Diurnal metabolism of dopamine in dystrophic retinas of homozygous and heterozygous retinal degeneration slow (rds) mice

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Abstract

Dopamine metabolism was studied in dystrophic retinal degeneration slow (rds) mice which carry a mutation in the rds/peripherin gene. RDS mutations in humans cause several forms of retinal degeneration. Dopamine synthesis and utilization were analyzed at various time points in the diurnal cycle in homozygous rds/rds retinas which lack photoreceptor outer segments and heterozygous rds/+ retinas which have short malformed outer segments. Homozygous retinas exhibited depressed dopamine synthesis and utilization while the heterozygous retina retained a considerable level of activity which was, nevertheless, significantly lower than that of normal retinas. By one year, heterozygous rds/+ retinas which had lost half of the photoreceptors still maintained significant levels of dopamine metabolism. Normal characteristics of dopamine metabolism such as a spike in dopamine utilization at light onset were observed in mutant retinas. However, light intensity-dependent changes in dopamine utilization were observed in normal but not rds/+ retinas. The findings of this study suggest that human patients with peripherin/rds mutations, or other mutations that result in abnormal outer segments that can still capture light, might maintain light-evoked dopamine metabolism and dopamine-dependent retinal functions during the progression of the disease, proportional to remaining levels of light capture capabilities. However, visual deficits due to reduced light-evoked dopamine metabolism and abnormal patterns of dopamine utilization could be expected in such diseased retinas. © 2000 Elsevier Science B.V. All rights reserved.

Theme: Sensory systems

Topic: Retina and photoreceptors

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1. Introduction

Retinitis Pigmentosa (RP) is a clinical diagnosis that encompasses a group of heterogeneous hereditary disorders that manifest progressive loss of photoreceptors and blindness. The prevalence of the disorder is about 1/4000, which makes it a common cause of visual impairment in all age groups [2]. Mutations in multiple genes have been shown to cause blindness due to photoreceptor cell-death [34,52]. Over 50 mutations in the RDS gene have been implicated in dominant forms of RP and macular degeneration [28,47].

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2. Material and methods

2.1. Animals

Handling of animals conformed with principles regarding the care and use of animals adopted by the American Physiological Society and Society for Neuroscience.

Normal BALB/c mice, mutant homozygous rds/rds (020/A) mice, and heterozygous rds/+ mice were studied. A breeding colony of BALB/c and rds/rds mice is maintained at the Animal Facility of the University of Texas Health Science Center. Heterozygous rds/+ mice (F1) were produced by breeding BALB/c and rds/rds mice. The mice are housed in light-proof rooms on a 12 h dark/light cycle, with light on at 8 AM. Throughout this paper, “day” refers to the light phase and “night” refers to the dark phase of the imposed dark/light cycle. Lighting was provided by fluorescent tubes. Light intensity at cage levels was 3–5 foot candles (fc). Experiments were carried out in a laboratory under selected illumination levels. Overhead illumination was provided by cool white fluorescent bulbs at a room temperature of 21°C. Illumination was measured with a digital illuminance meter (DX-200, INS Enterprise Inc., Taiwan). The mice were euthanized by cervical dislocation. Following enucleation, the anterior structures and lens were removed and the retina was dissected from the posterior eye cup. Procedures in the “dark” were carried out under dim red light (Kodak safety light filter no. 1).

2.2. Electron microscopy

Eyes were placed in 4% formaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for fixation. After 30 min, the eyes were bisected along the vertical meridian and the two hemispheres were fixed for an additional 3 h. The tissue was then treated in 1% OsO4, dehydrated and embedded in an epoxy resin. Thin sections were cut at different sites along the central–peripheral axis of the retina and stained with uranyl and lead salts before viewing under the electron microscope.

2.3. Enzyme inhibitions

Synthesis of dopamine from l-tyrosine is a two step process: (1) hydroxylation by tyrosine hydroxylase (TH) to produce l-3,4-dihydroxyphenylalanine (l-DOPA); and (2) decarboxylation of DOPA by aromatic l-amino acid decarboxylase (AAAD) to produce dopamine. DA synthesis and utilization were analyzed after inhibition of AAAD with m-hydroxybenzylhydrazine (NSD-1015; Sigma Chemical Co.). Inhibition of AAAD results in accumulation of DOPA, which is used as an estimation of in situ TH activity and DA synthesis. Utilization of DA is reflected by the decline in DA levels following inhibition of AAAD. The drug was dissolved in phosphate-buffered saline (pH 7.0) and injected intraperitoneally (150 mg/kg body weight). Groups of 5–7 mice were injected and each mouse was placed, individually, in a clear plastic cage and illuminated for 30 min at 60 fc, unless noted otherwise. The retinas were then isolated and frozen in liquid nitrogen.

2.4. Catecholamine analysis

DA, DOPAC and DOPA levels were analyzed by high pressure liquid chromatography (HPLC) with electrochemical (EC) detection. Two retinas of each mouse were pooled for one sample. HPLC analysis was carried out as previously described [39]. Briefly, frozen retinas were homogenized in ice cold 0.1 M perchloric acid containing...
10 μM ascorbic acid and 20 ng/ml 3,4-dihydroxybenzylamine (DHBA), an internal standard. Homogenates were centrifuged and aliquots of each supernatant fraction were injected into a Beckman Ultrasphere-ODS reverse phase column. DA and metabolites were eluted with a mobile phase consisting of 100 mM phosphoric acid, 0.1 mM EDTA, 0.45 mM sodium octylsulphate and 6% acetonitrile at pH 2.6–2.7 and were detected amperometrically. Chromatographic peaks were identified by relative retention times compared to those of external standards. Concentrations were determined by comparing peak areas of unknowns with those of standards. Values are corrected for the recovery of DHBA. Retinal protein was determined by the method of Lowry [31].

3. Results

DA metabolism in the retina was determined by the following measurements: (a) steady state levels of DA and DOPAC, the main DA degradation product, and (b) DA utilization and synthesis (in situ TH activity) in mice treated with AAAD inhibitor NSD-1015.

3.1. DA metabolism in young rds/+ and rds/rds mice

One to two month old BALB/c, rds/+ and rds/rds mice were studied. By this age, photoreceptor differentiation is completed, photoreceptor cell loss is not yet measurable in the rds/+ retinas [18], and only the initial wave of photoreceptor cell loss occurred in the rds/rds retina [38,44]. The mutation phenotypes, as expressed in the organization of the photoreceptor outer segments, are depicted in Fig. 1. While normal BALB/c photoreceptors outer segments consist of stacks of photopigment-containing disc membranes (Fig. 1A), in the homozygous rds/rds mutant photoreceptors disc membranes are not formed (Fig. 1B). The heterozygous rds/+ outer segments are characterized by malformed disorganized disc membranes (Fig. 1C).

3.1.1. Steady state levels of DA and DOPAC in night and day periods

Steady state levels of DA at 2 h into the night period (in darkness) and 2 h into the day period (in light) are shown in Fig. 2. There was no difference in steady state levels between dark and light in each of the three genotypes.

![Fig. 1. Ultrastructure of photoreceptor outer segments in normal and mutant retinas. (A) Normal BALB/c photoreceptors. The rod outer segment is characterized by stacks of photopigment-containing disc membranes. ×14 250. (B) Dystrophic homozygous rds/rds photoreceptors. Outer segments are not formed. Only rudimentary membranous structures are seen at the tip of the connecting cilium (arrow). ×17 750. (C) Heterozygous rds/+ photoreceptors. Disorganized clusters of disc membrane are seen. ×11 250. (ROS: rod outer segment; RIS: rod inner segment; C: connecting cilium.)](image-url)
Fig. 2. Steady state levels of DA and DOPAC in 1–2 month old BALB/c, rds/+ and rds/rds retinas. Mice were euthanized 2 h into the night period and 2 h into the day period. Catecholamines were determined by HPLC–EC. Each value is the mean ± S.E.M. from measurements of 6–7 mice (n=6–7). DA: There is no difference in steady state levels in night and day between BALB/c and rds retinas. Levels in rds/1 are higher both in night and day from levels measured in BALB/c and rds/rds retinas. DOPAC: In BALB/c and rds/+ retinas, levels in the day are much higher than those at night. Significant differences are seen in steady state levels of DOPAC between the 3 genotypes, both in night and day. Levels of DOPAC in rds/+ retina in day are significantly higher than the daytime levels in rds/rds retinas. (a) P < 0.004 vs. rds/1 dark; P < 0.001 vs. rds/rds dark, (b) P = 0.002 vs. rds/+ light; P < 0.001 vs. rds/rds light, (c) P < 0.001 vs. rds/rds light.

Between genotypes, steady state levels in rds/+ were higher both in dark (12%) and light (29%) compared to levels measured in normal BALB/c retina. Steady state levels of DOPAC were low in the three genotypes in the dark and increased upon illumination (Fig. 2). Differences in steady state levels of DOPAC between the 3 genotypes were measured both in night and day, with highest levels measured in the BALB/c retinas both in night and day. In comparison with normal BALB/c retina, levels of DOPAC in homozygous rds/rds retina were 73% lower in the night and 81% lower in day. In the heterozygous rds/+ retinas, levels of DOPAC were 47% lower in night but only 21% lower in day, indicating a much higher response to light as compared with the rds/rds retina.

3.2. DA utilization and synthesis at light onset

Since major cellular events such as disc shedding occur at light onset at the beginning of the day, DA utilization and synthesis in NSD-1015 treated mice were analyzed at light onset and 2–2.5 h into the day period.

The highest levels of DA utilization and synthesis were measured in normal mice at light onset when fully dark-adapted photoreceptors respond maximally to light. DA utilization in the BALB/c retina during the first 0.5 h in light was 73% higher than levels measured at 2.5 h into the day period. In the rds/+ retina, DA utilization in the first 0.5 h in light was 56% higher than levels at 2.5 h into the day period (Fig. 4). In the rds/rds retina, although DA utilization in light was limited in comparison to BALB/c and rds/+ retinas, residual DA levels at 0.5 h in light (0.98 ± 0.064 ng DA/2 retinas, n=7) are 21% lower than levels measured at 2 h into the day period (1.2 ± 0.004 ng DA/2 retinas, n=9, P < 0.001), indicating an elevated utilization at light onset. Similar to utilization, DA synthesis was highest at light onset. In comparison with levels at light onset, the BALB/c retinas showed a 36% decrease at 2.5 h into the day and the rds/+ retina showed a 45% decrease in DA synthesis (Fig. 4). No significant change in DA synthesis was measured in the rds/rds retina.

3.3. DA utilization and synthesis under low and high illumination levels

The consequences of the abnormal outer segments on the retinal sensitivity to light were analyzed by measure-
Fig. 3. DA utilization and synthesis in 1–2 month old normal and mutant retinas. Mice were studied 2 h into the day period. DA and DOPA levels were measured after injection of NSD-1015 (150 mg/kg) and illumination at 60 fc for 30 min. Control littermates were not treated. Each value is the mean±S.E.M from measurements of 5–8 mice (n=5–8). DA: Utilization, as determined by reduction in DA levels, was high in treated normal BALB/c mice, lower in treated rds/+ mice and very limited in rds/rds mice. DOPA: DA synthesis, as determined by accumulation of DOPA, was highest in treated BALB/c retinas. In the rds/+ retinas, synthesis was lower than in normal retinas but significantly higher than in rds/rds retinas. (a) \( P<0.001 \) vs. rds/+ treated; (b) \( P<0.001 \) vs. rds/rds treated, (c) \( P<0.001 \) vs. rds/+ treated; (d) \( P<0.001 \) vs. rds/rds treated.

4. DA metabolism in one year old rds/+ and rds/rds mice

Eleven to twelve month old BALB/c, rds/+ and rds/rds mice were studied. By this age most of the photoreceptors were lost in the rds/rds retina and about 50% of photoreceptors were lost in the rds/+ retina. The remaining photoreceptors in the rds/+ retinas carry abnormal rod outer segments similar to those seen in young rds/+ mice (Fig. 1).

3.4.1. Steady state levels of DA and DOPAC in night and day periods

Steady state levels of DA and DOPAC were studied 2 h into the night period (BALB/c and rds/+) and 2–3 h into the day period (BALB/c, rds/+ and rds/rds). There were no significant differences in the steady state levels of DA between the 3 genotypes (Fig. 6). The slightly higher (14%) steady state level of DA in rds/+ in the dark follows a pattern similar to that measured in young rds/+ retinas (see Fig. 2). Considerable differences in steady state levels of DOPAC between the 3 genotypes were measured in the day period, with levels in BALB/c retina the highest. Levels of DOPAC in illuminated rds/+ retinas were 35% lower as compared with BALB/c retinas, while levels in illuminated rds/rds retinas were 72% lower. Large night–day differences in DOPAC levels persisted in retinas of one year old rds/+ mice. Since night/day differences in DOPAC levels were already shown to be very small in young rds/rds retinas (Fig. 2), night levels of DOPAC in old rds/rds retinas were not analyzed because of limited availability of old rds/rds mice.

3.4.2. DA utilization and synthesis in day period

DA Utilization and synthesis were studied 3–5 h into the day period (Fig. 7). Both utilization and synthesis were maintained at relatively high levels in the old rds/+ mutant (Fig. 7). In normal retina, an 81% decline in DA from steady state levels was measured in NSD-1015 treated retina. A considerable decline (68%) was measured also in the heterozygous rds/+ retina whereas in the homozygous rds/rds retina only a 27% decline in DA levels was measured. DA synthesis, as depicted by DOPA accumulation, revealed corresponding differences between the different genotypes. BALB/c retina demonstrated the highest rate of synthesis. In comparison to normal, DA synthesis was 17% lower in the rds/+ retina and 80% lower in the rds/rds (Fig. 7).
photoreceptor cell death [39]. In the present study of the rds/+- retina we have found that the heterozygous retina, which is characterized by the presence of short and disorganized outer segments, is capable of considerable levels of DA turnover in comparison with the homozygous rds retina. However, DA metabolism is still significantly reduced in comparison to normal retinas. The reduced DA utilization and synthesis as measured in the present study in the 1–2 month old rds/+ retinas, prior to the onset of significant photoreceptor cell loss, is an indication of reduced light capture by the mutant retina. Electroretinogram (ERG) measurements of rds/+ retina revealed reduced rod ERG in 2 month old mice which might be a reflection of abnormalities in outer segment size and organization [10]. Thus, there appears to be a general correlation between structural integrity of outer segments, light capture as measured by the electroretinogram and light-evoked DA metabolism.

The correlation between outer segment structural integrity, the amount of light capture and DA turnover is a result of light-evoked regulation of key enzymes in DA synthesis. Increased DA synthesis in light is due to activation of TH, the rate limiting enzyme in DA synthesis [21,22], and induction of AAAD [17]. TH activation and DA release are linked to light by the removal of tonic inhibition of dopaminergic cells by GABA and direct light-dependent activation of dopaminergic cells via glutamate released by bipolar cells [5,6,16,26,33]. Interestingly, in spite of the large differences in DA turnover in the normal, rds/rds and rds/+ retinas, steady state levels of DA were similar in the three genotypes both in dark and light. Moreover, normal steady state levels of DA were maintained in old rds/+ and rds/rds retina, which suffered major photoreceptor loss. This might be a result of an efficient feedback mechanism by which reduced light-evoked synthesis and utilization are calibrated to maintain the DA storage at full capacity.

Further insight into the relationship between outer segment structural integrity and light sensitivity as related to DA metabolism during the first 0.5 h in light, at the beginning of the day period, and after 2 h into the day period. Mice, 2 month old, were treated with NSD-1015 and illuminated for 30 min at 60 fc before sacrifice. The first group of mice was treated with NSD-1015 in the dark, at the end of the night period, illuminated for 30 min at light onset and sacrificed after 0.5 h into the day period. Other groups were treated at 2 h into the day period and sacrificed 30 min later, at 2.5 h into the day period. Each value is the mean±S.E.M from measurements of 6–7 mice (n=6–7). DA: In BALB/c and rds/+ retinas, utilization at light onset, at the beginning of the day period, was much larger than that measured at 2.5 h into the day period. DOPA: In BALB/c and rds/+ retinas, accumulation was higher in the first 0.5 h into the day period compared to that at 2.5 h. (a) P=0.006 vs. 2.5 h, (b) P<0.001 vs. 2.5 h, (c) P<0.001 vs. 2.5 h, (d) P<0.001 vs. 2.5 h.

4. Discussion

In a previous study we showed that light evoked DA turnover is greatly reduced in rds/rds retinas that are devoid of outer segments and undergo an early onset
Fig. 5. DA utilization and synthesis in BALB/c and rds/+ retinas at 10 and 60 fc illumination. Two month old mice were kept under 10 fc illumination for the first 3–4 h of the day period. They were subsequently treated with NSD-1015 and exposed for 30 min to either 10 fc or 60 fc illumination. Each value is the mean±S.E.M. from measurements of 6–7 mice (n=6–7). DA: While utilization in BALB/c retinas increased significantly as light intensity increased from 10 to 60 fc, there was no difference in DA utilization in the rds/+ retina. DOPA: Exposure to higher illumination levels resulted in a significant increase in DOPA accumulation in the rds/+ retina but not in the BALB/c retina. (a) P<0.001 vs. 10 fc, (b) P=0.016 vs. 10 fc.

Fig. 6. Steady state levels of DA and DOPAC in one year old BALB/c, rds/+ and rds/rds retinas. Mice were euthanized 2 h into the night period and 2 h into the day period. Each value is the mean±S.E.M. from measurements of 5–6 mice (n=5–6). DA: There were no differences in steady state levels of DA between the 3 genotypes. DOPAC: Graded differences in DOPAC levels in the day period were seen between the genotypes, with normal BALB/c retinas the highest. Note that the old rds/+ retinas demonstrate a considerable night/day change in DOPAC levels. (a) P=0.001 vs. rds/+; P<0.001 vs. rds/rds, (b) P<0.001 vs. rds/rds.
Fig. 7. DA Utilization and synthesis in one year old BALB/c, rds/+ and rds/rds retinas. Mice were analyzed 2–3 h into the day period. Each value is the mean±S.E.M. from measurements of 5–6 mice (n=5–6). DA: Utilization levels, as determined by decline in DA levels in NSD-1015 treated mice, were highest in BALB/c retina. Significant utilization was seen also in treated rds/+ retinas but relatively limited utilization in treated rds/rds retinas was observed. DOPA: Synthesis, as reflected by DOPA accumulation in treated retinas, was considerable in the old rds/+ retinas, whereas that in treated rds/rds retinas was small. (a) \( P<0.002 \) vs. rds/+ treated; \( P<0.001 \) vs. rds/rds treated, (b) \( P<0.001 \) vs. rds/rds treated, (c) \( P<0.016 \) vs. rds/+ treated; \( P<0.001 \) vs. rds/rds treated, (d) \( P<0.001 \) vs. rds/rds treated.

regulation of target retinal neurons in an illumination-dependent manner.

Previous analysis of DA metabolism in normal BALB/c retina revealed a dramatic spike in DA synthesis and utilization at light onset, at the beginning of the day period [40]. In the present study, significantly higher levels of DA synthesis and utilization at light onset were measured also in the rds/+ retina. Furthermore, even the rds/rds retina with very diminished light capture and a comparatively low level of DA utilization during the light period, showed a significant increase in DA utilization at light onset. Thus, in mutant retinas with major outer segment abnormalities, the transition between the night and day phases of the diurnal cycle is translated into a transient increase in DA release that separates the initial period in light from the rest of the day period. It appears that a residual signal transduction capability, mediated by remaining photopigment molecules in the rudimentary discs and the plasma membrane of rds/rds photoreceptors [37,53], might be sufficient to elicit a light-evoked spike of DA release in dopaminergic amacrine cells at light onset. Alternatively, non-traditional photoreceptors, such as those thought to regulate circadian photoreceptions [55], might influence DA neuronal activity.

The observed spike in DA utilization at light onset coincides with peak outer segment disc shedding in the mouse retina [3]. Although direct evidence for a role for DA in regulation of disc shedding in the mammalian retina is not available, studies of amphibian retinas suggested that changes in DA release during the day period might be involved in regulation of disc shedding [4]. In the rds/+ retina, unlike normal albino mice, a peak in disc shedding was recorded near the end of the light period [45]. Hence, if DA is involved in regulation of disc shedding, the sharp change in DA turnover which was measured in the rds/+ at light onset might not correspond with peak shedding in this retina.

By one year, steady state levels of DA in the mutant rds/+ and rds/rds retinas were maintained at the same levels as in 1 yr old normal BALB/c retina. Furthermore, the ratios of synthesis and utilization in the mutants as compared to normal retinas at 1 yr (Fig. 7), was close to that measured in young mice (Fig. 2). Thus, in 1 yr old rds/+ and rds/rds mice, loss of photoreceptors and lifelong light deprivation did not exacerbate the deficiencies which were already measured in young mutant mice. The maintenance of relatively high levels of light-evoked DA turnover in 1 yr old rds/+ retina is of interest since, by 1 yr, about half of the photoreceptors were lost in the rds/+ retina. A possible explanation might be found in alterations...
in synaptic connectivity. Increase in the areas of synaptic contact between rod terminals and second order neurons was observed in the rds/+ mutant [24]. It was suggested that the increased synaptic contact might compensate for chronic cell loss and help maintain a threshold level of signal transmission [24].

An important issue is whether the course of the disease in the homozygous rds/rds retinas is affected by the very low release of DA from dopaminergic cells. In view of the possible role of DA in photoreceptor metabolism either directly through interaction with D2/D4 receptors on the photoreceptor cell [11], or indirectly through its interaction with other neurotransmitters [19] and growth factors [9], reduced availability of DA in dystrophic retinas might affect photoreceptor viability. Previously, a survival promoting effect of DA was demonstrated in a study where administration of a D2 receptor agonist, bromocriptine, protected photoreceptors against light damage in normal rat retinas and degenerative photoreceptor cell death in dystrophic RCS rat retinas [8]. However, similar protection was not observed in rds/rds retinas that were treated daily, between postnatal day 14 and 28, with D2 receptor agonists (bromocriptine; quinpirole) or D1 receptor agonists (SKF 38393; 6-Chloro-PB-hydrobromide). None of these prevented the 30–40% photoreceptor cell death observed in 1 month old rds/rds retinas [Nir, unpublished findings].

Since human RP patients with rds mutations are mostly heterozygous, data obtained with the rds/+ mice might be predictive of the levels of DA metabolism of RP patients with abnormal photoreceptors. Indications for the direct role of DA deficiency on visual functions in humans were obtained from observations of Parkinson patients. Depleted retinal DA stores as measured in post mortem eyes [36], were linked to impaired retinal processing as determined by psychophysical and electrophysiological studies [7,20]. It can be expected, therefore, that in RP patients, in addition to direct loss of visual functions due to photoreceptor pathology, further visual deficits might be caused by abnormal light-evoked retinal DA synthesis and utilization.

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