Studies on Oscillatory Potentials in Electroretinogram as a Marker of Visual Abnormalities in Vitamin A Deficiency.

A Dissertation Submitted to
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Abbreviation

APCI	Atmospheric Pressure Chemical Ionization
AT-	all trans-
Bas	basophil
BN rat	Brown Norway rat
CNS	central nervous system
D1	dopamine 1 receptor
D2	dopamine 2 receptor
Eos	eosinophil
ERG	electroretinogram
H&E	hematoxylin and eosin
Hct	hematocrit
Hgb	hemoglobin
LUC	large unstained cell
Lym	lymphocyte
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume

Mon	monocyte
MRM	multiple reaction monitoring
Neu	neutrophil
OPs	oscillatory potentials
PDE	phosphodiesterase
Plt	platelet
RAR	retinoic acid receptor
RAREs	retinoic acid response elements
RBC	red blood cell
Ret	reticulocyte
RXR	retinoid X receptor
VAD	vitamin A deficiency
Vit A	vitamin A
Vit A (–) diet	AIN93G-Vitamin A (–)
Vit A (+) diet	AIN93G
WBC	white blood cell

Contents

CHAPTER I

Preface

Vitamin A which is a fat-soluble vitamin is also called retinoid and has three active forms (retinal, retinol, and retinoic acid) in the body. The active forms of retinoid plays an essential role in numerous biological processes, including vision, cellular proliferation, differentiation, organ development, and immunity (1, 2). Therefore, loss of retinoids, known as vitamin A deficiency (VAD) disease, induces a variety of symptoms. Visual impairment is one of the typical symptoms of VAD disease. In the eye, loss of retinoid mainly influences development or maintenance of the retina (2). Retinoids delivered to the retina, as all-trans (AT-) retinol, enter the outer segments of the photoreceptor cells where it is transformed to 11-cis retinal, known as chromophore, and combined with the opsin to form the light sensitive rhodopsin (3). The 11-cis retinal is isomerized to AT-retinal by absorption of a photon and released from the rhodopsin in order to activate downstream molecules, such as transducin and phosphodiesterase (PDE), related to photo-transduction (4). Because this is the first reaction in phototransduction, loss of retinoids in retina induces photoreceptor cell

dysfunctions and causes visual abnormalities consisting of prolonged dark adaptation or night blindness with disintegration of the outer segments of photoreceptor cells (5). Therefore, it is very important for patients with VAD to understand functions of photoreceptor cells. In order to evaluate the function of photoreceptor cells and diagnose visual impairment by VAD, electroretinogram (ERG) recording is both clinically and non-clinically useful. Although the retina is constituted from several cell layers remaining each different function, ERG recording enables to evaluate function of each retinal cell layer, separately (Fig I-1). For example, the function of photoreceptor cells can be assessed by measuring amplitude or implicit time of peak of ERG a-wave (3). In human and animals with VAD, ERG is reported to show that a-wave reduces depending upon the stage of the disease (3, 5). As above described, effects of VAD on the function, histology, or ERG a-wave have been well investigated and clarified to date. On the other hand, there is a symptom which could not be explained only by photoreceptor dysfunctions in VAD disease. Previous studies have reported some patients with VAD resulting from intestinal diseases sometimes show reduced contrast sensitivity (6). Contrast sensitivity is the visual ability to see objects that may not be outlined clearly or that do not stand out from the background (7). Reduced contrast sensitivity is also noted in patients with diabetes, Parkinson disease,

or cataract (8, 9) and may cause decreased visual function in spite of normal visual acuity. Previous studies have demonstrated that ERG in patients with diabetes shows changes in oscillatory potentials (OPs) correlating with reduced contrast sensitivity (8). OPs, one of the ERG components, is reflecting activities of inner retinal layer cells including amacrine and ganglion cells (3), shown as Fig I-1. The inner retinal layer cells function in visual transduction between outer retinal layer cells to optic nerve through some neurotransmitters including dopamine, gamma-aminobutyric acid (GABA), glycine, acetyl choline, and so on (10). Recently, Gastinger MJ et al. reported that cholinergic and dopaminergic amacrine cells are lost during the early stages of retinal neuropathy in diabetic mouse model (11). This report suggests amacrine cell dysfunctions are implicated in reduced contrast sensitivity. However, there is no article explaining relationships between VAD disease and amacrine cell function or OPs.

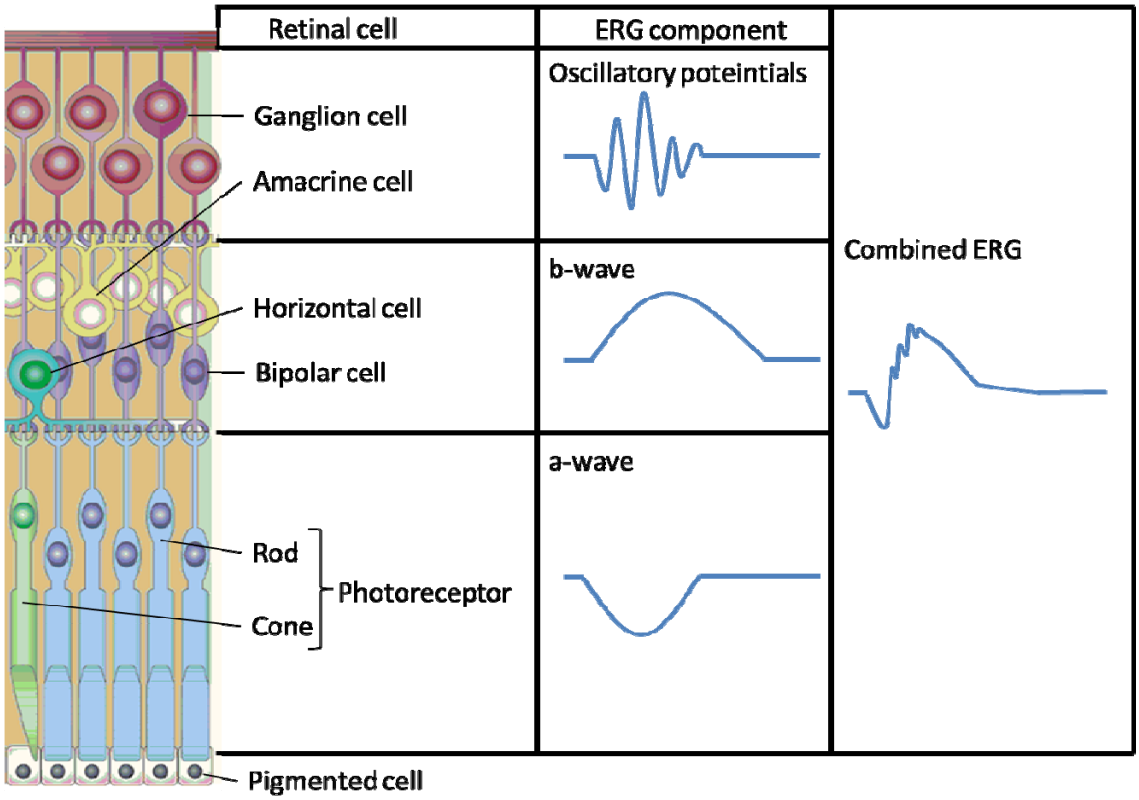
On the other hand, retinoic acid receptor (RAR) and retinoid X receptor (RXR) which mediate effects of active form of retinoids are known to express in inner retinal layer cells including amacrine cells (12, 13). AT-retinoic acid or 9-cis retinoic acid which are active forms of retinoid regulate dopamine pathways in the central nervous systems (CNS) by binding to these receptors (14). Dopamine receptors have been reported to

express in retinal cells including amacrine cells (15, 16). These suggest that retinoids have something rolls in visual transduction of not only photoreceptor but also the amacrine cells possible through dopamine pathways. Therefore, I estimated that loss of retinoid may influence the function of amacrine cells as well as photoreceptor cells and induce reduced contrast sensitivity with changes in OPs in ERG recording.

In the present study, in order to investigate the effects of VAD on amacrine cell function, I firstly produced a VAD model by feeding to rats vitamin A deficient (Vit A (-)) diet (CHAPTER II). Subsequently, retinal functions of amacrine cells as well as photoreceptor cells were electro-physiologically evaluated by ERG recording. Then, I also investigated the involvement of dopamine pathway in retinal function or visual transduction using the VAD model (CHAPTER III).

Figures

Figure. I-1 Diagram of retinal cell layer and ERG components reflecting functions or activities or each cells (Modified from Nature Reviews Neuroscience 2, 333-342 (May 2001)).



When light enters to retina, the light energy changes shape of retinal (from 11-cis retinal to AT-retinal) binding to rhodopsin. This change in shape begins a cascade of events that result in a change in the voltage across the membrane of the photoreceptor (hyperpolarization). Thus, photoreceptor cells become negatively charged when stimulated by light, and its function is exhibited by negative wave as a-wave in ERG. Once the photoreceptor cell has transduced the light to the change in membrane

potential, it then conveys this by its synapse onto a bipolar cell. The bipolar cells receive information from photoreceptors, and their axons transmit information to the amacrine or ganglion cells. Under dark adaptation, the photoreceptor cell stimulates depolarizing bipolar cells, as called ON bipolar cell, which releases K^+. The K^+ is received by Muller cells which across all layer of the retina, and course from outer side to inner side of the retina. Therefore, function of bipolar cells is reflected by a positive wave as b-wave in ERG. OPs consisting of approximately four wavelets have physiologic origin that differs from the a- and b-waves. Activity of inhibitory feedback circuits in the inner retinal layer including amacrine cells as the origin of OPs has been proposed (3, 17).

CHAPTER II

Establishment of Rat Model with Early Vitamin A deficiency Disease and Notification of Dysfunction of Inner Retinal Layer Cells.

1. SUMMARY

In the present study, rat model with early VAD disease was produced in order to investigate the visual impairments by loss of retinoid.

VAD state was produced by feeding a Vit A deficient diet to Brown Norway rats for 10 weeks. The effects of VAD were analyzed by evaluating the retinoid levels in plasma and retina. Retina was investigated by ophthalmological and histopathological examination at Week 10 or after a 5 week recovery period. Other examinations including clinical observation, body weights, food consumption, and hematology were also conducted.

VAD was detected first as a decrease in plasma all-trans retinol at Week 6. A reduced body weight gain with slight decrease in food intake, peri-papillary opacification indicative of papilledema without histopathologic changes were also

observed, known as early symptoms of VAD. After 5 weeks of recovery, body weight gain and food intake returned to control levels.

The findings observed in the present study are known as early symptoms of VAD. Therefore, the present VAD rat model without severe physical condition was considered to be appropriate to detect primary effects of VAD on retinal function.

2. INTRODUCTION

The Effects of VAD on retinal function and general condition in rats have been reported by several studies. Dowling JE et al. has reported that a small a-wave and a slight reduction of b-wave in ERG recording are induced by feeding albino rats a Vit A deficient diet for 8 weeks (5). In that study, the VAD rats showed no measurable retinoid in the blood and decreased body weight gain from approximately 4 to 5 weeks after initiation of feeding Vit A deficient diet. By the beginning of the 8 week, body weight loss, xerophthalmia, xerosis, and keratomalacia were observed. The other research has explained that the body weight gain is a sensitive indicator of Vit A status and serum Vit A levels below 20 ng/mL signify an adequate stage of deficiency to conduct the experiment (17). Although it is necessary to provide low retinoid level, progressive stage of VAD may influence general conditions in rats and induces secondary changes in retinal functions by poor physical conditions. Accordingly, monitoring general condition including body weights was very important to avoid occurrence of severe physical condition and to make an appropriate VAD model for evaluation the primary effects of VAD on retinal function. Therefore, I conducted general observation including clinical observation and measurement of body weights, food consumption, and plasma AT-retinol, and hematology. Ophthalmologic

examination was also done before notification of body weight loss or decline in plasma AT-retinol. Reversibility of these parameters was also evaluated after the 5 week recovery period.

3. MATERIAL AND METHODS

3.1. Animals

Thirty six male Brown Norway (BN) rats were purchased at 21 days of age from Charles River Laboratories International, Inc. Both eyes of all rats were checked by slit lamp and binocular indirect ophthalmoscope at pretreatment period. Rats which had no abnormal findings in both eyes were selected and divided into VAD and control groups. A diet with or without Vit A was fed to the control and the VAD group, respectively. An AIN93G, Vit A (+) diet, or an AIN93G-Vitamin A (–), Vit A (–) diet, were used. Both feed diets were obtained from Oriental Bio service Kanto. Compositions of the diets were showed in Table II-1, II-2, and II-3. Each animal was housed individually in a stainless steel cage, in a clean air room with a 12-hour light/dark cycle. The room had controlled illumination (less than 100 lux), temperature (23 to 24 °C), and humidity (53% to 71%). This study was performed in compliance with Laboratory Animal Policy at Eisai, and was approved by the Laboratory Animal Care and Use Committee at Eisai Co., Ltd. All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

3.2. Study design

The first week of feeding the Vit A (–) diet was defined as Week 1. To make VAD rats,

the Vit A (–) diet was fed to 18 male BN rats for 10 weeks. Subsequently, Vit A (+) diet was fed for 5 weeks (Week 11 to 15) in order to examine recovery from VAD. The Vit A (+) diet was given to 18 males in a control group for 15 weeks. In both VAD and control groups, each 12 main study and 6 recovery rats were euthanized at Weeks 10 and 15, respectively, by exsanguination from the abdominal aorta under isoflurane anesthesia, for sampling of eyes. After euthanasia, the retina was collected. Euthanasia to collection of the retina was carried out under dim red light. All animals were weighed weekly. Blood was collected from the jugular vein of each of 12 rats (euthanized at Week 10) at Weeks 6, 8, and 10 to measure plasma concentration of AT-retinol. Prior to euthanasia, ophthalmologic examination was performed on all surviving rats. At Week 10, each of 6 right retinas in both VAD and control groups were used for the measurement of retinoids. The retinal retinoids were also measured using each of 6 right retinas in both groups at Week 15. Each of 6 left eyes in both groups was applied on histopathologic examination.

3.3. General observations

All surviving animals were observed for clinical signs at least once a day and weighed weekly throughout experimental period. Amount of residual food was also recorded once a week.

3.4. Hematology

At necropsy, blood samples were collected from the jugular vein under isoflurane anesthesia using EDTA-2K as anticoagulant. The following parameters were examined.

Parameter (Abbreviation)	**Method**
Red blood cell (RBC)	Laser flow cytometric method[a]
Hematocrit (Hct)	Laser flow cytometric method[a]
Hemoglobin (Hgb)	Modified cyanmethemoglobin method[a]
Mean corpuscular hemoglobin (MCH)	Laser flow cytometric method[a]
Mean corpuscular volume (MCV)	Laser flow cytometric method[a]
Mean corpuscular hemoglobin concentration (MCHC)	Laser flow cytometric method[a]
White blood cell (WBC)	Laser flow cytometric method[a]
Differential WBC* *Basophil (Bas), Eosinophil (Eos), Lymphocyte (Lym), Monocyte (Mon), Neutrophil (Neu), and Other (Large unstained cell [LUC])	Peroxidase method and Laser flow cytometric method[a]
Platelet (Plt)	Laser flow cytometric method[a]
Reticulocyte (Ret)	Laser flow cytometric method[a]

a: ADVIA 120 Hematology System, Siemens Health Care Diagnostics

3.5. Retinoid determinations in Plasma and Retina

Reagents and standard derivatives

HPLC-grade solvents and reagents, acetonitrile, methanol, propanol, and formic acid were purchased from Wako Pure Chemicals (Osaka, Japan). Ultra-pure water was prepared by using Millipore Milli-Q TOC system (Billerica, MA, US). The standard

reagent of retinyl acetate was obtained from Cica reagent (Tokyo, Japan). Internal standard solutions (1 mg/mL) were prepared in acetonitrile. To prepare working sample solutions for LC-MS, retinyl acetate stock solution was diluted to a final concentration of 5.0 μg/mL.

Liquid chromatography/mass spectrometric analysis

An Applied Biosystems AB5000 mass spectrometer (Foster City, CA, US) with a SHIMADZU prominence UFLC (Kyoto, Japan) was used as the LC-APCI-MS system. The Waters Shield RP18 (2.0 x 150 mm) (Milford, MA, US) with Phenomenex security guard (C18, 2.0 x 4.0 mm) (Torrance, CA, US) was selected as the stationary phase. The composition of mobile phase A was water/acetonitrile/formic acid (95:5:0.1) and mobile phase B was propanol/acetonitrile (80:20). Linear gradients of mobile phase B were as follows: 0 min-1%, 0.5 min-1%, 1.5 min-25%, 30 min-99 %, 33 min-99%, 33.1 min-1%, 40 min-stop. The flow rate of the mobile phase was 250 μL/min with 450 μL/min of post-column addition of Methanol. AB5000 mass spectrometer was used in Atmospheric Pressure Chemical Ionization (APCI) - multiple reaction monitoring (MRM) mode. Each condition of MRM channel is listed below.

Retinoids	Q1 (Da)	Q2 (Da)	Dwell time (msec)	DP (volts)	CE (volts)	CXP (volts)
Retinol (retinol esters)	269.2	93.2	150	66	27	12
Retinal	285.1	161.3	150	71	15	18
Retinoic acid	301.2	123.3	150	66	25	18

CE: Collision Energy, CXP: Collision Cell Exit Potential, DP: Declustering Potential

Most of the sample pretreatment process was done under dim red light. For the plasma sample pretreatment, 50 μL of plasma samples were moved to shaded brown tubes (1.5 mL) and mixed with 190 μL of acetonitrile and 10 μL of 5.0 μg/mL retinyl acetate in acetonitrile. Mixed solutions were centrifuged 9500 × g for 20 min. The supernatant was moved to a plastic vial. For the tissue sample pretreatment, retina was homogenized in 2.0 mL of water/acetonitrile (25:75). A hundred μL of 5.0 μg/mL retinyl acetate in acetonitrile was added to 2.0 mL of tissue homogenates. Two hundred and fifty uL of samples were moved to shaded brown tubes (1.5 mL). Mixed solutions were then centrifuged at 8600 × g for 20 min. Supernatants were moved to shaded plastic vials.

The amount of plasma retinoid was calculated based on the amount of retinyl acetate. X-cis retinal includes 11- or 13-cis retinal in this study, because it was technically difficult to measure the retinal, separately.

3.6. Ophthalmologic examination

The ophthalmologic examinations with slit lamp or binocular indirect ophthalmoscope were performed on both eyes of all animals at pretreatment period and Weeks 10 and 15. Before observation, the pupils were dilated by topical application of 0.5% tropicamide and 0.5% phenylephrine hydrochloride (Mydrin®-P, Santen Pharmaceutical Co., Ltd., Osaka, Japan). Fundus was photographed by a Kowa RC-2 fundus camera (Kowa, Tokyo, Japan).

3.7. Histopathologic examination

Each of 6 left eyes in both control and VAD groups was preserved in glutaraldehyde-formaldehyde and processed to slide for microscopic examination of paraffin embedded sections stained with hematoxylin and eosin (H&E) at Weeks 10 and 15.

3.8. Statistical analysis

Student's t-test were repeated for comparison of the body weights, food consumption, and concentration of plasma retinoid. Concentration of retinal retinoids was analyzed

with Student's t-test. In each analysis, differences were considered to be significant when $P < 0.05$.

4. RESULTS

4.1. General observations

Body weights and food consumption within the study period are shown in Figure II-1 and II-2. Significantly lower body weights with slight decrease in food consumption were observed from Week 8 in VAD rats. During the recovery period, body weights of VAD rats increased with similar growth rate compared to the control group. However, the difference in body weights between the control and VAD rats at Week 10 (44.5 g) was maintained at Week 15 (55.3 g). Food consumption was comparable to control levels during recovery periods. At clinical observation, there was no adverse clinical sings throughout the experimental period.

4.2. The concentration of plasma all trans-retinol

Concentration of plasma AT-retinol is shown in Figure II-3. The plasma AT-retinol levels in the control group were stable throughout the experimental period of 6 to 10 weeks with the average concentration of about 800 ng/mL (minimal–maximal concentrations: 576–1012 ng/mL). At Week 6, in the VAD group, the average concentration of plasma AT-retinol decreased to 393 ng/mL (min–max: 261–516 ng/mL). The average plasma concentrations of AT-retinol in VAD rats decreased further to 100 ng/mL (min–max: 57–161 ng/mL) and 36 ng/mL (min–max: 30–44 ng/mL) at Week 8 and 10, respectively.

4.3. Retinal retinoids

The levels of all measurable retinal retinoids were significantly lower in VAD rats than those of control rats at Week 10 (Figure II-4 (a)). AT-retinol, AT-retinal, and AT-retinoic acid in VAD group decreased by about 50% or less. Meanwhile, 9-cis retinol and retinal decreased by 85% and 54%, respectively. X-cis retinal containing 11- and 13-cis retinal decreased by 46%. These changes have almost returned to control levels at Week 15 (after 5 week recovery period) (Figure II-4 (b)).

4.4. Hematology

The results of hematology at Week 10 and 15 were summarized in Table II-4. There were no changes in hematologic parameters.

4.5. Ophthalmologic examination

Binocular indirect ophthalmoscope observation of the fundus revealed peri-papillary opacification, and was found in all VAD rats at Week 10 (Table II-5 and Figure II-5). At Week 15, the region of opacification tended to become smaller, but did not disappear. There were no other VAD related changes in anterior and posterior chambers and optic media during the experimental period.

4.6. Histopathologic examination

Each of the 6 left eyes of control and VAD rats were histopathologically observed at Weeks 10 and 15 under H&E staining. There were no findings in the retina including

photoreceptor cells, inner retinal layer cells, and papilla.

5. DISCUSSION

In VAD group, feeding a Vit A deficient diet to rats was stopped at Week 10, because reduced body weight gain which is thought as a good marker for VAD state in rats (17) was observed with slight decrease in food consumption in VAD rats at Weeks 8 to 10. At Week 10, retinoid level in plasma and retina also significantly decreased. For these reasons, Vit A deficient period for 10 weeks is considered appropriate to induce low VAD state in BN rats. Also, peri-papillary opacification was noted in all VAD rats at ophthalmologic examination. This finding is considered to be one of the properties of papilledema (18). In VAD animals, occurrence of papilledema is reported as a result of increased cerebrospinal fluid pressure (19). These results indicate that VAD disease was certainly induced in BN rats given Vit A (-) diet for 10 weeks and a VAD model was successfully established in the present study.

The VAD model is considered to be with no severe physical condition, because there was not body weight loss, adverse VAD-related clinical sings, or changes in hematologic parameters throughout the experimental period. In addition, there were not histopathologic changes in the retina of VAD rats and xerophthalmia, xerosis, or keratomalacia indicative of an advanced VAD state (5). In the previous study, albino rats given Vit A deficient diet for 8 weeks showed disintegration of outer segment of

photoreceptor cells. The discrepancy can be explained by difference between accessibilities of retinal damage of pigmented or non-pigmented species. Generally, ocular melanin may protect the retina, probably by the absorption of light. Because of this, albino rat retinas are particularly more sensitive to the deleterious effects than pigmented rat retinas (20).

Decreased retinal retinoids and body weight gain with food consumption observed up to Week 10 were comparable to control levels after feeding Vit A (+) diet to the VAD rats for 5 weeks.

These results indicate that the VAD rats in the present study were considered to be in a reversible and early stage of VAD disease and have an appropriate state to investigate the primary effects of VAD on retinal function.

6. FIGURES AND TABLES

Table II-1. Composition of AIN-93G (per 100 g)

Vitamin Free Casein	20.00000 g
L-cystine	0.30000 g
Cornstarch	39.74860 g
αCornstarch	13.20000 g
Sucrose	10.00000 g
Soybean oil	7.00000 g
Cellulose	5.00000 g
AIN-93G mineral mixture	3.50000 g
AIN-93 vitamin mixture	1.00000 g
Choline bitartrate	0.25000 g
tert-butylhydroquinone	0.00140 g

Table II-2. Composition of AIN-93G Vitamin A (-) (per 100 g)

Vitamin Free Casein	20.00000 g
L-cystine	0.30000 g
Cornstarch	39.74860 g
αCornstarch	13.20000 g
Sucrose	10.00000 g
Soybean oil	7.00000 g
Cellulose	5.00000 g
AIN-93G mineral mixture	3.50000 g
AIN-93 vitamin mixture (w/o Vitamin A)	1.00000 g
Choline bitartrate	0.25000 g
tert-butylhydroquinone	0.00140 g

Table II-3. Composition of AIN-93 vitamin mixture (per 100 g)

Vitamin D3(400000 IU/g)	0.02500 g
Vitamin E (50%)	1.50000 g
Vitamin B1	0.06000 g
Vitamin B2	0.06000 g
Vitamin B6	0.07000 g
Vitamin B12 (0.1%)	0.25000 g
Vitamin K1	0.00750 g
d-Biotin	0.00200 g
Folic acid	0.02000 g
Ca-Panthothenate	0.16000 g
Niacin	0.30000 g
Sucrose	97.54550 g

Table II-4. Hematology in control and vitamin A deficient rats.

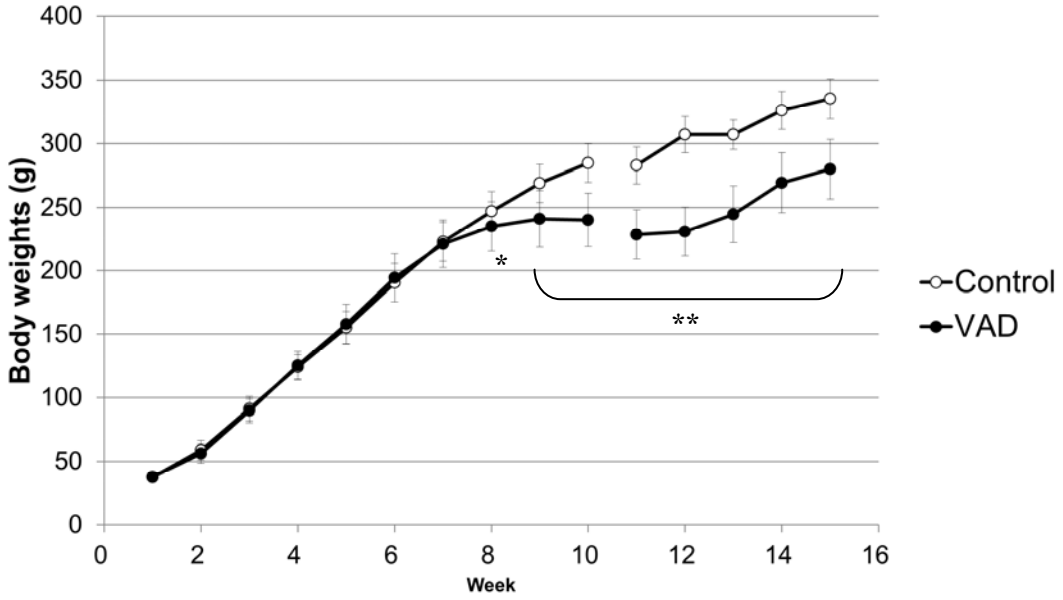
Week 10									
		RBC	Hgb	Hct	MCV	MCH	MCHC	Ret	WBC
Unit		10^6 /uL	g/dL	%	fL	pg	%	10^9 /uL	10^3 /uL
Mean value	Control	7.88	13.3	41.0	52.0	16.9	32.5	176.7	18.19
	VAD	8.74	14.3	44.3	50.7	16.4	32.3	141.1	17.33
		Neu	Lym	Mon	Eos	Bas	ULC	Plt	
Unit		10^3 /uL	10^3 /uL	10^3 /uL	10^3 /uL	10^3 /uL	10^3 /uL	10^3 /uL	
Mean value	Control	1.26	15.75	0.32	0.47	0.10	0.29	831	
	VAD	2.92	13.47	0.24	0.16	0.09	0.45	1052	
Week 15									
		RBC	Hgb	Hct	MCV	MCH	MCHC	Ret	WBC
Unit		10^6 /uL	g/dL	%	fL	pg	%	10^9 /uL	10^3 /uL
Mean value	Control	8.07	13.1	40.8	50.6	16.3	32.2	226.1	15.27
	VAD	8.09	13.1	41.3	51.0	16.2	31.8	297.2	15.52
		Neu	Lym	Mon	Eos	Bas	ULC	Plt	
Unit		10^3 /uL	10^3 /uL	10^3 /uL	10^3 /uL	10^3 /uL	10^3 /uL	10^3 /uL	
Mean value	Control	1.78	12.48	0.32	0.37	0.06	0.26	815	
	VAD	1.38	13.33	0.30	0.22	0.07	0.22	897	

Table II-5. Summary of peri-papillary opacification at Week 10 and 15

	Control group	VAD group
Week 10		
Right eye	0/18	18/18
Left eye	0/18	18/18
Week 15		
Right eye	0/6	6/6
Left eye	0/6	6/6

Peri-papillary opacification found at Weeks 10 and 15 are summarized in this table.

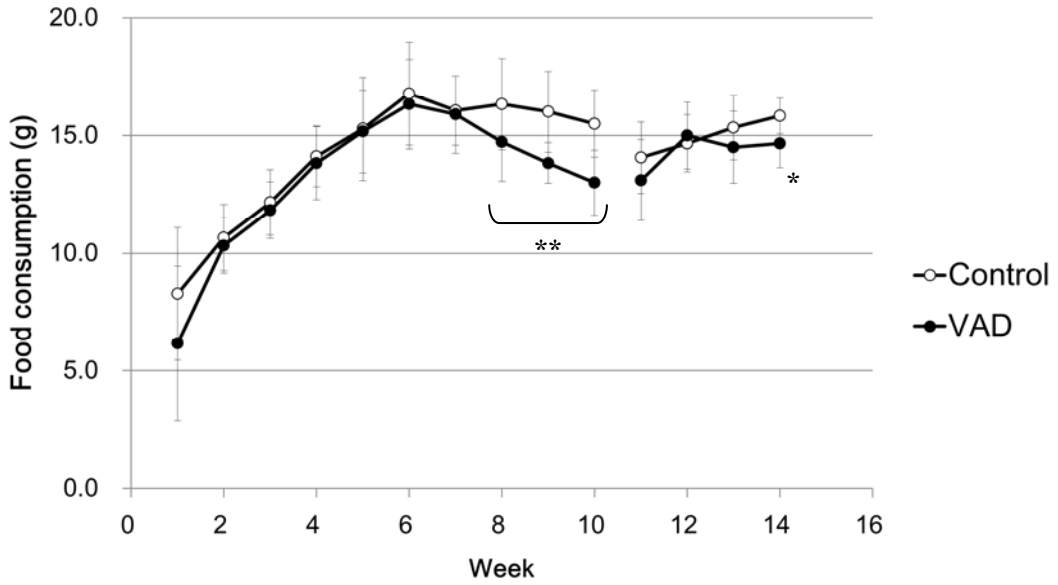
Figure II-1. Comparison of body weight between control and vitamin A deficient rats.



Plots of body weight for control and vitamin A deficient rats during experimental period.

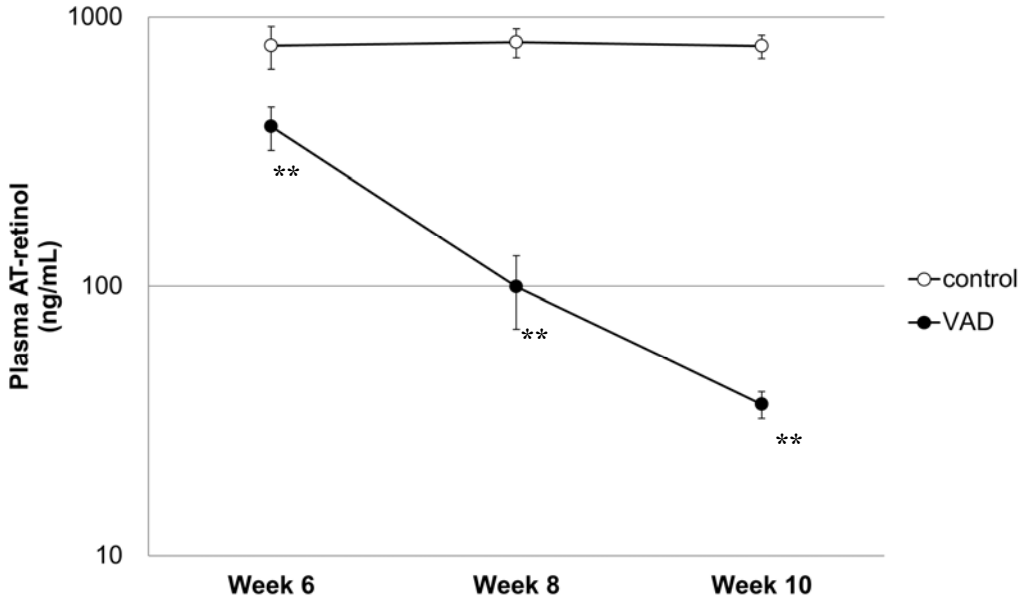
Mean values of 18 rats are shown. Bars, SD. *: $p<0.05$ **: $p<0.01$

Figure II-2. Comparison of food consumption between control and vitamin A deficient rats.



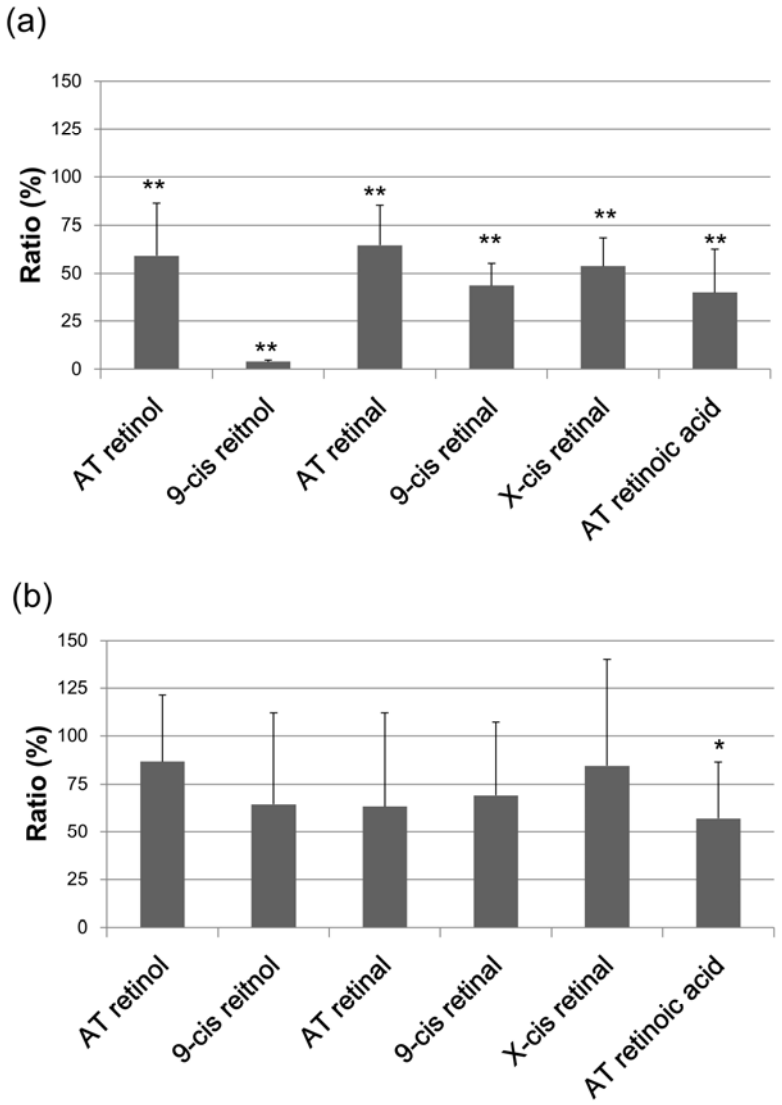
Plots of food consumption of control and vitamin A deficient rats during experimental period. Mean values of 18 rats are shown. Bars, SD. *: $p<0.05$ **: $p<0.01$

Figure II-3. Comparison of plasma all-trans retinol between control and vitamin A deficient rats.



Plots of concentration of plasma all-trans retinol for control and vitamin A deficient rats at Weeks 6, 8, and 10. Mean values of 12 rats are shown. Bars, SD. **: $p<0.01$

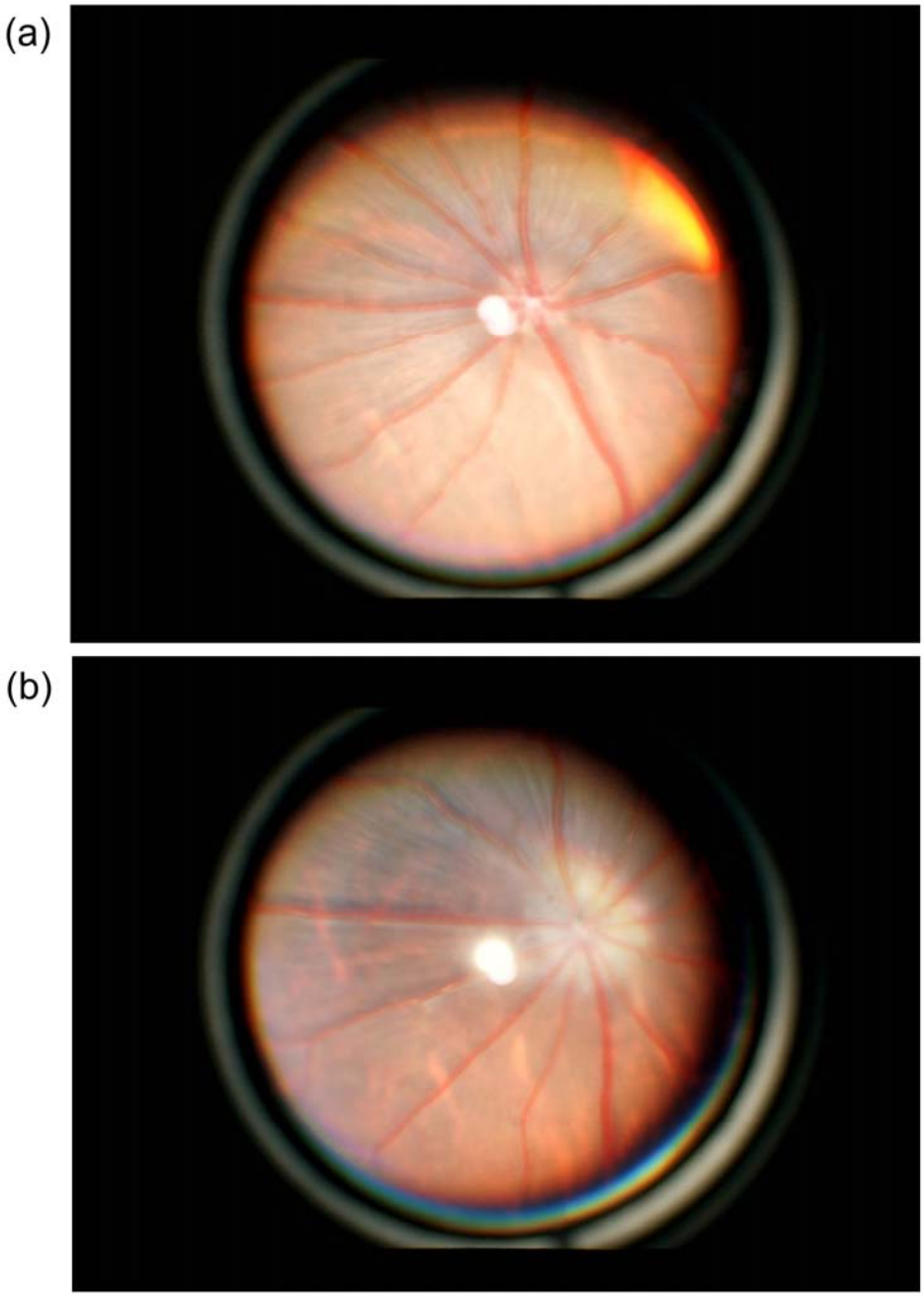
Figure II-4. Comparison of retinoids in retina between control and vitamin A deficient rats.



(a) The ratios of measurable retinal retinoids in retina of vitamin A deficient (n=6) rats at Week 10 to those of control (n=6) are shown. (b) The ratios of retinal retinoids in vitamin A deficient rats at Week 15 to those of control rats are shown. Bars, SD.

*: $p < 0.05$, **: $p < 0.01$ versus control with student's t-test.

Figure II-5. Fundus photographs at Week 10.



(a) and (b) show fundus photographs in control and VAD rats, respectively.

CHAPTER III

Investigation of Retinal Function of the Vitamin A Deficient Rat model and Involvement of Dopamine Pathway in Inner Retinal Layer Cell Functions.

1. SUMMARY

As I described in CHAPTER I, I hypothesized that loss of retinoid influences the function of amacrine cells as well as photoreceptor cells and induce reduced contrast sensitivity with changes in OPs in ERG recording. In order to verify the hypothesis, I electro-physiologically investigated retinal functions of the VAD model produced in CHAPTER II in the present study. As the result, ERG recording at Week 10 showed reduced OPs as well as a- and b-waves at multi-intensities of light stimulation. Further analysis indicated that the ratio of the changes in OPs was more significant than those of a- and b-waves. After 5 weeks of recovery, these changes returned to control levels. These results suggest that inner retinal cell functions which are reflected in OPs were reduced by VAD. OPs are considered to become more sensitive markers of visual impairment by VAD since the rat model is in early stage of VAD disease.

Subsequently, I compared expression of dopamine receptors and locomotor activity,

as an alternative indicator of dopamine pathway activity, between control and the VAD model with reduced OPs in order to investigate the involvement of dopamine pathway in function of inner retinal layer cells. Although comparison of locomotor activity showed decreased rearing count in the VAD rats, there was no significant reduction in the VAD rats in analysis of gene expression of dopamine receptors.

2. INTRODUCTION

Retinal Function of the Vitamin A Deficient Rat model

ERG in laboratory animals with VAD has been recorded in previous studies. In an analysis of ERG with multi-intensity of light stimulus (light intensity: 0.02 to 2000 cds/m^2), the b-wave was greatly reduced at less than approximately 20 cds/m^2 of light intensity, and the a-wave was barely perceptible even at the highest light intensity by feeding albino rats a Vit A deficient diet for 8 weeks in a previous report (5). In that study, disintegration of outer segment of the photoreceptor cell was histopathologically observed, and there were body weight loss, xerophthalmia, xerosis, or keratomalacia indicative of an advanced VAD state. Severe physical condition generally induces decreased blood circulation and reduces the supply of nutrients to eye, and then may secondary affect ERG components. Therefore, I electro-physiologically analyzed retinal function in the VAD model without severe physical condition under the latest protocol recommended by ISCEV in order to investigate the primary effect of VAD on retinal function (21).

Involvement of Dopamine Pathway in Inner Retinal Layer Cell Functions

Subsequently, I researched mechanisms of effects of VAD on function of inner retinal

layer cells. The inner retinal layer cells function in visual transduction between outer retinal layer cells to optic nerve through some neurotransmitters including dopamine, GABA, glycine, acetyl choline, and so on (10). On the other hand, retinoic acid, one of the active forms of retinoid, is known to be implicated in regulation of gene expression. The retinoic acid is mediated primarily by two families of nuclear hormone receptors, RAR and RXR (18). RAR can be activated by AT-retinoic acid and 9-cis retinoic acid, while RXR is activated by 9-cis retinoic acid. Activated retinoid receptors function as transcription factors, activating specific retinoic acid response elements (RAREs) for transcriptional regulation of target genes.

Dopamine receptor is one of the genes have the RAREs in the promoter region and is known to be regulated by retinoic acid (22, 23, 24). Dopamine receptors are present in the amacrine cells and are known to be involved in visual signal transduction (6, 23, 24, 25). This was supported by a previous study that dopamine blockers lead to decline in OPs (10, 20). RAR and RXR also present in amacrine cells (12, 13). These may suggest that retinoids are associated with dopamine pathway in visual transduction in inner retinal layer cells. In order to assess this hypothesis, gene expression of dopamine receptors in the retina and spontaneous locomotor activity, an indicator of dopamine level in the CNS, were additionally measured to afford collateral evidence for

the effects of VAD on the dopamine system. Reversibility of these parameters was also evaluated after the 5 week recovery period.

3. MATERIAL AND METHODS

3.1. ERG recording

Before ERG recording, rats from each group were dark-adapted overnight and anesthetized with a combination of 40 mg/kg ketamine and 0.2 mg/kg xylazine by intramuscular injection. The pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine HCl. Rats were placed on an electric heating set at 25 °C to prevent a decrease in body temperature until recording. Dark adaptation was maintained throughout the recording. ERG was recorded from the right eye of each animal using a contact lens type electrode (LS-W; Mayo, Aichi, Japan). The recording electrode was placed in contact with the cornea, while a reference electrode was placed in the mouth. ERG responses were amplified, band-pass filtered between 0.5 to 200 Hz, by using a computer-assisted signal analysis system (MEB2200, Neuropack; Nihon Kohden, Tokyo, Japan). Light stimuli from 0.0003 to 30 cd•s/m^2 were provided by a SLS-3100 (Nihon Kohden, Tokyo, Japan). ERG response amplitudes and implicit times for a- and b-waves were determined based on the ISCEV standard (26). OPs were also recorded from the right eye between 0.0003 and 3.0 cd•s/m^2 of light stimuli with similar methods of the ISCEV standard (26). A bandpass filter was set to 50–500 Hz. The amplitudes and implicit time of OPs were determined as previously described (27). The amplitude of OPs was measured from the baseline to the peak of each wavelet, and

the implicit time was measured from the stimulus onset to the peak of each wavelet. Each ERG response was recorded with intervals of more than 30 seconds and was averaged for three flashes at each light stimuli level.

3.2. Locomotor activity

Spontaneous locomotor activity was measured using an automated motion analysis system (SCANET SV-20; MELQUEST, Toyama, Japan). Animals (n = 6 for control and VAD rats) were tested in the SCANET cage for 30 min. The following parameters were measured with similar methods as shown in previous study: Horizontal movements and the frequency of vertical movements caused by rearing (28).

3.3. Quantitative PCR analysis

Gene expression was investigated with quantitative reverse transcription PCR analysis (qRT-PCR). Total RNA was extracted from whole unilateral retina using RNeasy mini kit (Qiagen, Hilden, Germany) at Week 10; and, converted to cDNA using a SuperScript® III (life technologies, Carlsbad, CA, US). Gene expression levels were quantified using an ABI7900HT Sequence Detection System (life technologies, Carlsbad, CA, US). The amounts of mRNA of Dopamine Receptor 1 (D1) and 2 (D2) were determined using specific probe sets (Assay ID: dopamine receptor D1A: Rn03062203_s1, dopamine receptor D2: Rn01418275_m1) of TaqMan Gene

Expression Assays (life technologies, Carlsbad, CA, US). PCR was performed on 10 ng of the obtained cDNA. PCR conditions were 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 60 °C for 1 min.

3.4. Statistical analysis

Student's t-test was used to compare amplitude and implicit time of ERG components and the relative amplitude of b-wave and OPs to a-wave. Wilcoxon analysis was applied to locomotor activity data. In each analysis, differences were considered to be significant when $P < 0.05$.

4. RESULTS

4.1. ERG recording

Typical ERG wave forms which were recorded at 0.0003 to 30 cd•s/m^2 at Week 10 are shown in Figure III-1. The reduction of OPs in the VAD model could be noticed clearly from these traces. The relationship between light intensity and amplitude or implicit time of a- and b-waves is shown in Figure III-2 (a) and (b), respectively. The amplitude of b-wave was significantly decreased to approximately 50% of control value at light intensity higher than 0.003 cd·s/m^2 at Week 10. The amplitude of a-wave was also significantly decreased at light intensity higher than 0.3 cd·s/m^2. There were significant reductions in implicit time of b-waves in VAD rats compared to control at light intensity higher than 3.0 cd•s/m^2. Slight prolongations of implicit time of a-wave were observed at light intensity less than 0.03 cd·s/m^2 with statistical significance. However, there were no differences in the implicit time of a-wave between VAD and control rats at light intensity higher than 3.0 cd·s/m^2. There were no differences in the amplitude and implicit time between VAD and control rats at Week 15 (Figure III-2 (c) and (d)).

OPs of the VAD model were apparently smaller than those of control rats at Week 10 (Figure III-1 (c) and (d)). Summary of amplitudes and implicit times of OPs recorded at 0.03 to 3.0 cd•s/m^2 is shown in Table III-1. (OPs recorded at less than 0.01 cd•s/m^2

were not listed on these tables because they could not be detected in most control animals.) The mean amplitudes of OP1, 2, and 3 and summed OPs (ΣOPs = OP1 + OP2 + OP3 + OP4) in VAD rats were significantly decreased to 35% to 71% of control rats at Week 10. In several VAD rats, loss of all OP wavelets was observed at less than 0.3 cd•s/m^2. At 3.0 and 30 cd•s/m^2, loss of a few OP peaks was noted. The mean implicit time of OP1 and 2 were slightly prolonged at multi-intensity at Week 10. These changes returned to control levels at Week 15. Relative amplitudes of b-wave (b/a) and ΣOPs (ΣOPs/a) to a-wave at 3.0 cd•s/m^2 are shown in Figure III-3. While the b/a of VAD rats was comparable to the control value, ΣOPs/a was significantly decreased at Week 10 (Figure III-3 (a)), and returned to the level of control after the recovery period (Figure III-3 (b)). These data suggest that VAD influenced OPs more strikingly.

4.2. Locomotor activity

In the VAD model, horizontal movements tended to decrease, and the frequency of rearing was significantly lower at Week 10 compared with control rats (Figure III-4, Horizontal movement on Week 10, $p = 0.31$; rearing on Week 10, $p = 0.04$). At Week 15, no apparent differences between VAD and control rats were observed in these parameters (Figure III-4, Horizontal movement on Week 15, $p = 0.438$; rearing on Week

15, $p = 0.485$).

4.3. Gene expression of dopamine receptors

The mRNA of D1 and D2 in retina were investigated with qRT-PCR at Week 10 (Figure III-5). The expression levels of both D1 and D2 in VAD rats were slightly decreased compared with control rats, but there was no statistical significance (D1: $p = 0.13$, D2: $p = 0.16$).

5. DISCUSSION

Retinal Function of the Vitamin A Deficient Rat model

It has been previously shown that VAD causes dysfunction of photoreceptor cells (29), where VAD induces a decrease in 11-cis retinal which is necessary for the synthesis of rhodopsin. This leads to insufficient phototransduction from light to electric signals and decline of the a-wave in ERG. The present results were consistent with these changes. However, the VAD model showed that only functional impairments of the retina were induced by feeding Vit A(-) diet to BN rats for 10 weeks since there was no histopathologic change in CHAPTER II and reversible ERG change in a- and b-waves and OPs.

In outer retinal layer cells, the electric stimulus is transmitted to and activates bipolar cells by hyperpolarization of photoreceptor cells. The activation of bipolar cells is reflected in the b-wave in ERG (3). Accordingly, it is considered that the magnitude of the b-wave depends on that of the a-wave. No difference in the b/a ratio between control and the VAD model in the present study suggests that the there was no interference in the signal transmission from photoreceptor cells to bipolar cells by VAD. The signal from bipolar cells subsequently activates the inner retinal layer cells such as amacrine cells. The reduction of a-waves may lead to the decreased input to bipolar

cells, and then reduce signal transduction from bipolar cells to amacrine cells which generate OPs. Therefore, there may be a corresponding decrease in OPs following the reduction of a-wave. In the present study, there was decreased amplitude of a-wave with strong light intensities in the VAD model with decreases in OPs at Week 10. However, analysis showed a significant reduction of ΣOPs/a in VAD rats at Week 10, despite having no changes in b/a, indicating a greater magnitude of reduction in OPs than the reduction secondary to a-wave reduction alone.

Involvement of Dopamine Pathway in Inner Retinal Layer Cell Functions

In the CNS, AT- retinoic acid and 9-cis retinoic acid are known to regulate gene expression of dopamine receptors via nuclear receptors, RAR and RXR (22, 23, 24, 30). AT-retinoic acid was decreased with statistical significance at Week 10. In addition, the greater decrease in 9-cis retinoic acid was suggested by the remarkable reduction of endogenous precursors, 9-cis retinol and retinal in CHAPTER II (31). The retinoid receptor mutant mice show decreases in locomotor activity which is an indication of dopamine activities in the CNS (14). The significant reduction in rearing count in the present study indicates that the similar decrease in dopamine receptors by retinoids is present in the VAD model. The other previous study have reported that dopamine acts

as one of the important chemical messengers among amacrine cells or between amacrine cells–ganglion cells in the retina and activates the dopamine pathway via D1 and D2 (9). Based on the decreases in retinal retinoid and rearing count, I speculated that a VAD state induces down-regulation of dopamine receptors in the inner retinal layer cells with decreases in OPs. There was no statistically significant difference in mRNA levels of D1 and D2 in the retina between the VAD model and control rats, although there were tendencies of reduction in these levels. This may indicate that additional factors contribute to the decline of OPs in VAD rats.

6. FIGURES AND TABLES

Table III-1. Summary of the amplitude and implicit time of each OP wavelet and summed OPs at weeks 10 and 15

(A) Week 10

		Amplitude (μV)					Implicit time (msec)			
		OP1	OP2	OP3	OP4	ΣOP	OP1	OP2	OP3	OP4
0.03 cd·s/m^2										
control	AV	34.6	83.8	45.8	6.8	170.9	30.1	35.6	42.0	52.4
	SD	5.2	12.4	18.7	10.9	34.9	1.2	1.0	1.2	3.6
VAD	AV	**15.3****	**37.8****	**21.6****	2.6	**77.2****	**34.8****	**40.6****	**47.2****	55.7
	SD	12.9	29.7	16.7	5.3	59.4	2.6	2.7	3.2	5.1
0.3 cd·s/m^2										
control	AV	75.8	134.8	44.8	3.4	258.8	26.1	31.6	39.1	50.0
	SD	17.8	30.6	12.7	3.4	58.8	2.3	2.2	2.2	3.3
VAD	AV	**32.6****	**68.6****	**31.8***	5.5	**130.9****	**28.9****	**34.6****	41.0	50.0
	SD	19.6	26.4	21.3	8.9	64.0	2.8	2.6	3.4	5.3
3.0 cd·s/m^2										
control	AV	120.1	161.6	50.4	3.2	335.4	24.3	30.3	38.3	48.1
	SD	27.2	38.3	13.9	2.6	78.8	2.2	2.4	2.5	1.7
VAD	AV	**45.4****	**77.3****	**33.1****	5.1	**161.0****	**26.9***	**32.6***	39.3	47.1
	SD	25.3	34.9	19.6	6.7	73.9	3.3	2.9	4.1	4.2
30.0 cd·s/m^2										
control	AV	129.5	181.5	54.5	5.8	371.4	22.4	28.7	37.1	48.0
	SD	28.3	45.8	15.1	3.4	87.6	2.7	2.6	2.6	2.8
VAD	AV	**45.4****	**84.3****	**36.1****	4.6	**170.4****	**24.6***	**30.5***	37.5	45.7
	SD	23.2	35.9	20.9	5.6	73.8	2.5	2.4	3.0	3.9

AV: Average, SD: Standard Deviation

(B) Week 15

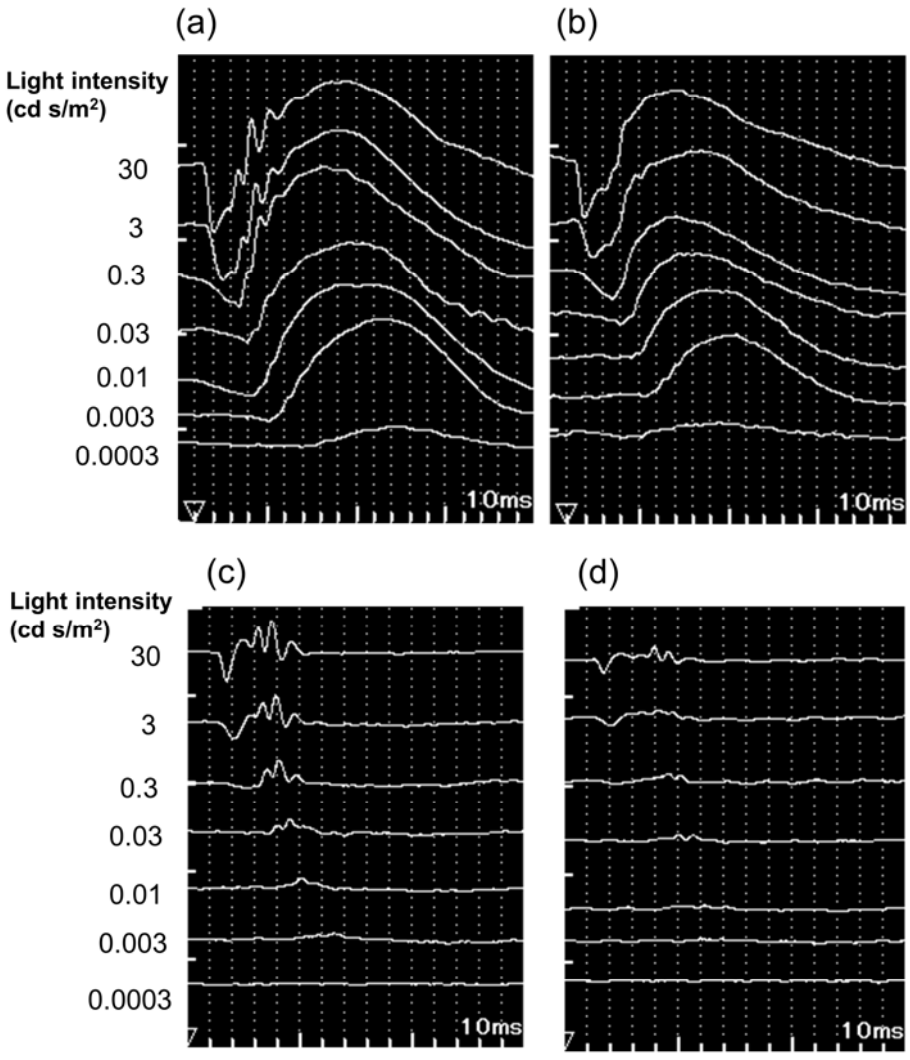
		Amplitude (μV)					Implicit time (msec)			
		OP1	OP2	OP3	OP4	ΣOP	OP1	OP2	OP3	OP4
0.03 cd·s/m^2										
control	AV	36.2	67.2	38.2	3.3	144.8	29.9	35.2	41.5	51.1
	SD	20.0	40.8	21.7	5.3	80.2	0.9	1.2	1.1	2.1
VAD	AV	30.3	67.7	41.7	4.0	143.7	28.9	34.8	41.3	51.0
	SD	17.5	37.4	25.7	5.5	80.0	0.7	0.5	0.7	2.1
0.3 cd·s/m^2										
control	AV	76.8	139.7	45.3	10.3	272.2	25.0	30.5	38.3	47.6
	SD	16.7	32.0	14.5	6.9	59.5	0.8	0.7	1.8	1.8
VAD	AV	73.8	139.0	40.5	6.0	259.3	25.0	30.3	38.4	49.4
	SD	16.9	27.6	11.1	3.6	55.1	0.5	0.4	0.8	0.7
3.0 cd·s/m^2										
control	AV	126.8	174.8	54.3	8.5	364.5	23.4	29.1	37.5	46.6
	SD	19.8	40.3	13.8	4.8	72.5	0.6	0.6	1.6	1.7
VAD	AV	117.8	165.2	45.3	2.5	330.8	23.0	28.9	37.5	47.9
	SD	24.7	26.9	14.6	3.6	66.4	0.4	0.7	0.7	1.3
30.0 cd·s/m^2										
control	AV	137.2	194.5	54.7	6.3	392.7	21.5	27.5	36.1	46.5
	SD	24.2	43.8	15.0	5.5	79.8	0.5	0.6	0.8	1.4
VAD	AV	124.7	181.5	45.8	3.2	355.2	21.1	27.2	36.0	46.6
	SD	26.4	34.4	12.8	1.5	70.5	0.3	0.5	0.5	1.0

AV: Average, SD: Standard Deviation

The amplitude and implicit time of each OP wavelet and summed OPs in vitamin A deficient rats were compared with those of control rats. The mean and standard deviation of each wavelet and ΣOPs were shown at Weeks 10 and 15 in (A) and (B),

respectively. Bold figures mean the values have statistical significance. *: $p < 0.05$ and **: $p < 0.01$ versus control with student's t-test.

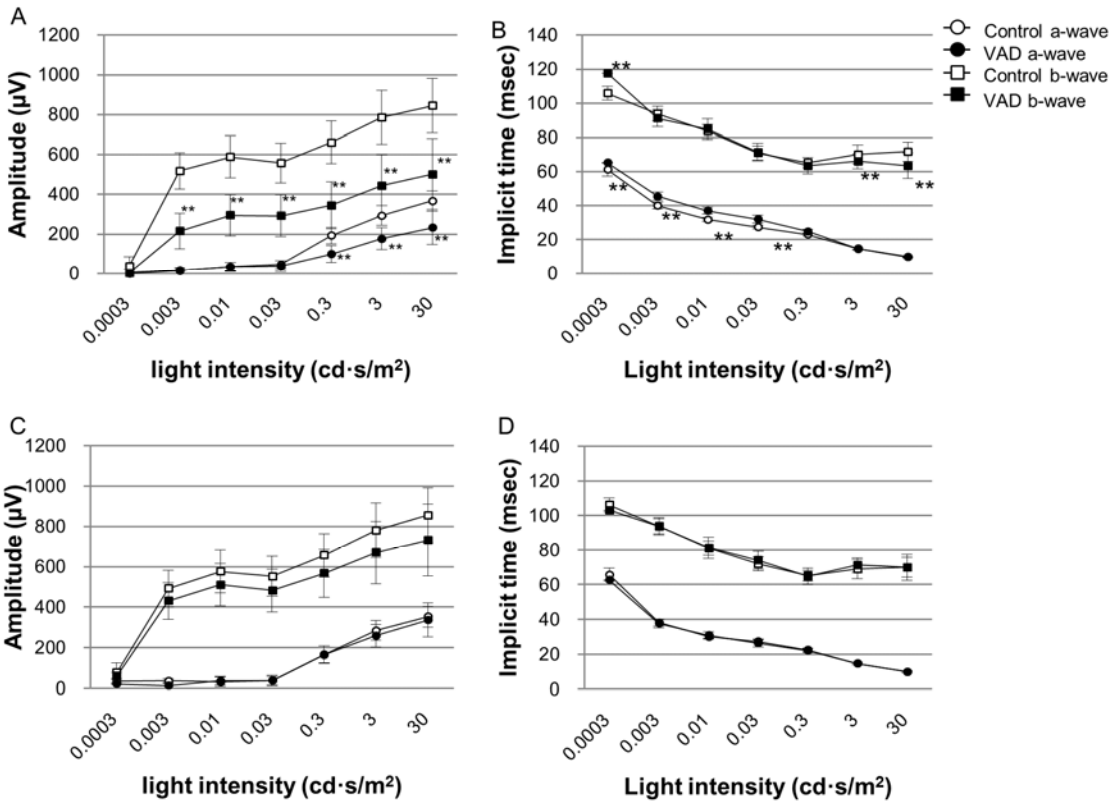
Figure III-1. Typical ERG wave forms of control and vitamin A deficient rats at Week 10.



Dark adapted ERG wave forms produced by 0.0003 to 30 cd•s/m^2 of light stimuli in (a) control and (b) vitamin A deficient rats are shown. Wave forms of OPs produced by 0.0003 to 3.0 cd•s/m^2 of light stimuli are shown in (c) control and (d) vitamin A

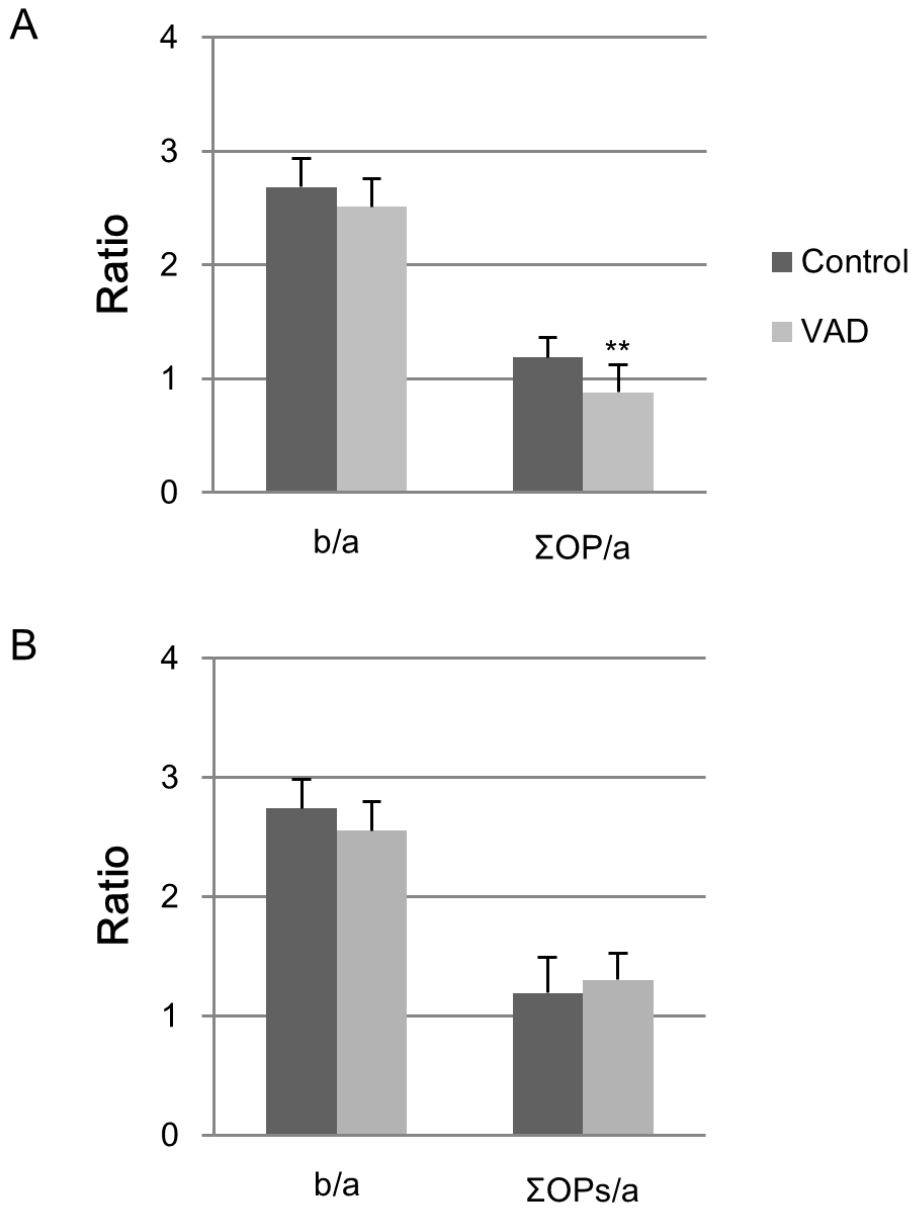
deficient rats.

Figure III-2. The relationships of the amplitude and the implicit time of ERG components to light intensity at Weeks 10 and 15.



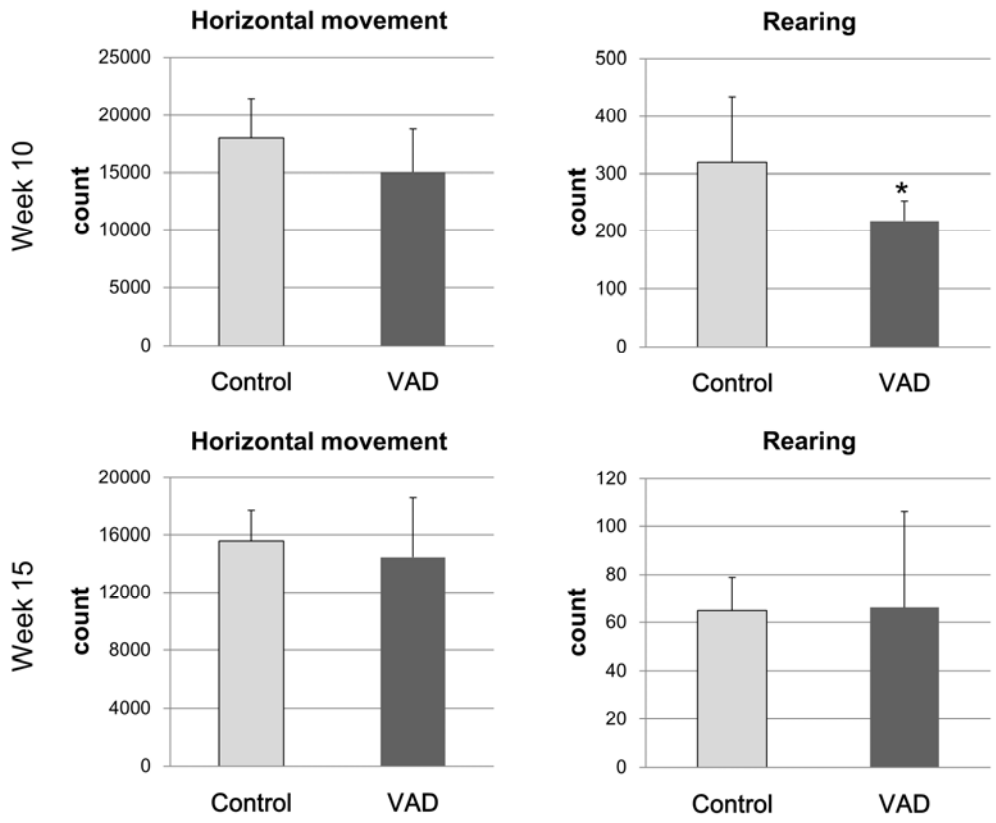
Mean responses of 6 to 8 rats to seven flashes of 0.0003 to 30 cd•s/m^2 are shown. Bars, SD. (a and c) Amplitude and (b and d) implicit time of a- and b-waves at Weeks 10 (a and b) and 15 (c and d). **: $p<0.01$

Figure III-3. The effect of vitamin A deficiency on the relative amplitude of b-wave and ΣOPs to that of a-wave.



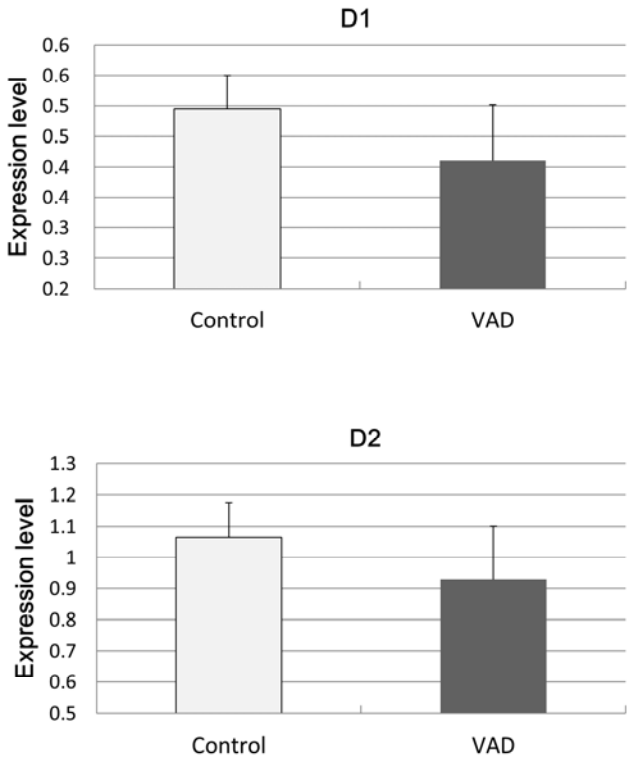
Mean values of b/a and ΣOPs/a at Week 10 (a) and 15 (b) are shown. Bars, SD. **: $p < 0.01$ versus control with student's t-test.

Figure III-4. Comparison of locomotor activities between control and vitamin A deficient rats.



Mean values reveals total frequency of horizontal movements (A) and the frequency of rearing (B) with the SD at Weeks 10 (A) and 15 (B). *: $p < 0.05$ versus vehicle control with Wilcoxon statistical analysis.

Figure III-5. The expression levels of both dopamine 1 and 2 receptors in control and vitamin A deficient rats at Week 10.



Mean values of mRNA levels of dopamine 1 and 2 receptors are shown with SD (n=3)

CHAPTER IV

Concluding remarks

VAD is well known to cause visual impairments including prolonged dark adaptation and night blindness based on dysfunction of photoreceptor cell. Associated with this mechanism, human and animals with VAD show that a-wave reduces depending upon the stage of the VAD disease in ERG recording (3, 5). Similar results were observed in the present model with early stage of VAD disease. Additionally, there was reduced OPs indicative of inner retinal layer cell dysfunction in this research. Amacrine cells are releasing and receiving several neurotransmitters, including dopamine, GABA, glycine, and acetylcholine in order to stimulate other amacrine or ganglion cells (10, 30, 32). The significant reduction of ΣOPs/a in the VAD model possibly indicates that VAD influences not only functions of photoreceptor cells but also signal transduction of inner retinal layer cells via these transmitters. Here, I speculated that a down-regulated dopamine pathway is mainly implicated in the inner retinal layer cell dysfunction since retinoic acid is involved in regulation of dopamine receptors. However, there was no statistical significant difference in mRNA level of D1 and D2 in retina between normal and the VAD model rats. This may indicate that other factors contribute to the decline

of OPs in VAD rats, although the possibility of the involvement of dopamine pathway cannot be entirely ruled out since there were reduction of rearing count and the tendency of decreased mRNA levels of D1 and D2. For example, a previous report indicated that retinoic acid is required for activation of an enzyme that converts glutamate to GABA (33). The decline of OPs in the VAD model may be affected by a variety of factors associated with abnormalities in the levels of neurotransmitters, and decreased photoreceptor output.

In the present study, I have shown for the first time that VAD affects the generation of OPs. The decreased OPs in the present study possibly reflect reduced contrast sensitivity in patients with VAD and may also be applied for prediction of, or for early detection of, other conditions with reduced contrast sensitivity. The model used in the present study may also afford an animal model for the research associated with OPs abnormality.

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