Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.5, No.6, 195-202, 2018

The Japanese Society of Toxicology

Fundamental Toxicological Sciences
URL : http://www.fundtoxicolsci.org/index_e.html

Minireview

The temporal turning window for rat behavioral phenotypes by rotenone

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(Received October 15, 2018; Accepted October 27, 2018)

ABSTRACT — Availability of animal models of human neuronal disease has been contributing to reveal their etiology. Particularly, dopaminergic neurodegeneration has been shown by exposure of rats to chemicals, such as rotenone, corresponding to the rat models of attention deficit hyperactivity disorder (ADHD) and Parkinson's disease. A single chemical of rotenone causes two behavioral phenotypes, both of which are completely opposite; hyperactivity or hypoactivity. They were created by different timing of chemical exposure, neonatal periods or adulthood, suggesting that there would be a temporal turning point of two behavioral phenotypes. Therefore, we examine a turning point of these behavioral phenotypes by measuring the spontaneous motor activity of rotenone models. We estimate the turning window of both behavioral phenotypes would be around between three weeks and four weeks of age in the rat dopaminergic neurodegeneration. Gene set enrichment analysis extracts a cytokine network in both rat models.

Key words: Dopaminergic neurons, Rotenone, Behavioral phenotypes, ADHD, Parkinson's disease, Encephalic lethargica

INTRODUCTION

Hyperactivity among children was first described by von Economo (1931) in cases of encephalic lethargica. Hyperactivity, sleep disorders and antisocial personality disorder are all associated with this disease in childhood and Parkinsonism was observed in adult cases (von Economo, 1931). This suggests that the etiology of hyperactivity in children could involve the potentially irreversible degeneration of dopaminergic neurons since Parkinson disease is caused by the selective loss of dopaminergic neurons.

Currently, hyperactivity is associated with ADHD or autism. The etiology seems to be, in part, associated with dopaminergic tone. Dopamine transporter may be involved in the pathogenesis of ADHD, as methylphenidate, which increases the synaptic concentration of dopamine by blocking the dopamine transporters, has been used for the treatment of ADHD (Elia *et al.*, 1999). Genetic studies have associated certain alleles of the human D4 receptor gene with the occurrence of ADHD (LaHoste *et al.*, 1996).

Thus, although the molecular mechanism is unknown, the developmental deficit of several components of the dopaminergic system may underlie motor hyperactivity.

The early developmental deficit might be attributed to the disease in later life. Through detailed reconstructions of neonatal and medical histories of birth cohorts in the United Kingdom, Dr. David Barker (1938-2013) proposed the concept that parameters of fetal, infant, and childhood growth may be predictors of disease in later life (Bateson *et al*., 2004). The plausibility of extending the Barker hypothesis to encompass brain development and to explore the impacts of toxic chemicals on brain development was argued (Landrigan *et al.*, 2005). The expanded Barker's hypothesis proposed that the environmental origins of neurodegenerative disorders in later life might be early in life during windows of developmental vul-

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nerability. The vulnerability to environmental factors is dependent on the period of their exposure: *in utero* and in early postnatal life may be most sensitive. Therefore, children are victim of environmental violence and predictors of disease in later life. In the case of dopaminergic deficit in the development, one could assume that it might result in Parkinson's disease in the adulthood since the etiology of the disease is the selective loss of dopaminergic neurons in the substantia nigara pars compacts (BenMoyal and Soreq, 2006). As mentioned above, an animal model of Parkinson's disease has been developed by selective dopaminergic degeneration with neurotoxicants, pesticides, or endocrine-disrupting chemicals in adult animals. It is tempting to investigate what would happen in adult animals with developmental deficit in dopaminergic neurons; according to the hypothesis, early exposure to neurotoxic chemicals should reduce the number of dopaminergic neurons in critical areas of the brain to levels below those needed to sustain function in the face of the neuronal attrition associated with advance age.

Therefore, it is very important to determine the temporal turning period of behavioral phenotypes exerted by dopaminergic dysfunction .

MEASUREMENTS OF SPONTANEOUS MOTOR ACTIVITY FOR BEHAVIORAL PHENOTYPES

The pioneer work of the animal model for hyperactivity was carried out by Dr. Shaywitz *et al.* (1976), who demonstrated that rat pups treated with 6-hydroxydopamine (6-OHDA) via intracisternal administration at 5 days of age developed increased motor activity, leading to cognitive difficulties in shuttle-box learning between 2-4 weeks of age. These observations were strikingly similar to the clinical syndrome of minimal brain dysfunction, called ADHD at present, found in children. In 6-OHDA-treated rat pups, brain dopamine was depleted, suggesting that brain dopamine may be involved in the pathogenesis of the disorder.

Depletion of brain dopamine is also seen in the patients with Parkinson's disease. Parkinson's disease is a progressive neurodegenerative movement disorder (Parkinson, 1817; Mizuno *et al.*, 1998; Strange, 1992; BenMoyal and Soreq, 2006; Cannon and Greenamyre, 2013; Kalia and Lang, 2015). Clinically, most patients present with the cardinal symptoms of bradykinesia, resting tremor, rigidity, and postural instability. The animal model of Parkinson's disease was developed by Dr. Greenamyre's research group, who demonstrated that adult rats treated with rotenone, a pesticide developed tremor, rigidity, and postural instability (Betarbet *et al*., 2000; Panov *et al.*, 2005). The major symptoms of Parkinson's disease result from the profound and selective loss of dopaminergic neurons in the substantis nigra pars compacta (Langston *et al.*, 1983).

Thus, spontaneous motor activity of these two kinds of animal models could be quantitative index. We employed Supermex® system to measure spontaneous motor activity, as described previously (Masuo *et al.,* 1997, 2002, 2004a, 2004b, 2007; Masuo and Ishido, 2011; Ishido *et al*., 2002, 2004a, 2004b, 2004c, 2004d, 2005, 2007, 2011, 2017; Ishido and Shimaya, 2016). A Supermex® sensor head comprising paired infrared pyroelectric detectors was used to measure the radiated body heat of each animal. The system detected any object with a temperature at least 5°C higher than that of background within a cone-shaped area (6-m diameter. 110° vertex). Motion was monitored in several directions using an array of Fresnel lenses placed above the cage, so movement in the x-, y-, and z-axes could be determined. Activity was measured at 15-min intervals for 22-24 hr under a 12-hr light-dark cycle. Food and water were fully given at the beginning of counting and the rats were not disturbed during the assessment period. Measurements from 16 animals were recorded concurrently.

ROTENONE AND BEHAVIORAL PHENOTYPES

At least, it seems to be possible to produce rat hyperactivity disorders and rat Parkinson's disease by a single dopaminergic toxin. The opposite behavioral phenotypes could be exerted by different exposure timing of a single dopaminergic chemical.

Rotenone is derived from plants and commonly used as a pesticide (Ray, 1991). The structure of rotenone is shown in Fig.1. Toxicological characteristics are acute oral LD_{50} is 102 ± 12.6 mg/kg (for male rat) and 2 year oncogenity is negative in the rat. Rather, recent report shows anti-cancer property of rotenone in the rat (Heinz,

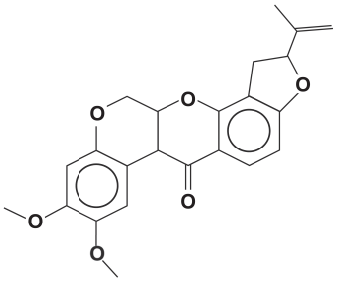

Fig. 1. A structure of rotenone.

et al., 2017). Rotenone is rapidly degraded in soil and water with a half-life of 1-3 days.

As mentioned above, rotenone has been shown to be a dopaminergic toxin, developing Parkinson's disease with adult rats (Betarbet *et al.*, 2000; Ishido and Shimaya, 2016) and we demonstrated that exposure of rat pups to rotenone causes hyperactivity during juvenile and adulthood due to dopaminergic lesions by rotenone (Ishido *et al*., 2017).

DEVELOPMENT OF DOPAMINERGIC NEURONS

Dopamine is a highly conserved neurotransmitter (Cooper *et al.*, 2003). The central and peripheral dopamine systems are involved in a number of complex functions such as regulation of blood pressure, movements, goal-directed behavior, cognition, attention and reward. From anatomical studies of the dopamine system, there are three major categories based on the length of the efferent dopamine fibers: ultrashort systems, intermediate-length systems, and long systems. The long systems are the long projections linking the ventral tegmental area (A10) and substantia nigra pars compacta (A9) dopamine cells with three principal sets of targets: the neostriatum, the limbic cortex (mesocortical projection), and other limbic structure (mesolimbic projection).

Dopamine synthesis originates from tyrosine, and its rate-limiting step is the conversion of L-dihydroxyphenylalanine(L-DOPA) by tyrosine hydroxylase. DOPA is subsequently converted to dopamine by L-aromatic amino acid decarboxylase at rate so rapid that DOPA levels in the brain are negligible. Endogenous mechanisms for regulating the rate of dopamine synthesis in dopamine neurons primarily involve modulation of tyrosine hydroxylase activity.

Ontogenetic studies in the rat showed that midbrain dopamine neurons appear between embryonic days E12-15, near the midbrain-hindbrain junction (rhombic isthmus) (Martinez and Simeone, 1999). These neurons begin to express tyrosine hydroxylase by E12.5, and then extensively migrate from the rhombic isthmus in a rostro-ventral direction to the ventral midbrain. Development of the ventral midbrain requires the orchestration of a number of genes, such as Engrailed1, 2, Pax2, Pax5, Wnt1, Sonic hedgehog, and Fgf8. Also, Ptx3 and Nurr1 are implicated in specification of the mesencephalic dopamine system. Nurr1 are required for induction of the tyrosine hydroxylase. A recent study identified two genes, Lmx1a and Msx1, as major upstream regulators of the dopaminergic neural subtype specification (Anderson *et al.*, 2006). Developmental processes such as differentiation and synaptogenesis are still incomplete in the 5-day old rat (Rice and Barone, 2000). Thus, it is highly expected that exposure during critical periods of neural development to environmental chemicals cause deficit in neural networks.

Dysfunction of dopamine systems has been associated to several neurodegenerative disorders. Tyrosine hydroxylase has been reported to be an oxidatively labile enzyme (Borges *et al.*, 2002). Animal models of dopaminergic neurodegeneration have been reported for Parkinson's disease through treatment with 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP) (Langston *et al.*, 1983) and some pesticides such as rotenone (Betarbet *et al*., 2000), maneb, and paraquat (Thiruchelvam *et al*., 2000), suggesting that oxidative stress is a primary mediator of the dopaminergic neurodegeneration.

Rotenone has a lipophilic nature (Fig. 1) and therefore crosses biological membranes easily, resulting in inhibiting mitochondrial complex I.

RAT HYPERACTIVITY BY ROTENONE

We examined the effect of a single dose of rotenone (3 mg/kg) on rat spontaneous motor activity through oral route at 5 days of age. Rotenone-treated rats were significantly more hyperactive than vehicle-treated rats in the nocturnal phase, but not in the diurnal phase at 8~11 weeks of age (Fig. 2A). It was notable that the hyperactivity of rotenone-treated rats in a novel environment was most prominent throughout nighttime. The rhythmic pattern was similar between groups, indicating that the day-night cycle was retained. The total activity of rotenone-treated rats for 12 hr in the dark was 1.3~1.4 times higher than that of control rats ($p < 0.05$, Student's t-test after ANOVA).

RAT HYPOACTIVITY BY ROTENONE

Since motor impairment is a feature of Parkinson's disease, we focused our initial efforts on evaluating spontaneous motor activity, as above. In the nocturnal phase, the rotenone-treated rats exhibited greater hypoactivity than vehicle-treated rats, as shown in Fig. 2B. In the diurnal phase, no significant differences in motor activity were detected between control and treated rats. The rhythmic pattern was similar between groups, indicating that the day-night cycle was retained. Hypoactivity was persistent in rotenone-treated rats during dark periods, with treated rats exhibiting an average of 49% less activity than vehicle-treated control rats.

Immunohistochemical analysis was then conducted for tyrosine hydroxylase, which is a rate-limiting enzyme for

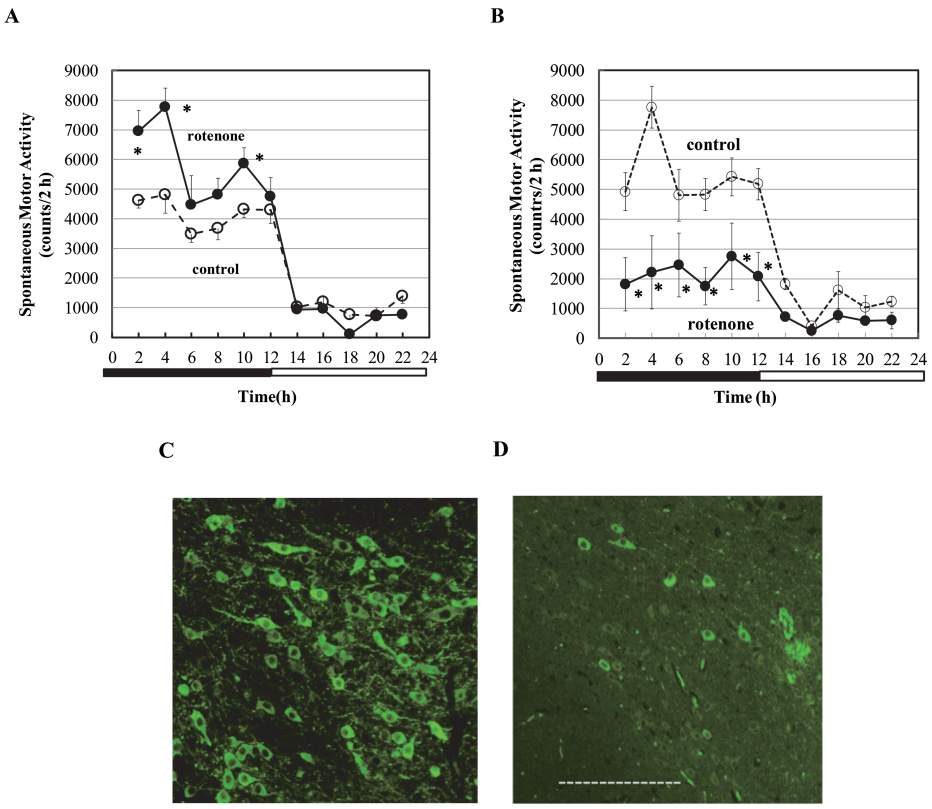

Fig. 2. Typical Patterns of behavioral traits of rat models for hyperactivity disorders (A) and Parkinson's disease (B). Following administration of 3 mg/kg of rotenone (*filled circle*) at 5 days (*A*) or 7 weeks (*B*) of age, spontaneous motor activity was measured, using the Supermex® system (Muromachi Kikai, Tokyo, Japan). Control rats were given olive oil alone (*open circle*). *Significantly different from control rats ($p < 0.05$, Student's t test after ANOVA). (*C*) and (*D*) Immunohistochemistry for tyrosine hydroxylase. Brain sections of control (*C*) or rotenone-treated rats (*D*) were stained with anti-tyrosine hydroxylase antibody. Scale bar = 200 μm. Adapted from ref. Ishido and Shimaya, 2016 and Ishido *et al.*, 2017.

catecholamine synthesis. Anti-tyrosine hydroxylase antibody provided strong staining in the substantia nigra of control rats (Fig. 2C). In contrast, staining was markedly reduced in rotenone-treated rats, indicating degeneration of dopaminergic neurons (Fig. 2D).

THE TEMPORAL TURNING POINT FOR RAT BEHAVIORAL PHENOTYPES PRODUCED BY ROTENONE

Figure 3 (*left*) shows the effects of the timing of rotenone exposure on rat spontaneous motor activity. Rats were exposed to 3 mg/kg rotenone on 5, 6, 14, or 21 days of age and their spontaneous motor activity was measured at 11 weeks of age. The highest spontaneous motor activity was significantly seen by rotenone exposure at 5 days of age, compared to other ages tested, suggesting the critical window for hyperactivity of the chemical toxicity.

Figure 3 (*center panel*) shows the effects of rotenone (3 mg/kg) exposure on 4 weeks of age on rat spontaneous motor activity. The chemical significantly decreased it about 12.5%. Furthermore, it was 49% decrement by exposure of 7-weeks old rats to 3 mg/kg rotenone (Fig. 3, *right*).

Thus, the turning period of behavioral phenotypes was exerted by rotenone around 3~4 weeks of age in the rat.

THE GENE EXPRESSION PROFILES OF RAT BEHAVIORAL PHENOTYPES

Since gene expression profiling is a basic approach to reveal the molecular alteration of behavior, we first investigated the cellular effects of rotenone on gene expression profiling of hyperactive rats, using the Affymetrix GeneChip Rat Genome 230 2.0 Array. Gene expression levels were calculated as the ratio of expression level in the

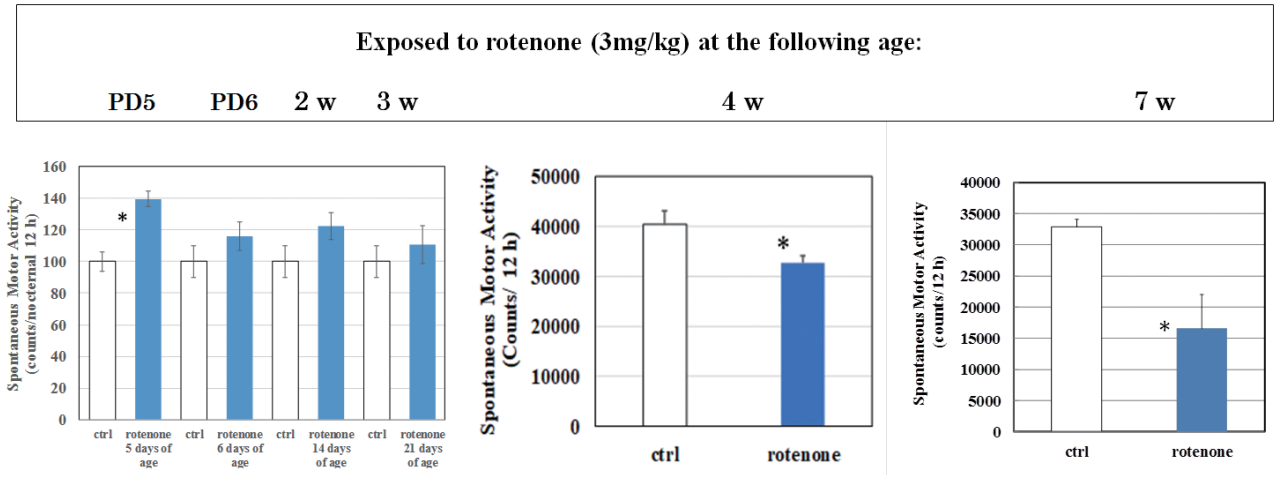

Fig. 3. A temporal turning point of two behavioral phenotypes by rotenone in the rat. Rotenone (3 mg/kg/day; *blue bar*) was exposed at indicated periods such as 5, 6, 14, 21 days of age (*left panel*), 4 weeks of age (*center panel*), and 7 weeks of age (*right panel*). Control rats were given olive oil alone (*white bar*). Then, their spontaneous motor activity was measured, using Supermex® system, as Fig. 2. Spontaneous motor activity during nighttime (12 hr) are indicated as mean ± S.E. (n = 5). Asterisks indicate significantly different from control rats ($p < 0.05$). PD; postnatal days: w; weeks: ctrl; control. Adapted from ref. Ishido and Shimaya, 2016 and Ishido *et al*., 2017.

Table 1. Typical gene expression in the midbrain of a rotenone model of hyperactivity disorders.

	Gene name	Expression ratio (log_2 ratio)
Increment	Tyrosine-protein kinase B	5.7
	Galectin-related inter-fiber protein	3.2
	Troponin T	2.9
	ATP binding cassett	2.4
	Dyein heavy chain	2.0
	Echinoderm microtubule protein like4	1.9
	Protein kinase C delta	1.4
	Leukocyte immunoglobulin-like receptor	1.2
	Dehydrogenase/reductase member7	1.0
Decrement	Chemokine receptor 3	-5.3
	Insulin-like growth factor binding protein 5	-3.6
	Tenascin N	-3.2
	sulfotransferase	-3.1
	p53-like inducible protein	-2.7
	WNT1 inducible protein	-2.6
	Sarcospan	-1.5
	Anaphase promoting complex subunit1	-1.4
	Heat shock protein 70	-1.3
	Calcitonin receptor-like	-1.2
	RING finger protein Abhydrolase domain containing 6	-1
	Kuppel-like factor2	-1

Adapted from ref. Ishido *et al*., 2017.

exposed rat brain to that in the control rat brain. Table 1 lists the typical changes of levels in gene expression of molecules in the midbrain at 11 weeks of age (Ishido *et al*., 2017). It is noteworthy that relatively many genes related to apoptosis/cell cycle were altered in their expression; Baz1b, Prkcd, Dhrs7, Rnf138, Anapc1, Ssn, Ste, and Igfbp5. It is also notable that the molecules involved in regulation of ATPase activity were altered in gene expression; Tnnt2, Abcg5, and Dnah7.

To compare the transcriptome of rat hyperactivity dis-

Table 2. Gene expression in the midbrain of rotenone model of Parkinson's disease.

	Gene name	Expression ratio (log_2)
Increment	RT1 class Ib, Ia locus Aw2	3.7
	Sulfotransferase family 1A	2.9
	HMG-CoA synthase	2.4
	Peroxisomal membrane protein 4	2.3
	Glycerol-3-phosphate dehydrogenase 1	2.2
	Glucocorticoid regulated kinase	2.2
	Par-6 partitioning defective 6 homolog	1.8
	Lipocalin 7	1.7
	Phospholipase A2	1.7
	Cyclin-dependent kinase inhibitor	1.6
	Connective tissue growth factor	1.6
Decrement	Beta-glo	-2.2
	Hemoglobin	-2.1
	Aminolevulinic acid synthase	-1.7
	Amylase 1	-1.6
	Tenascin C	-1.5
	Complement component 3	-1.5

Adapted from ref. Ishido and Shimaya, 2016.

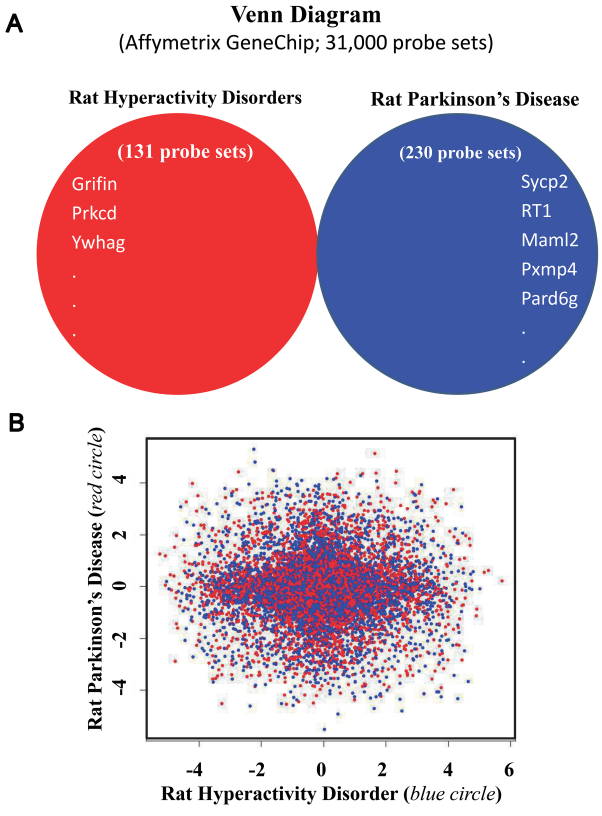

Fig. 4. Venn diagram *(A)* and regression analysis *(B)* of transcriptomes of rat hyperactivity disorders and Parkinson's disease. Note that there are no overlapping genes between two kinds of rat models as indicated.

orders in this study, we created the rotenone model of Parkinson's disease (Greene *et al*., 2010; Greene, 2012; Ishido and Shimaya, 2016). Total RNA was isolated from the midbrain of 11-week-old rats and then transcribed to cDNA. Table 2 summarizes changes in gene expression of major molecules in the midbrain. Notably, MHC molecules exhibited the largest increase in response to rotenone (Huh *et al*., 2000). Our gene set analysis also suggests that connective tissue growth factor might be a mediator of events driven by TGF-β1, which is consistent with a previous finding (Grotendorst, 1997). Lipocalin 7 was also reported to be sensitive to TGFβ1 (Brown *et al*., 2010). Notably, Richter *et al*. (2009) reported that gene expression of hemoglobin was down-regulated by the mitochondrial inhibitor rotenone, which is consistent with our present data. Our transcriptome analysis also revealed that other mitochondrial proteins such as HMG-CoA synthase and glycerol-3-phosphate dehydrogenase 1 appear to be affected by rotenone exposure (Table 2).

Figure 4A shows the Venn diagram that was no overlapping between transcriptomes in two rat dopaminergic degeneration models (Hyperactivity disorders vs Parkinson's disease). Figure 4B also shows no regression between both. However, gene set enrichment analysis extracts a cytokine network such as IL6 and TNFαin hyperactivity disorders model or IL-10, IL-6, IL-1B, TNFα, and TGF-β1 in Parkinson's disease model, respectively (Fig. 5).

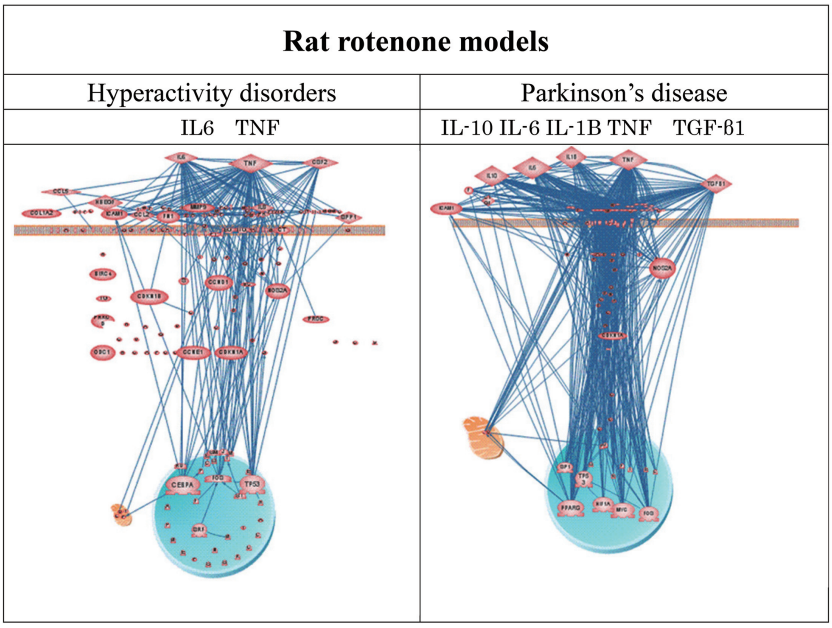

Fig. 5. Schematic representation of gene network deduced from transcriptome of midbrain of rotenone-treated rats.DNA array data of rat hyperactivity disorders (*left*) and Parkinson's disease (*right*) were analyzed using Pathway Studio software (Ariadne Genomics, MD, USA).

CONCLUSION

The spontaneous motor activity was measured with a Supermex® system since hyperactivity is a central feature of ADHD and motor impairment is that of Parkinson's disease. The turning point of both behavioral phenotypes would be between three weeks and four weeks of age in the rat dopaminergic neurodegeneration. Estimation of the turning window for behavioral phenotypes will facilitate to evaluate the DOHaD hypothesis on dopaminergic dysfunction.

ACKNOWLEDGMENT

This work was supported by NIES grant and by KAKENHI.

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

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