

VITAMIN A DEFICIENCY INDUCES MOTOR IMPAIRMENTS AND STRIATAL CHOLINERGIC DYSFUNCTION IN RATS

M. CARTA,^a R. STANCAMPIANO,^a E. TRONCI,^b
M. COLLU,^c A. USIELLO,^d M. MORELLI^b
AND F. FADDA^{a*}

^aDepartment of Applied Science for Biosystems, Section of Nutrition and Human Physiology, University of Cagliari, Via Porcell 4, I-09124 Cagliari, Italy

^bDepartment of Toxicology, University of Cagliari, P.zza B. Loddo, I-09124, Cagliari, Italy

^cDepartment of Neuroscience, University of Cagliari, Cittadella Universitaria, 09042 Monserrato, Cagliari, Italy

^dGEINGE-Biotecnologie Avanzate, Via Comunale Margherita 142, 80145 Naples, Italy

Abstract—Vitamin A and its derivatives, retinoids, are involved in the regulation of gene expression by binding two nuclear receptor families, retinoic acid receptors and retinoid X receptors. Retinoid receptors are highly expressed in the striatum, revealing an involvement of this system in the control of movement as demonstrated by previous observations in knockout mice. To further assess the role of retinoids in adult striatal function, the present study investigated the effect of vitamin A deprivation on rat motor activity and coordination, the rate of synthesis and release of dopamine, the functioning of D1 and D2 receptors and their expression in the striatum. Moreover, the content of acetylcholine in the striatum was measured. Results show that 24 weeks of post-natal vitamin A deprivation induced severe locomotor deficits and impaired motor coordination. Vitamin A deprivation rats showed a significant hyperactivity following D1 receptor stimulation by *R*(+)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine or amphetamine and reduced catalepsy in response to haloperidol treatment. This different response to the above drugs is not due to a change in striatal DA release or synthesis between vitamin A deprivation and control animals. *In situ* hybridization experiments showed identical level of expression for the D1 and D2 receptor transcripts. On the other hand, the striatal tissue content of acetylcholine was reduced significantly by about 30% starting from the initial manifestation of motor deficits. We suggest that the locomotor impairment could be imputable to the dysfunction in striatal cholinergic interneurons. Our results stress the basic role of vitamin A in the maintenance of basal ganglia motor function in the adult rat brain. © 2006 Published by Elsevier Ltd on behalf of IBRO.

Key words: retinoids, dopamine, acetylcholine, amphetamine, striatum, motor control.

*Corresponding author. Tel: +39-070-6758938; fax: +39-070-6758917. E-mail address: ffadda@unica.it (F. Fadda).

Abbreviations: ACh, acetylcholine; ANOVA, analysis of variance; ChAT, choline acetyltransferase; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HPLC, high performance liquid chromatography; NSD 1015, *m*-hydroxybenzylhydrazine; RARs, retinoic acid receptors; RXRs, retinoid X receptors; SKF 81297, *R*(+)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; TH, tyrosine hydroxylase; VAD, vitamin A deprivation/deficient/deficiency.

0306-4522/06/\$30.00+0.00 © 2006 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2006.01.027

Retinoids play an important role in the development and maintenance of the CNS, where, by binding to their nuclear receptors, they induce gene transcription by interacting with distinct promoter sequence in target genes, thus controlling the rate of synthesis of several proteins.

The identification of the differential expression of the two retinoid receptors, namely retinoic acid receptor (RAR) and retinoid X receptor (RXR), each of them existing in three subtypes, alpha, beta and gamma, in different brain areas, has opened a new field of research investigating their role in cerebral functions (Zetterstrom et al., 1999).

Studies with retinoid receptors knockout mice revealed the importance of these receptors in long term potentiation and long term depression, electrophysiological processes that are thought to be important in learning and memory processes (Chiang et al., 1998). Other studies confirmed these observations in vitamin A deficient (VAD) mice (Miseret et al., 2001). Furthermore, for the first time our group found an impairment in spatial memory task and decreased acetylcholine (ACh) release in rat hippocampus (Cocco et al., 2002). More recently, deterioration of spatial memory test has been confirmed by Etchamendy et al. (2003).

Other studies using knockout mice for retinoid receptors which are highly expressed in striatum, RAR β , RAR γ , RXR β and RXR γ , have also revealed an involvement of this system in the control of movement. In particular double null mutant mice RAR β -RXR β , RAR β -RXR γ and RXR β -RXR γ , exhibited a significant reduction in forward locomotion assessed with the open field test and in motor coordination as measured by the rotarod test (Krezel et al., 1998).

The striatum is a retinoid-metabolizing region enriched in retinol binding proteins as well as in nuclear retinoic receptors (Zetterstrom et al., 1999). Thus, retinoids may have an important role in the control of basal ganglia signaling (Zetterstrom et al., 1999; Saga et al., 1999; Krezel et al., 1998). It has been suggested (Krezel et al., 1998; Goodman, 1998; Eichele, 1997) that altered vitamin A signaling could be implicated in the etiology of neuropsychiatric diseases, such as schizophrenia and Parkinson's disease, in which the striatal dopamine (DA) systems have an important role.

To determine whether retinoids are actively involved in the adult striatal functioning, we experimentally induced postnatal VAD in rats. This approach makes normal embryogenesis and postnatal development possible.

In the present study, we have analyzed the impact of VAD on coordination and on spontaneous and DA-mediated motor activity. Furthermore, given the fundamental

role of DA in basal ganglia-mediated motor behaviors, we studied the integrity of dopaminergic transmission in VAD rats by measuring in the striatum the rate of synthesis and extracellular levels of DA and the integrity of DA D1 and D2 receptors by means of mRNA level evaluation. Moreover, since retinoic acid has been shown to regulate the expression of choline acetyltransferase (ChAT) in cell cultures (Berrard et al., 1995; Berse and Blusztajn, 1995; Diebler et al., 1998) we measured the striatal content of ACh.

EXPERIMENTAL PROCEDURES

Animal preparation

Three week old Sprague–Dawley male rats (Harlan, Italy) were used (weight 35–50 g). Animals were housed at a constant temperature of 22 ± 2 °C and 60% relative humidity, on a 12-h light/dark cycle (lights on at 7:00 a.m.) laboratory food and water was available *ad libitum*. Experiments were carried out in accordance with the European Communities Council Directive (86/609/ECC) for the Care and Use of Laboratory Animals and were approved by the animal Care Committee of Cagliari University. The minimal numbers of animals were used to achieve statistical significance, and all efforts were made to minimize animal suffering.

One group of rats received the diet lacking vitamin A (VAD). The vitamin A-free diet (Laboratori. Piccioni, Segrate, MI, Italy) had the following composition: (for 100 g of food, dry weight): casein vitamin-free 18%, sucrose plus maize starch 68.4%, cellulose 2%, hydrogenated coconut oil 4.6%, Hegsted salt 4.8%, yeast 2%, plus a vitamin integration lacking vitamin A 0.2%. A second group of rats (controls) was fed with the same vitamin A-free diet described above plus vitamin A integration (5 IU retinol/g).

Retinol analysis

In a separate group of rats, blood was drawn from the abdominal aorta following anesthetization by chloral hydrate. Serum was separated and stored at -70 °C until analysis. Serum was analyzed for total retinol concentration by the method of Ross (1986).

Surgery, microdialysis procedure and high performance liquid chromatography (HPLC) detection

Control and VAD rats were anesthetized with pentobarbital (50 mg/kg i.p.) and mounted in a stereotaxic frame (David Kopf Instrument, USA). Using a standard stereotaxic procedure a microdialysis probe was surgically implanted in the lateral striatum (A +0.7; V -7; L -3.2; from bregma) according to the atlas of Paxinos and Watson (1986). After surgery, the dialysis probe was continuously perfused at a rate of 0.5 μ l/min with Ringer solution containing: (mM) KCl 3, NaCl 145, CaCl₂ 1.3, MgCl₂ 1, in aqueous phosphate buffer, pH 7.3. The day following surgery, the flow rate was set to 2 μ l/min. After about 60 min of stabilization, 20 min fractions were collected, in vials containing 10 μ l 0.1 M perchloric acid, for the determination of DA and 3,4-dihydroxyphenylacetic acid (DOPAC). After three basal samples, six animals from each group were injected with amphetamine 2 mg/kg i.p. and for another six, amphetamine was added to the Ringer solution giving a final concentration of 10 μ M. Forty microliter dialysate samples were injected directly into a HPLC with electrochemical detection (Antec Layden, Holland). The flow cell was equipped with a glass carbon working electrode, and an Ag/AgCl reference electrode. The potential was set at +0.65 V. The mobile phase (0.1 M NaH₂PO₄, 550 mg/l (w/v) octyl sulfate sodium, 30 mg/l EDTA, 2 mmol/l KCl, 15% (v/v) methanol, adjusted to pH 4.5 with H₃PO₄), was delivered at a flow rate of 500 μ l/min to a reverse phase C18 column (4.6 mm \varnothing , 150 mm length, CHROMPACK).

Determination of DOPA level in the striatum

DA synthesis was measured as DOPA accumulation, following inhibition of DOPA decarboxylase activity by *m*-hydroxybenzylhydrazine (NSD 1015), injected i.p. in the dose of 100 mg/kg, 30 min before kill. The brains were rapidly removed, dissected on ice and the striata were collected. Striata were frozen at -80 °C before analysis. Tissue samples were homogenized in 500 μ l of perchloric acid 0.5 M and centrifuged. The levels of DOPA in the supernatant (after filtration) were detected by HPLC method with electrochemical detector. The mobile phase (0.1 M NaH₂PO₄, 550 mg/l (w/v) octyl sulfate sodium, 30 mg/l EDTA, 2 mmol/l KCl, 10% (v/v) methanol, adjusted to pH 3 with H₃PO₄), was delivered at a flow rate of 500 μ l/min to a reverse phase C18 column (4.6 mm \varnothing , 150 mm length, CHROMPACK).

Determination of ACh level in the striatum

Animals were killed by focused microwave irradiation (5 kW, 0.8 s). To determine the striatal content of ACh, the brain was removed and the striata were collected and processed as reported above. The levels of ACh in the supernatant (after filtration) were detected by HPLC method (Antec Layden). The flow cell was equipped with a platinum working electrode, and an Ag/AgCl reference electrode. The potential was set at +0.5 mV. The mobile phase (0.2 M phosphate buffer, pH 7.5) was delivered at a flow rate of 400 μ l/min to a ACh/Ch analytical column incorporating a prepacked enzyme reactor (3 mm \varnothing , 100 mm length, Varian).

Rotarod test

The animals were trained for three consecutive days, twice a day, two minutes per session, to allow them to learn the test and reach a stable performance. On the fourth day the test was performed twice, taking the time spent by the animals on the rotarod between two consecutive falls. The best performance for each session was taken to get the mean fall latency. The speed of rotation was 6 r.p.m.

Determination of motor activity

Spontaneous activity was measured for one hour in individual activity cages equipped with intersecting photocells (San Diego Instruments, San Diego, CA, USA).

Drug-induced motor activity was measured after one hour habituation (with amphetamine and R(+)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SKF 81297; purchased from Sigma-Aldrich, Milano, Italy).

Motor activity was determined as total number of photocells beam interruption (activity counts) and recorded over a period of 120 min. Counts were referred to 10 min periods.

Vertical catalepsy was tested after 30 min from haloperidol injection (1 mg/kg s.c.; Sigma-Aldrich). The test was carried out hooking the forearms of the rats on to a horizontal bar at height of 15 cm, and the duration of maintenance of this position was measured for up to 6 min.

In situ hybridization studies

In order to perform *in situ* hybridization studies, rats were injected with amphetamine, killed 1 h later with CO₂ and their brains were rapidly removed, frozen in dry ice-cooled isopentane and stored at -20 °C. Cryostat coronal sections (12 μ m) were mounted on glass slides coated with gelatin, dried on a warm plate and stored at -20 °C. Slides were then warmed to room temperature, post-fixed in 4% paraformaldehyde/0.9% NaCl solution for 10 min, dehydrated in an ascending series of alcohols, delipidated in chloroform, rehydrated in a descending series of alcohols, air-dried, and stored at -20 °C. Sections were hybridized with [³⁵S]-labeled ribonucleotide probe complementary to mRNA encoding for D₁ or

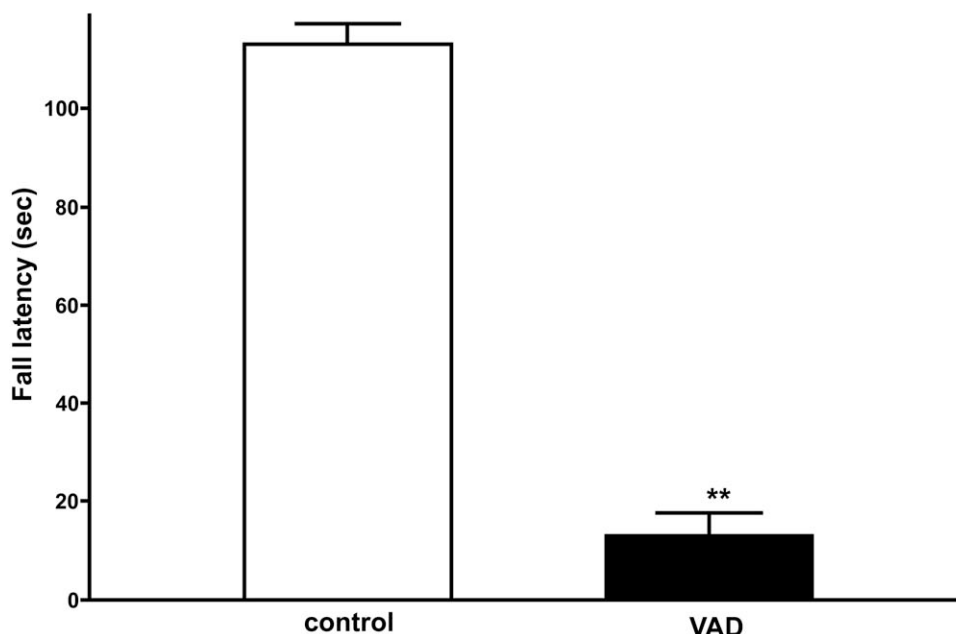


Fig. 1. Rotarod performance of control and VAD rats. Each column represents mean value \pm S.E. of 10 rats. ** $P < 0.01$ between control and VAD.

D_2 receptor. cDNA sequence complementary to bs 405/901 of rat mRNA for D_1R (acc. no. M35077) and to bs 541/1321 of rat mRNA for D_2R (acc. no. X17458) were used. Plasmids were linearized with *XhoI* restriction enzyme. Antisense ribonucleotide probe for both D_1 and D_2 receptor was generated using T3 RNA polymerase, in the presence of [35 S]UTP. Each slide was hybridized with 100 μ l of buffer containing 2×10^6 cpm of radioactively labeled probe. Hybridization was carried out at 55 $^{\circ}$ C for 12 h. The next morning slides were washed ($1 \times$ SSC, room temperature; Rnase A, 20 mg/ml, for 15 min; 4×20 min in $0.2 \times$ SSC at 60 $^{\circ}$ C, brief rinse in water), air-dried, and apposed to X-ray film.

Sections from nucleus accumbens shell and core and rostral, middle and caudal striatal levels for each rat (9.2 mm anterior to the interaural line; Paxinos and Watson, 1986) were examined for mRNA evaluation. To obtain the average density of autoradiogram gray values, quantitative analysis of labeling was performed using the image analysis program Scion Image. The average gray value of white matter was subtracted from striatal value in order to correct for background labeling. For measurement of D_1 and D_2 receptor mRNA levels the striatum was divided into two quadrants: lateral and medial.

Statistical analysis

Analysis of variance (ANOVA) for repeated measures and Newman-Keuls post hoc tests was used to analyze the data of basal, drug-induced motility, and microdialysis experiments; Student's *t*-test was used for the data concerning rotarod, catalepsy, determination of DOPA and ACh. Differences in D_1 and D_2 receptor mRNA levels between groups were determined by one-factor ANOVA, followed by Tukey HSD test.

RESULTS

Serum levels of retinol

Serum retinol concentrations were significantly decreased in 24 week-old VAD rats when compared with controls and were 3.2 ± 0.6 μ mol/l and 0.22 ± 0.2 μ mol/l in control and VAD rats, respectively. Values are mean \pm S.E.M., $n = 4$; $P < 0.01$ (Student's *t*-test).

Behavioral experiments

Rats receiving the VAD diet developed a progressive impairment in the motor function. After 24 weeks of VAD these animals showed an abnormal posture, with forepaws and hind paws flattened to the ground and an abnormal gait. As expected VAD rats were unable to perform the rotarod test, a specific test to verify balance and motor coordination. Thus, even at low speed (6 r.p.m.), VAD rats had serious problems staying on the apparatus (fall latency 13 s), whereas all control animals were able to complete the 2 min test (fall latency 113 s) (Fig. 1).

Although the serum retinol concentration was significantly reduced following 24 weeks' VAD, at the time of behavioral test, VAD rats displayed intact day-vision capability during the rotarod tests in a well-illuminated room. Animals did not exhibit any sign of blindness when observed in the open field in which some obstacle was set inside.

Despite these impairments, VAD rats showed a slight but significant hyperlocomotion in a new environment during the first 20 min of exploration (Fig. 2). Furthermore, amphetamine (2 mg/kg), which is known to increase synaptic DA level, induced a strong hyperactivation in VAD rats compared with controls (Fig. 3). In these rats, 5 mg/kg of amphetamine produced a longer lasting stereotypic behavior than the control rats (data not shown). After one hour habituation, no motor activation, except in the first 10 min, was elicited in either group by saline injection.

D1 agonist-induced motor activity

To assess the functionality of D_1 receptors we tested the effect of SKF 81297, a D_1 selective agonist. Thus, SKF 81297 (1 mg/kg s.c.) induced a significant hyperactivation in VAD rats with respect to controls (Fig. 4).

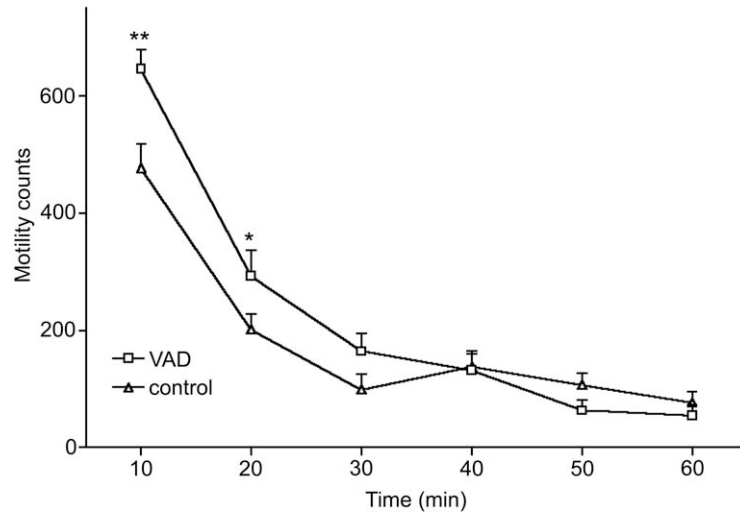


Fig. 2. Time course of the spontaneous motility of control and VAD rats. Spontaneous motor activity was recorded for a period of 60 min. Each point represents mean value \pm S.E. of 12 rats. * $P < 0.05$ and ** $P < 0.01$ between control and VAD in corresponding time point.

D2 receptors antagonist-induced catalepsy

Haloperidol, a D2 receptor antagonist (1 mg/kg s.c.), induced catalepsy in normal animals, but had a much more blunted effect in VAD rats (Fig. 5).

Microdialysis experiments

Given the role of dopaminergic transmission in the control of movement, we performed microdialysis experiments to verify the integrity of this system.

As shown in Fig. 6, both basal and amphetamine (2 mg/kg i.p.) induced striatal extracellular levels of DA were not statistically different between VAD and control animals. DOPAC levels were also unchanged (data not shown) revealing that no modification in the turnover of DA was present in VAD rats. We observed that the direct

infusion of amphetamine (10 mM) through the microdialysis probe induced turning behavior in VAD rats, but had no effect in control animals (data not shown), revealing that the differences in the motor response were not due to a different drug absorption between the two groups.

TH activity

To verify the activity of the tyrosine hydroxylase (TH), the rate limiting enzyme in the synthesis of DA, we injected NSD 1015 which, inhibiting the central decarboxylases, blocks the conversion of DOPA to DA. As shown in Fig. 7, tissue DOPA concentrations in VAD rats are not statistically different with respect to control animals, revealing that the activity of TH is not affected by vitamin A availability.

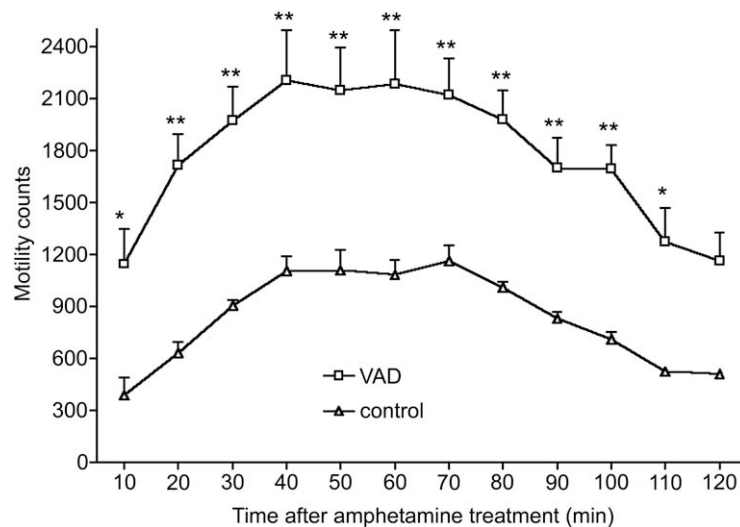


Fig. 3. Time course of the motility induced by amphetamine (2 mg/kg i.p.) in control and VAD rats. The drug-induced motor activity was recorded for a period of 120 min. Each point represents mean value \pm S.E. of 12 rats. * $P < 0.05$ and ** $P < 0.01$ between control and VAD in corresponding time point.

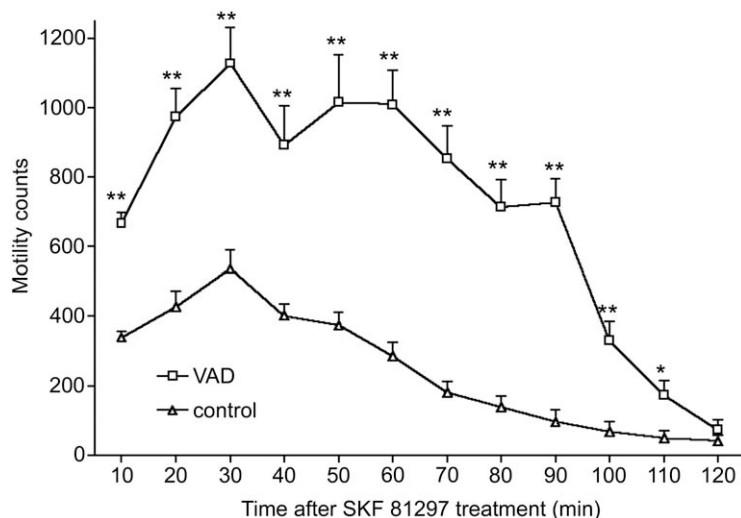


Fig. 4. Time course of the motility induced by SKF 81297 (1 mg/kg s.c.) in control and VAD rats. The drug-induced motor activity was recorded for a period of 120 min. Each point represents mean value \pm S.E. of 12 rats. * $P < 0.05$ and ** $P < 0.01$ between control and VAD in corresponding time point.

Expression of D1 and D2 receptors

Previous reports have shown an interaction between retinoic receptors and the promoter region controlling the transcription of the D2R gene (Samad et al., 1997). For this reason we performed *in situ* hybridization, to investigate if the different responses to D1 and D2 receptor stimulation were due to a different expression of these receptors in VAD rats compared with controls. This experiment revealed no difference in the striatal and accumbal expression either for D1 or D2 transcripts between the two groups of animals (Fig. 8). Evaluation of mRNA levels was made in rostral, middle and caudal striatal levels, obtaining the same result. Sections of the rostral striatum were presented in Fig. 8 in order to show both striatal and accumbal areas.

Striatal ACh content

In the light of previous reports showing a regulation of ChAT by retinoic acid, the striatal content of ACh was measured.

Fig. 9 illustrates the striatal ACh concentrations in VAD and control rats. The ACh content in the striatal tissue was significantly decreased in VAD rats respect to control animals by 29.6% ($P < 0.01$), revealing that ChAT activity might be affected by VAD.

DISCUSSION

The results reported in the present study show for the first time that postnatal VAD induces severe locomotor deficits in rats. Indeed, after 24 weeks of VAD, rats showed severe

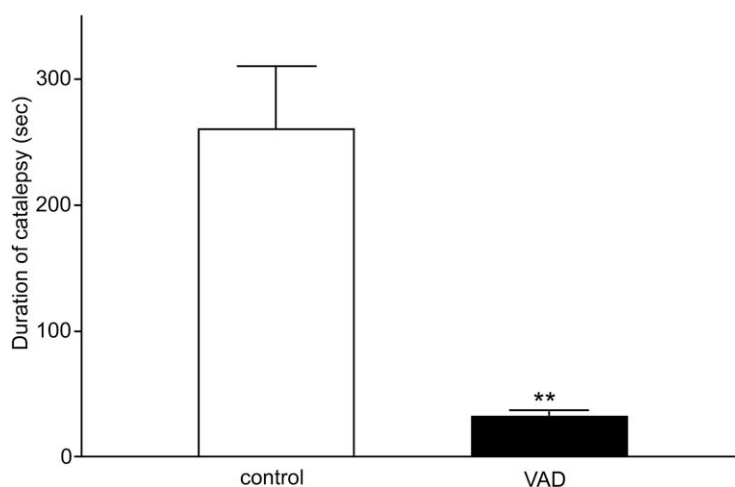


Fig. 5. Catalepsy induced by haloperidol treatment (1 mg/kg s.c.) in control and VAD rats. Each column represents mean value \pm S.E. of nine rats. ** $P < 0.01$ between control and VAD.

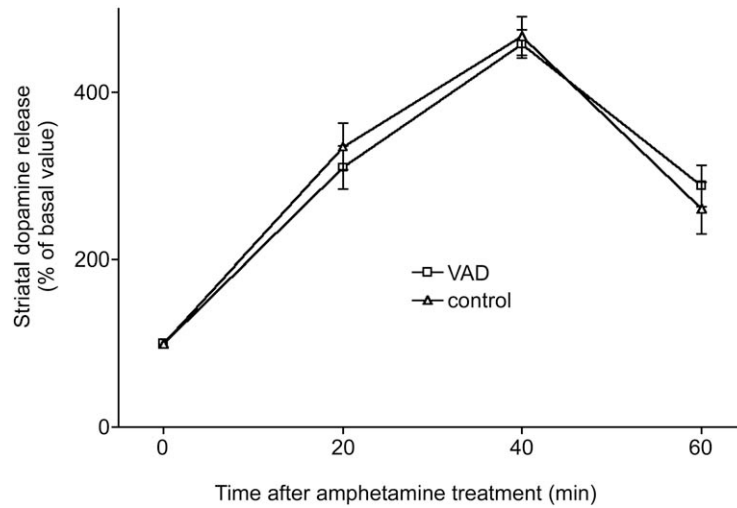


Fig. 6. Effect of administration of amphetamine (2 mg/kg i.p.) on extracellular level of DA in control and VAD rats. Basal value of DA was 25.4 ± 3.1 pg/40 μ l. Data points represent the mean \pm S.E. percentage change from baseline (T=0) of six rats.

deficits in balance and motor coordination to prevent them from performing properly in the rotarod test. VAD rats does not appear to affect DA release, synthesis or content in striatum as well as the expression of D1 and D2 transcripts in the same brain area, suggesting that the observed motor impairment could be due to a striatal cholinergic dysfunction. In fact, the striatal tissue content of ACh was reduced to about 70% of the controls already at the appearance of the first signs of motor impairment. Despite the observed motor impairment, VAD rats showed a slight, but significant hyperlocomotor response to a novel environment. At the time of testing, the animals showed no signs of blindness in a well-illuminated room when observed in the open field (see above). Given the importance of DA in the control of movement, we first focused on this system to find possible dysfunctions that might explain the motor disturbance observed in our rats. DA signaling is mainly mediated by D1 and D2 DA receptors in the striatum. Thus, to clarify the

possible role of these receptors in the locomotor dysfunction induced by VAD, we examined the function and expression of D1 and D2 receptors. VAD rats showed a significant hyperactivity following D1 receptor stimulation by the D1 receptor agonist SKF 81297 or by amphetamine treatment. Moreover, blockade of the D2 receptors by haloperidol, induced less catalepsy in VAD than in control rats.

Surprisingly, microdialysis and HPLC experiments revealed that this different response to amphetamine is not due to differences in striatal DA release or synthesis, as measured by DOPA accumulation following DOPA decarboxylase inhibition. Moreover, the total tissue content of DA, DOPAC and homovanillic acid was also unchanged (data not shown) as measured in another group of rats.

The absence of dopaminergic dysfunction is in agreement with the lack of retinoic receptors in the substantia nigra, the brain region from which the dopaminergic neu-

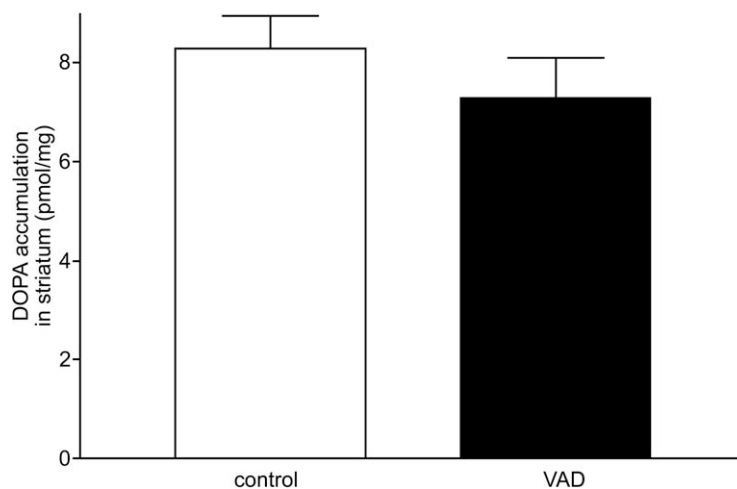


Fig. 7. DOPA accumulation in the striatum. Rats were treated with NSD 1015 (100 mg/kg i.p.), an aromatic L-amino acid decarboxylase inhibitor, 30 min before kill. Each value is the mean \pm S.E. of six rats.

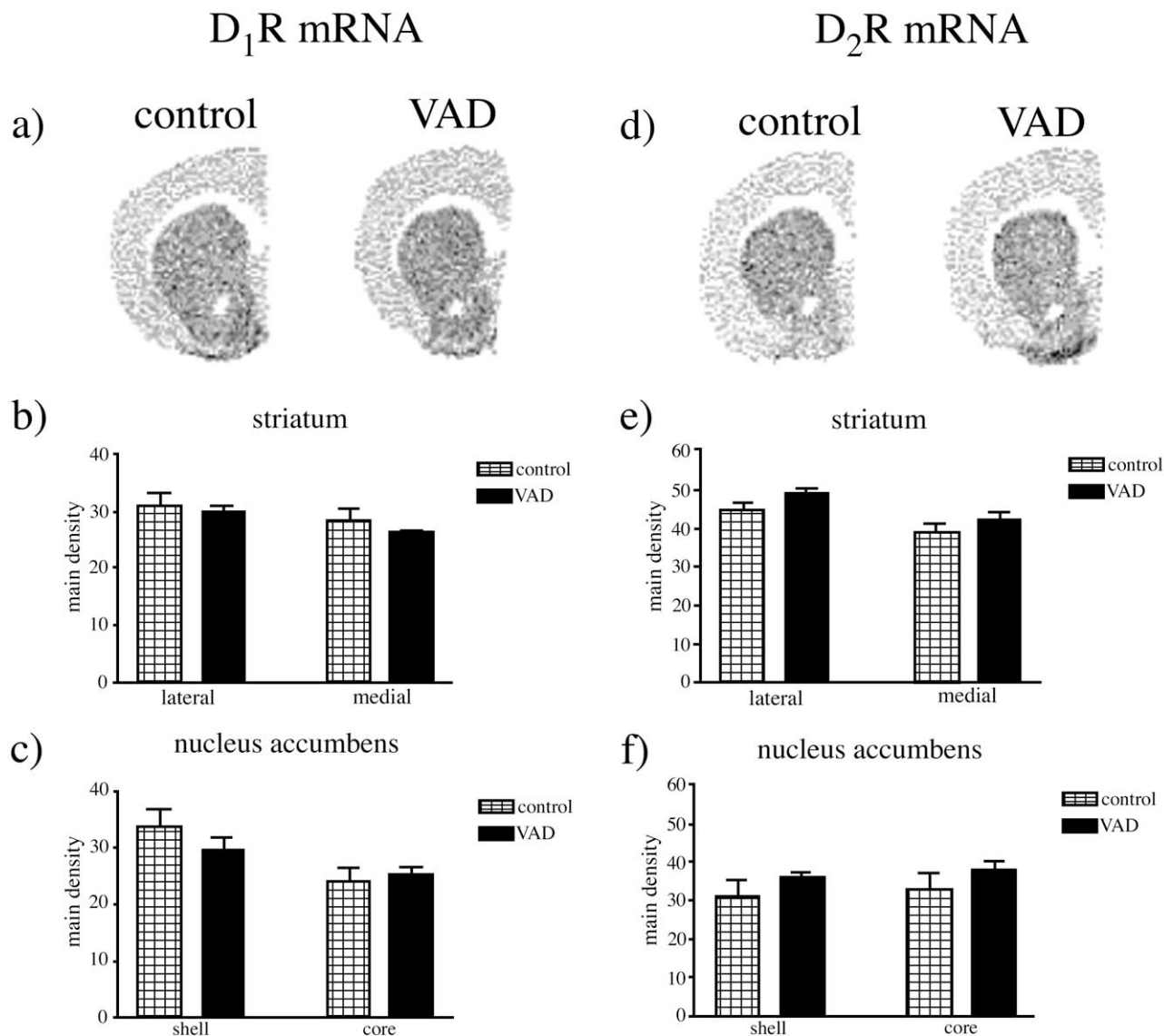


Fig. 8. Autoradiograms showing *in situ* hybridization for D₁ and D₂ receptor mRNA in control and VAD rats (a–d). Histograms report quantification of D₁ (b, c) and D₂ (e, f) receptor mRNA levels in the lateral and in the medial striatum and in the shell and core of the nucleus accumbens, measured as mean density values (mean ± S.E.M.). Both groups showed similar levels of D₁ and D₂ receptor mRNA in all areas analyzed. *N* = 6 for control group; *N* = 7 for VAD group.

rons arise and with the observation of [Krezel et al. \(1998\)](#), who showed, by means of the rotarod test, similar dysfunction in balance and motor coordination in retinoid receptor knockout mice that had normal expression of TH.

In situ hybridization experiments have shown identical levels of expression for the D₁ and D₂ receptor transcripts, in spite of the fact that an interaction of RXR with the transcription of the D₂ receptor gene has been reported ([Samad et al., 1997](#)). Indeed, [Krezel et al. \(1998\)](#) showed only a slight reduction (20–30%) of D₁ and D₂ receptor transcripts in retinoid receptor knockout mice. However, only a moderate hypoexpression (25–30%) of brain retinoid signaling has been reported in 27 week-old VAD rats ([Etchamendy et al., 2003](#)). Unlike the knockout model, this approach makes normal embryogenesis and postnatal de-

velopment possible. Furthermore, other substances such as docosahexaenoic acid have been shown to have a low affinity for RXRs and can probably mimic some actions of vitamin A ([de Urquiza et al., 2000](#); [Lengqvist et al., 2004](#); [Crawford et al., 2003](#)), possibly accounting for the normal expression of D₁ and D₂ receptor transcripts found in our rats. It could be argued that differences at the protein level for the D₁ and D₂ receptors might exist, accounting for the abnormalities observed in our animals. However, we believe this is very improbable, the retinoid receptor being a nuclear receptor which is known to regulate protein synthesis at the transcriptional level; therefore, if any difference exists, this should appear also at the level of the transcript for the protein encoded by that gene. Nevertheless, D₁ and D₂ pathways seem to be involved in the

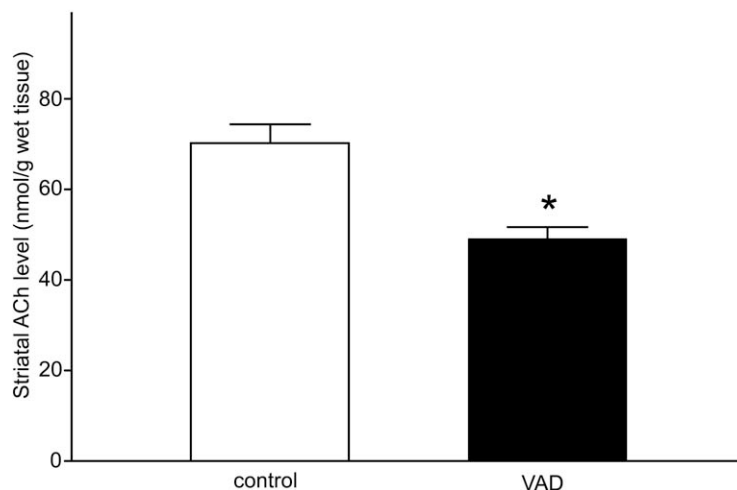


Fig. 9. ACh content in the striatum in control and VAD rats. Each value is the mean \pm S.E of eight rats. * $P < 0.01$ between control and VAD.

motor disturbance in VAD rats, as shown by the abnormal response induced by D1 and D2 receptor agonists and antagonist.

The fact that the dopaminergic system was not changed in VAD rats, prompted us to investigate the state of the striatal cholinergic interneurons. In fact, previous reports have demonstrated that retinoic acid regulates the expression of ChAT, the enzyme synthesizing ACh, and the vesicular acetylcholine transporter in cell culture (Bernard et al., 1993, 1995; Kobayashi et al., 1994; Berse and Blusztajn, 1995; Diebler et al., 1998).

Indeed, we found a significant reduction in the striatal levels of ACh after 20 weeks of VAD when the rats started to show abnormal posture and lack of normal motor coordination, suggesting a reduced ChAT activity in this area. In line with this result, Corcoran et al. (2004) have recently reported a marked reduction in the expression of ChAT in the forebrain of 6 month-old VAD rats. Accordingly, Cocco et al. (2002) found a diminished release of ACh in the hippocampal formation. Zetterstrom and co-workers (1999) were not able to show any clear expression of retinoic receptors in the large cholinergic interneurons. On the other hand, Corcoran et al. (2004) have suggested that retinoic acid is able to regulate ChAT expression in the forebrain by activating RAR α . However, this latter receptor does not appear to be expressed in the striatum. Nevertheless, the observed striatal cholinergic dysfunction demonstrates that retinoic acid regulates ChAT expression also in this area, although the retinoic receptor implicated in this event remains to be identified.

It is noteworthy that double null mutant mice RAR β -RXR β , RAR β -RXR γ and RXR β -RXR γ , despite a normal TH expression, showed a similar dysfunction in forward locomotion and motor coordination (Krezel et al., 1998), but in this work the cholinergic system has not been investigated. Moreover, Saga et al. (1999) found, in RXR γ 1 knockout mice, a selective alteration in ChAT expression in the striatal cholinergic interneurons, which was associated

with impairment of DA receptors function. Indeed, similarly to our VAD rats, these mutants exhibited an altered response to haloperidol administration.

It is well known that striatal cholinergic interneurons express both D1 and D2 receptors and modulate the activation of the direct as well as the indirect pathway (Calabresi et al., 2000; Kaneko et al., 2000; Alcantare et al., 2003).

ACh and DA exert opposing influences on striatal efferent neurons (Calabresi et al., 2000; Kaneko et al., 2000; Alcantare et al., 2003; Fiorillo and Williams, 2000). Moreover, activation of D1 receptors produces increased ACh release, while activation of D2 receptors has the opposite effect (Bertorelli and Consolo, 1990; Damsma et al., 1990; Consolo et al., 1993). It has been reported that the increase in ACh release by D1 receptor agonists is a mechanism which tends to counteract the action of D1 receptor stimulation on striatal output neurons, while the inhibition of ACh release by D2 activation should be a mechanism which tends to offset the inhibitory action that this pathway exerts on motor activation (Morelli et al., 1993). Indeed, anticholinergic drugs are often administered with the D2 antagonist haloperidol, in order to reduce extrapyramidal side effects (Chase, 1972; Levinson, 1991).

We suggest that the lack of ACh modulation of the direct and indirect pathway could be responsible for the motor dysfunction observed in VAD rats. In particular, a decreased cholinergic activity on the direct pathway could account for hyperactivation of the D1 pathway. In these animals in fact, extracellular ACh levels did not increase in response to DA D1 agonists as occurred in control animals, thus facilitating the activation of this pathway. Similarly, haloperidol induced a lesser degree of catalepsy in VAD rats due to the fact that, contrary to control animals, no increase in extracellular ACh levels could be elicited in response to D2 blockade. In line with our observations cholinergic cell-ablated mice in the nucleus accumbens showed increased sensitivity to cocaine administration

(Hikida et al., 2001). We suggest that the striatal cholinergic dysfunction may be responsible for an impaired transmission within the striatal circuits, in turn leading to the observed motor impairment.

We cannot exclude that other events are involved in the motor dysfunction shown by VAD rats. Nevertheless, in our opinion cholinergic dysfunction may play a fundamental role. Further studies should be performed in order to ascertain which brain areas are affected by the cholinergic dysfunction in VAD rats and to identify the retinoic receptors through which retinoic acid regulates ChAT synthesis. However, this goes beyond the purpose of the present study.

In conclusion, we have demonstrated for the first time the important role of vitamin A in the maintenance of the basal ganglia motor function in adult rats. Indeed, 24 week-old VAD rats showed a marked motor impairment which appears to be associated with a striatal cholinergic dysfunction.

VAD is a serious problem in many countries throughout the world. The World Health Organization estimates that over 200 million adults and children have moderate to severe VAD (WHO, 1995) with an increasing trend for morbidity and mortality. It has been estimated that 13 million subjects are affected by xerophthalmia (Underwood, 1998). Furthermore, the consequences of a deficiency of this micronutrient, as revealed by both the present study and other work performed recently, may seriously affect not only the vision, but also other important cerebral functions.

REFERENCES

- Alcantare A, Chen V, Herring BE, Mendenhall JM, Berlanga M (2003) Localization of dopamine D2 receptors on cholinergic interneurons of the dorsal striatum and nucleus accumbens on the rat. *Brain Res* 986:22–29.
- Berrard S, Faucon Biguet N, Houhou L, Lamouroux A, Mallet J (1993) Retinoic acid induces cholinergic differentiation of cultured newborn rat sympathetic neurons. *J Neurosci Res* 35:382–389.
- Berrard S, Varoqui H, Cervini R, Israel M, Mallet J, Diebler MFJ (1995) Coregulation of two embedded gene products, choline acetyltransferase and the vesicular acetylcholine transporter. *J Neurochem* 65:939–942.
- Berse B, Blusztajn JK (1995) Coordinated up-regulation of choline acetyltransferase and vesicular acetylcholine transporter gene expression by the retinoic acid receptor alpha, cAMP, and leukemia inhibitory factor/ciliary neurotrophic factor signaling pathways in a murine septal cell line. *J Biol Chem* 270:22101–22104.
- Bertorelli R, Consolo S (1990) D1 and D2 dopaminergic regulation of acetylcholine release from striata of freely moving rats. *J Neurochem* 54:2145–2148.
- Calabresi P, Centonze D, Gubellini P, Pisani A, Bernardi G (2000) Acetylcholine-mediated modulation of striatal function. *Trends Neurosci* 23:120–126.
- Chase TN (1972) Drug-induced extrapyramidal disorders. *Res Publ Assoc Res Nerv Ment Dis* 50:448–471.
- Chiang MY, Misner D, Kempermann G, Schikorski T, Giguère V, Sucov HM, Gage FH, Stevens CF, Evans RM (1998) An essential role for retinoid receptors RAR β and RXR γ in long-term potentiation and depression. *Neuron* 21:1353–1361.
- Cocco S, Diaz G, Stancampiano R, Diana A, Carta M, Curreli R, Sarais L, Fadda F (2002) Vitamin A deficiency produces spatial learning and memory impairment in rats. *Neuroscience* 115:475–482.
- Consolo S, Girotti P, Zambelli M, Russi G, Benzi M, Bertorelli R (1993) D1 and D2 dopamine receptors and the regulation of striatal acetylcholine release in vivo. *Prog Brain Res* 98:201–207.
- Corcoran JPT, Lin So P, Maden M (2004) Disruption of retinoid signalling pathway causes a deposition of amyloid β in the adult rat brain. *Eur J Neurosci* 20:896–902.
- Crawford MA, Golfetto I, Ghebremeskel K, Min Y, Moodley T, Poston L, Phylactos A, Cunnane S, Schmidt W (2003) The potential role for arachidonic and docosahexaenoic acids in protection against some central nervous system injuries in preterm infants. *Lipids* 38:303–315.
- Damsma G, Tham CS, Robetson GS, Fibiger HC (1990) Dopamine D1 receptor stimulation increases striatal acetylcholine release in the rat. *Eur J Pharmacol* 186:335–338.
- de Urquiza AM, Liu S, Sjöberg M, Zetterstrom RH, Griffiths W, Sjövall J, Perlmann T (2000) Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. *Science* 290:2140–2144.
- Diebler MF, Tomasi M, Meunier FM, Israel M, Dolezal V (1998) Influence of retinoic acid and of cyclic AMP on the expression of choline acetyltransferase and of vesicular acetylcholine transporter in NG108-15 cells. *J Physiol Paris* 92:379–384.
- Eichele G (1997) Retinoids: from hindbrain patterning to Parkinson disease. *Trends Genet* 13:343–345.
- Etchamendy N, Enderlin V, Marighetto A, Pallet V, Huguere P, Jaffard R (2003) Vitamin A deficiency and relational memory deficit in adult mice: relationships with changes in brain retinoid signalling. *Behav Brain Res* 145:37–49.
- Fiorillo CD, Williams JT (2000) Cholinergic inhibition of ventral mid-brain dopamine neurons. *J Neurosci* 20:7855–7860.
- Goodman AB (1998) Three independent lines of evidence suggest retinoids as causal to schizophrenia. *Proc Natl Acad Sci U S A* 95:7240–7244.
- Hikida T, Kaneko S, Isobe T, Kitabatake Y, Watanabe D, Pastan I, Nakanishi S (2001) Increased sensitivity to cocaine by cholinergic cell ablation in nucleus accumbens. *Proc Natl Acad Sci U S A* 98:13351–13354.
- Kaneko S, Hikida T, Watanabe D, Ichinose H, Nagatsu T, Kreitman RJ, Pastan I, Nakanishi S (2000) Synaptic integration mediated by striatal cholinergic interneurons in basal ganglia function. *Science* 289:633–637.
- Kobayashi M, Matsuoka I, Kurihara K (1994) Cholinergic differentiation of cultured sympathetic neurons induced by retinoic acid. Induction of choline acetyltransferase-mRNA and suppression of tyrosine hydroxylase-mRNA levels. *FEBS Lett* 337:259–264.
- Krezel W, Ghyselinck N, Samad TA, Dupè V, Kastner P, Borrelli E, Chambon P (1998) Impaired locomotion and dopamine signaling in retinoid receptor mutant mice. *Science* 279:863–867.
- Lengqvist J, Mata De Urquiza A, Bergman AC, Willson TM, Sjövall J, Perlmann T, Griffiths WJ (2004) Polyunsaturated fatty acids including docosahexaenoic and arachidonic acid bind to the retinoid X receptor alpha ligand-binding domain. *Mol Cell Proteomics* 3:692–703.
- Levinson DF (1991) Pharmacologic treatment of schizophrenia. *Clin Ther* 13:326–352.
- Misner DL, Jacobs S, Shimizu Y, de Urquiza AM, Solomin L, Perlmann T, De Luca LM, Stevens CF, Evans RM (2001) Vitamin A deprivation results in reversible loss of hippocampal long-term synaptic plasticity. *Proc Natl Acad Sci U S A* 98:11714–11719.
- Morelli M, Fenu S, Cozzolino A, Pinna A, Carta A, Di Chiara G (1993) Blockade of muscarinic receptors potentiates D1 dependent turning behavior and C-fos expression in 6-hydroxydopamine-lesioned rats but does not influence D2 mediated responses. *Neuroscience* 53:673–678.
- Paxinos G, Watson C (1986) The rat brain in stereotaxic coordinates, 2nd edition. San Diego: Harcourt Brace Jovanovich.

- Ross AC (1986) Separation and quantitation of retinyl esters and retinol by high-performance liquid chromatography. *Methods Enzymol* 123:68–74.
- Saga Y, Kobayashi M, Otha H, Murai N, Nakai N, Oshima M, Taketo MM (1999) Impaired extrapyramidal function caused by the targeted disruption of retinoid x receptor RXR γ 1 isoform. *Genes Cells* 4:219–228.
- Samad T, Krezel W, Chambon P, Borrelli E (1997) Regulation of dopaminergic pathways by retinoids: activation of the D2 receptor promoter by members of the retinoic acid receptor-retinoid X receptor family. *Proc Natl Acad Sci U S A* 94:14349–14354.
- Underwood BA (1998) From research to global reality: The micronutrient story. *J Nutr* 128:145–151.
- World Health Organization (1995) Global prevalence of vitamin A deficiency. MDIS working paper #2 (document WHO/NUT/95.3). Geneva, Switzerland: World Health Organization.
- Zetterstrom RH, Lindqvist E, Mata de Urquiza A, Tomac A, Eriksson U, Perlmann T, Olson L (1999) Role of retinoids in the CNS: differential expression of retinoid binding proteins and receptors and evidence for presence of retinoic acid. *Eur J Neuroscience* 11:407–416.

(Accepted 26 January 2006)
(Available online 13 March 2006)