**De novo transgenerational inheritance of male rat hyperactivity by rotenone**

Masami Ishido

**Center for Environmental Risk & Health Research, National Institute for Environmental Studies, Tsukuba 305-8506, Japan**

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**ABSTRACT** — There is growing evidence of transgenerational effects of a single exposure to chemicals, whose mechanism is implicated to be epigenetic. However, it is largely unknown whether psychiatric diseases such as ADHD or autism caused by environmental chemicals might be transmitted. Rotenone (3 mg/kg), a dopaminergic toxin was orally exposed to Wistar male pups at 5-day old. Their spontaneous motor activity was higher 1.3 fold than that of control rats at 11 weeks of age. At 26 weeks of age, the hyperactive rat (F0) was mated with Wistar female rats. We established the two strains of such mating and found the spontaneous motor activity of the offspring (F1) were much higher 1.5~2.0 fold than those of both control offspring and the parents. Thus, in this study I show the rat hyperactivity caused by neonatal rotenone lesions was transmitted to next generation, indicating the *de novo* inheritance.

**Key words:** ADHD, Rat hyperactivity, Transgeneration, Epigenetics, Rotenone

**INTRODUCTION**

Attention deficit hyperactivity disorder (ADHD) is characterized by behavioral and cognitive symptoms such as hyperactivity, inattention, disorganization, and impulsivity (Goldman et al., 1998; Gozal and Molfese, 2005; Gordon and Mitchell 2005; Rappley, 2005). The etiology is considered to be multifactorial. Interaction of genetic and environmental risk factors would be affected in expression of the disorder. Genetic studies have shown the association of 7-repeat alleles of D4 receptor gene with the occurrence of ADHD (Franke et al., 2012; LaHoste et al., 1996). Many environmental factors seem to cluster around pregnancy and birth, including maternal smoking, alcohol consumption and stress during pregnancy.

The animal model for hyperactivity disorders was produced by Shaywitz et al. (1976), who demonstrated that rat pups treated with 6-hydroxydopamine at 5 days of age developed increased motor activity, leading to cognitive difficulties in shuttle-box learning between 2–4 weeks of age. Following their protocol, we have also demonstrated rat hyperactivity by administration of endocrine disrupting chemicals, such as bisphenol A (Ishido et al., 2004a, 2007, 2011), *p*-octylphenol (Masuo et al., 2004), nonylphenol (Masuo et al., 2004), dibutylphthalate (DBP; Ishido et al., 2005), dicyclohexylphthalate (DCHP; Ishido et al., 2004b), diethylhexylphthalate (DEHP; Masuo et al., 2004), and *p*-nitrotoluene (Ishido et al., 2004c; Ishido and Usu, 2017a). The results of our animal experiments have been supported by many other epidemiological studies (Kim et al., 2009; Cho et al., 2010; Yolton et al., 2011; Harley et al., 2013; Chopra et al., 2014; Park et al., 2015; Huang et al., 2015; Philippat et al., 2017; Engel et al., 2018; Ku et al., 2020).

It was shown that vinclozolin, an endocrine disruptor exerted the effects on male fertility and that the effects were transmitted to F4 generation even only when the original gestating mother (F0) of F1 generation received
a transient chemical treatment (Anway et al., 2005). It might be mediated through epigenetic phenomena.

Here, their report allowed us to examine if rat hyperactivity caused by environmental chemical, rotenone toxicity would be transgenerated.

**MATERIALS AND METHODS**

**Materials**

Rotenone was purchased from Sigma-Aldrich (Tokyo, Japan). Olive oil was from nakarai tesque Corp. (Kyoto, Japan).

**Animals and treatments with chemicals**

All animal experiments were carried out in strict accordance with the Experiment and Related Activities in Academic Research Institutions guidelines, under jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the National Institute for Environmental Studies (NIES), Japan. In addition, this study was carried out in compliance with the ARRIVE guidelines. Pregnant Wistar rats were obtained from Clea Japan (Tokyo, Japan). They were maintained in home cages and fed with a standard laboratory chow (MF diet, Oriental Yeast Corp., Tokyo, Japan) and distilled water ad libitum at 22°C on a light-dark cycle (12 hr/12 hr) for at least one week. All animal care procedures were in accordance with NIES guidelines. About 50 male pups were born from 10 pregnant rats and 5–7 pups were randomly housed. The male pups were selected to be 10–14 g body weight at 5 days of age. They were weaned at 3 weeks of age.

Rotenone was suspended in 30% (w/w) milk-oil solution which was composed of nonfat milk (Meiji Co., Tokyo, Japan) and olive oil, and 3 mg/kg of rotenone was orally administered into the pups at 5 days of age. Control rats were administered with vehicle (30 μL) alone.

**Mating**

Control or the treated male rats (F₀) were mated with females from different litters, respectively at 26–30 weeks of age (Fig. 1). The offspring (F₁) were obtained, and their spontaneous motor activity was measured by Supermex system as below. Two F₁ strains were established, and designated as epi5A and epi5B, respectively.

**Measurements of spontaneous motor activity**

To examine the behavioral effects of rotenone, we employed the Supermex system (Muromachi Kikai, Tokyo, Japan). A Supermex sensor head consists of paired infrared pyroelectric detectors that measure the radiated body heat of the animal. This system detects any object with a temperature at least 5°C higher than background within a cone-shaped area with a 6 m diameter and a 110° vertex. The sensor monitors motion in multiple zones of the cage through an array of Fresnel lenses placed above the cage and movement of the animal in the X, Y, and Z axis can be covered. A Supermex has the ability to analyze up to 64 channels with an optional instrument, an interface for data recording (DI-064W). Output of the sensor signals representing the magnitude of the rat’s movement is transmitted by an interface device to a personal computer and is digitally converted and processed by the CompACT AMS software.

Spontaneous motor activity of the rats was individually measured in a home cage. We measured the activity counted by this system for 15 min intervals for a period of 22 hr. Food and water were fully available *ad libitum* from the beginning of measurement and rats were never disturbed in any way.

**Statistics**

Statistical analyses were carried out using the Microsoft Excel 365 software (Tokyo, Japan). Total activity in the nocturnal phase was analyzed by Student’s t-test after ANOVA (analysis of variance).
RESULTS AND DISCUSSION

To examine whether rat hyperactivity elicited by rotenone would be de novo inheritance or not, we first created hyperactive rats (F0) by neonatal rotenone lesion. Rotenone (3 mg/kg) was orally exposed to 5-day old male pups and their spontaneous motor activity was measured. Figure 2A shows that rotenone significantly increased motor activity through the nocturnal phase of the light-dark cycle.

We then examined the transgeneration of rat hyperactivity. Male control or male hyperactive rats (F0) were mated with female control rats from different litters, respectively. The offspring (F1) were obtained. Two F1 strains were established, designated as epi5A (blue filled square) and epi5B (blue filled circles). Data were represented as an fold of the basal SMA of control rats (ctrl) at diurnal periods. B: Male hyperactive rats were mated with female control rats, resulting in hyperactive rats (F1), whose SMA was represented with that of F0 rats. There are two strains of F1 generation, designated as epi5A (blue filled square) and epi5B (blue filled circles). Data were represented as an fold of the basal SMA of control rats (ctrl) at diurnal periods. C: SMA during nighttime of all generations was integrated, as indicated. Data were represented as a percentage of those of control rats (ctrl), and indicated as mean ± s.e. *Significantly different from control rats (p < 0.05).

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Conflict of interest--- The authors declare that there is no conflict of interest.

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Rat hyperactivity by rotenone through transgeneration

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