340 g). They were housed 6 per for females and 4 per for males in plastic cages with bedding and under regulated temperature ( $21 \pm 2^\circ\text{C}$ ) and humidity ( $50 \pm 10\%$ ), and a 12 h light/dark cycle (6 h–18 h). Food and water were provided *ad libitum*. All animals received care in compliance with the Guide to the Care and Use of Experimental Animals from the Canadian Council of Animal Care and the protocol was approved by our institutional animal research ethics committee.

As soon as the animals arrived at the animal facilities, they were randomly assigned to one or the two diets. All females were under the assigned diet for one month before breeding and this was maintained until Gestational Day 15 (G15), when all dams were fed Rodent Chow 5075, Charles River Laboratories, the usual diet for all laboratory rats in our facility. We changed the diet at GD15 in order to assure only a periconceptional deficit in folate, rather than a full length pregnancy deficit.

After one month of the assigned diet, a blood sample was taken from the saphenous vein of each female rat which were then placed in a new

cage with a male for breeding. Blood was immediately centrifuged at 5000 RPM for 20 min and the supernatant was aliquoted and stored at  $-80^\circ\text{C}$  until analysis for homocysteine.

We checked every morning for the presence of a vaginal plug in the cage, a sign of breeding during the night. When a plug was found, the female was placed in an individual cage and the day was considered as G1. A new female was introduced to the male until all females were bred. If there was no vaginal plug after 7 days in a row, the female was removed from the breeding cage and placed in an individual cage and the day was considered approximately as G3. However, none of these females (2 from control group and 1 from folate deficiency group) was found to be pregnant after being removed from the breeding cage in the absence of a vaginal plug and thus were excluded from the experiments.

The 4 dams in the control group and the 5 dams in the FD group which were actually pregnant were allowed to raise the entire litter, and dams and pups were kept in the same cage from birth until weaning at Post-natal day 21 (P21). After weaning, males and females were separated. For behavioral tests, we used 2 males (housed together after weaning) and 2 females (housed together after weaning) from each litter (16 pups were tested in the control group and 20 in the FD group). The number of rats is specified for each test, however, due to time and logistical constraints, we were unable to administer the longer behavioral tests on time (marble burying and prepulse inhibition) and thus a reduced number of animals was used in these tests. Dams and all untested siblings were euthanized at P21. Adult offspring were euthanized after behavioral testing at P50.

The 12 females were randomly assigned to 2 groups of 6 dams each: (i) control, fed with Basal Diet 5755 (folate 4.2 mg/kg; choline 1400 mg/kg) from TestDiet® until G15; (ii) peri-conceptional folate deficit (named FD group) fed with Diet 5BRC from TestDiet® until G15, which is similar to Basal Diet 5755 in all nutrients except for folic acid (0.2 mg/kg) and choline (750 mg/kg, which is a minimum daily intake for rodents), and the addition of SST 1%.

SuccinylSulfaThiazole (SST) is a non-absorbable antibiotic which prevents de novo synthesis of folate by gut bacteria in the rat; it is commonly used at this dose for the folate deficiency rat model (Fournier et al., 2002; Choi et al., 1998; Young-In et al., 2002; Young-In et al., 1994; Walzem and Clifford, 1988; Tagbo and Hill, 1977). We also used a diet with reduced amounts of choline because dietary choline is an indirect methyl group donor in one-carbon metabolism that could interfere with folate deficiency. We kept a minimum amount of choline in the diet in order to prevent the high teratogenicity induced by the dietary deficiency of methyl group donors.

### 2.2. Analysis of homocysteine levels

We used the Axis® Homocysteine Enzyme Immunoassay (EIA) kit for quantitative determination of total L-homocysteine in plasma from pre-conception blood in dams. For the analysis we used aliquots from 7 dams fed with standard diet and 7 dams from the FD group.

### 2.3. Behavioral testing

All behavioral tests were performed between 9 a.m. and 4 p.m. by the same person.

#### 2.3.1. Open Field ( $n = 16$ controls, $n = 20$ FD)

This test measures observable spontaneous motor activities. At P20, the rat was placed in the apparatus, a box with a 40 cm<sup>2</sup> base with a 40 lux light intensity and a video recorder placed above connected to a computer running ANY-Maze® software (Stoelting CO, USA). Each rat was initially placed in the same orientation and all trajectories were analysed during a 5 min session, including time of mobility and distance travelled overall, and specifically in the center of the box. The center of the box is less attractive for the animal because it feels more exposed, so altered trajectories in this specific area can reflect either

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