

Development and Regulation of Rhodopsin Kinase in Rat Pineal and Retina

Anthony K. Ho, *Robert L. Somers, and David C. Klein

Section on Neuroendocrinology, Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development, and *Section on Retinal Metabolism, Laboratory of Vision Research, National Eye Institute, National Institutes of Health, Bethesda, Maryland, U.S.A.

Abstract: Rhodopsin kinase, once thought to be a retinal enzyme, was recently found at high levels in the pineal gland. In the present study the developmental pattern and the regulation by environmental lighting of this enzyme in both tissues was studied in the rat. Enzyme activity was present in the neonatal pineal gland several days earlier than in the retina, and increased gradually up to 20 days of age and remained at that level thereafter; the retinal enzyme appeared to increase until day 60. Pineal and retinal rhodopsin kinase activities showed a 25% increase in the middle of the dark and the beginning of the light period, respectively. Exposure to constant light caused a 50% decrease in rhodopsin kinase levels in both tissues. However, only pineal rhodopsin kinase activity declined

followed bilateral superior cervical ganglionectomy. This indicates pineal rhodopsin kinase activity is similar to other pineal enzymes in that it is controlled by light acting through the sympathetic nervous system. In contrast, the light-induced decrease in retinal rhodopsin kinase may be due to the direct destructive effect of light on the retina. The finding of neural control of pineal rhodopsin kinase in the pineal gland of adult rats is consistent with a function of the enzyme in the neural regulation of pineal function. **Key Words:** Rhodopsin kinase—Pineal—Retina—Development—Regulation. Ho A. K. et al. Development and regulation of rhodopsin kinase in rat pineal and retina. *J. Neurochem.* 46, 1176–1179 (1986).

Rhodopsin kinase (RK), a soluble enzyme that selectively phosphorylates photon-activated rhodopsin (Bownds et al., 1972; Kuhn and Dreyer, 1972; Shichi and Somers, 1978), was originally thought to be localized exclusively in the retina. Recent studies have demonstrated that comparable amounts of this enzyme are also present in the mammalian pineal gland (Somers and Klein, 1984). The presence of this phototransduction-related protein in the pineal gland probably reflects the ancestral function of the pineal gland as a photoreceptor (Kappers, 1969; Collin, 1971; Oksche, 1984). Although the mammalian pineal gland has lost the capacity of direct photoreception, morphological features typical of photoreceptor cells have been found in the neonatal rat pineal gland but not in the adult rat gland (Clabough, 1973; Zimmerman and Tso, 1975). Accordingly, the first objective of this study was to investigate the developmental pattern

of RK in the rat pineal gland and compare it to that in the retina.

The second issue of interest was the regulation of RK. The biochemical activity of the mammalian pineal gland is regulated by environmental lighting acting indirectly through a neural circuit extending from the retina via a retinohypothalamic projection to the suprachiasmatic nuclei (SCN) (Klein and Moore, 1979). It contains a circadian clock which drives the pineal gland. Light acts to reset the clock and blocks the stimulation of the pineal gland by the SCN. From the SCN, the neural circuit passes through both central and peripheral structures including the superior cervical ganglia (SCG) (Moore, 1978). Neural stimulation of the pineal gland at night increases pineal serotonin *N*-acetyltransferase (NAT) activity 30- to 70-fold (Klein and Weller, 1970) and maintains the normal activity of hydroxyindole-*O*-methyltransferase (HIOMT).

Received July 22, 1985; accepted October 22, 1985.

Address correspondence and reprint requests to Dr. A. K. Ho at Room 8D-42C, Building 10, National Institutes of Health, Bethesda, MD 20892, U.S.A.

Abbreviations used: HIOMT, hydroxyindole-*O*-methyltransferase; LD, light-dark; LL, continuous lighting; NAT, *N*-acetyltransferase; RK, rhodopsin kinase; SCN, suprachiasmatic nuclei; SCG, superior cervical ganglia.

These two enzymes convert 5-hydroxytryptamine (serotonin) to melatonin. When this stimulation is blocked either by light acting on the retina or surgical removal of the SCG, pineal NAT activity does not increase at night in the dark and HIOMT activity decreases slowly over the course of weeks (Wurtman et al., 1963; Moore and Rapport, 1971; Klein and Moore, 1979; Sugden and Klein, 1983). In view of this, it was of interest to determine whether RK in the pineal is regulated by the same retinal-pineal neural circuit.

MATERIALS AND METHODS

Animals

The animals used were Sprague-Dawley rats (Zivic Miller Laboratories, Allison Park, PA, U.S.A.). Unless otherwise specified, they were housed under a diurnal lighting cyclic [light-dark (LD) 14:10, lights on at 0500 hours) with food and water ad libitum. In the developmental studies rats of known age or conception date were housed in the above conditions for 1 week before the experiment. Male rats were used for age 15 days or above and mixed sex litters at all other ages. On the day of the experiment, rats were killed by decapitation between 1200 and 1500 hours. Retinae and pineal glands were dissected, frozen on solid CO₂, and stored at -60°C until assayed for RK activities.

In the daily rhythm study, male rats (weight between 150 and 175 g on arrival) were used. The animals were maintained in LD 14:10 lighting schedule for 2 weeks before being killed at six time points throughout 24-h periods. Retinae and pineal glands were dissected and stored as above. A dim red light was used during the dark period to handle animals and to dissect tissues.

In the photoneural regulation study, bilateral superior cervical ganglionectomized (SCGX) rats or sham-operated animals were maintained in the LD 14:10 lighting schedules or the continuous lighting (LL) for 3 weeks before being killed between 1000 and 1200 hours. The surgical procedure was performed by the supplier.

RK assay

RK activity was determined by a published method (Shichi et al., 1983; Somers and Klein, 1984). Tissue samples were homogenized in a 10 mM PIPES buffer, pH 7, containing 4 mM dithiothreitol and 1 mM EDTA. In the developmental study pineal samples consisted of pools of eight glands at 3 days before and 2 days after birth, five glands at 5 days and 10 days after birth, three glands at 15 days after birth, and one gland each for older rats. Retina samples consisted of four pairs of retinae at 2 days after birth, three pairs at 5 days after birth, and one pair for the older rats. The homogenate was centrifuged at 12,000 g for 5 min, and the protein concentration of the supernatant was determined by a dye binding method, with bovine serum albumin as the standard (Bradford, 1976).

An ~5 µg sample of the supernatant soluble protein from the tissue samples was added to 50 mM potassium phosphate buffer, pH 6.8, containing 1 mM MgCl₂, 0.2 mM vanadate, 0.1 mM [³²P]ATP (1 × 10⁶ cpm/nmol). To initiate the reaction, 0.5 nmol of rhodopsin in urea-treated bovine rod-outer segments was added and incu-

bated at 30°C for 5 min. The final volume of the reaction mixture was 50 µl. To terminate the reaction 200 µl of 0.125 M potassium phosphate buffer, pH 6.8, 62.5 mM potassium fluoride, 12.5 M EDTA, and 6.25 mM ATP was added. The reaction tubes were then centrifuged at 12,000 g for 5 min and the phosphorylated opsin pellets were dissolved in 40 µl of 10 mM Tris acetate buffer, pH 7.4, containing 2% sodium dodecyl sulfate, 2% β-mercaptoethanol, and 5 mM EDTA. After the addition of 10 µl of 50% glycerol containing 0.05% bromophenol blue, the samples were applied to a 10% acrylamide-sodium dodecyl sulfate gel (Laemmli, 1970). Electrophoresis was for 30–40 min at 60 V and then 3.5 h at 165 V. The gel was then stained by Commassie Blue, dried, and autoradiographs prepared. The [³²P]phosphorylated opsin bands were identified, removed, and radioactivity was determined.

Statistical analysis

Data were analyzed by Duncan's Multiple-Range Test (Duncan, 1955).

RESULTS

Development

Pineal RK activity was detectable 2 days after birth, and then increased in a linear fashion until 20 days after birth. No significant changes were observed after this. In contrast, retinal RK activity was not detectable at 2 or 5 days after birth. Subsequently, retinal RK increased in a linear fashion up to 20 days and doubled between 20 and 60 days of age (Fig. 1).

Daily rhythm

Pineal RK activity at night was significantly

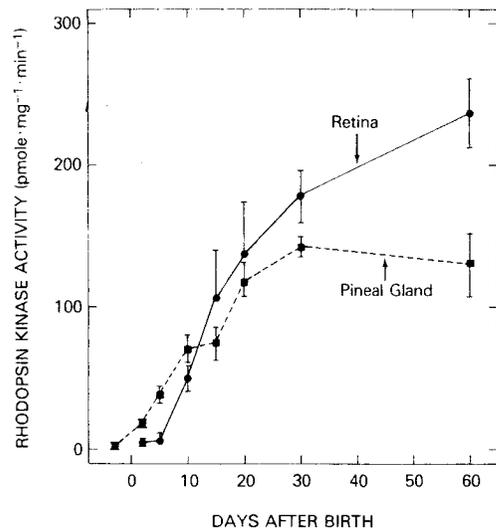


FIG. 1. Developmental pattern of RK activity in rat pineal gland and retina. Tissues were obtained from 3–60 days old rats. Pineal samples consisted of pools of one to eight glands; retina samples consisted of pools of one to four pairs of retinae. Each point represents the mean ± SEM of five determinations. For further details see Materials and Methods.

higher than the values obtained at 1100, 1300, and 1900 hours ($p < 0.05$). Also, retina RK activity at 0500 hours was significantly higher than all other retina RK values ($p < 0.05$) (Fig. 2).

Photoneural regulation

SCGX or continuous lighting for 3 weeks produced a significant 50% reduction in the pineal RK activity ($p < 0.01$). In the retina, however, only continuous lighting decreased RK activity ($p < 0.01$); SCGX had no effect (Fig. 3).

DISCUSSION

The adult mammalian pineal gland has evolved into a compact secretory organ (Kelly, 1962). However, histological investigations have shown that 4 days after birth, rat pinealocytes display some morphological features similar to those of developing photoreceptors (Zimmerman and Tso, 1975), features that are no longer apparent after 17 days of age. In contrast, the present results show that pineal RK activity has the opposite pattern. It is low at birth, increases continuously up to 20 days after birth, and remains at high level after maturation. This suggests that pineal RK is not associated with photoreceptor-like morphological features in the developing rat pineal gland.

Another interesting finding of the developmental study of RK in the pineal and the retina is that pineal RK activity was detectable earlier than that of the retinal RK, and was higher per milligram protein than the retinal RK activity up to 10 days after birth. This may indicate an earlier differentiation of the pineal gland during its development as compared to the retina and is in accordance with the findings in some submammalian species that the

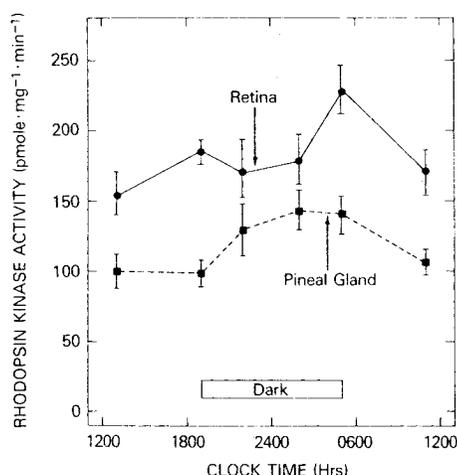


FIG. 2. Daily variations of the RK activities in the rat pineal gland and retina. Rats were housed under a diurnal lighting cycle (LD 14:10, lights on 0500 hours) for 3 weeks before the experiment. Each point represents the mean \pm SEM of five determinations.

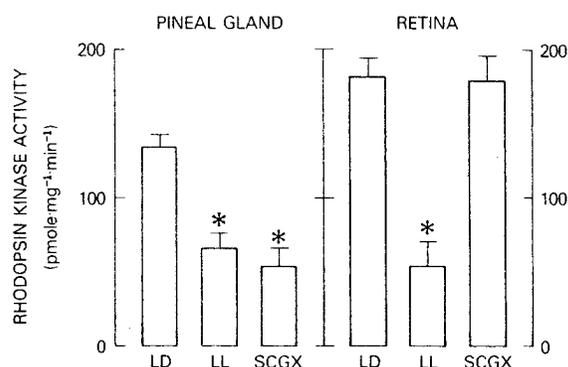


FIG. 3. Effects of constant light or SCGX on the RK activity in the rat pineal gland and retina. The tissues were obtained from sham operated (LD) or SCGX rats housed in diurnal lighting (LD 14:10), or sham-operated rats in constant light (LL) for 3 weeks. Each value represents the mean \pm SEM from six animals. *Significantly different from LD animals, $p < 0.01$.

development of the photosensitive pineal gland precedes that of the retina (van Veen et al., 1984).

Pineal RK activity in the middle of the dark period is significantly higher than the daytime levels. This, together with the observation that the pineal RK is decreased after exposing rats to constant light, or SCGX, indicates that RK in the pineal gland is regulated by photoneural mechanisms and responds slowly to neural stimulation. In this regard, pineal RK is very similar to another retina and pineal specific enzyme, HIOMT (Axelrod et al., 1965; Quay, 1965; Cardinali and Wurtman, 1972; Sugden and Klein, 1983).

It is unlikely that the observed changes in RK are due to changes in protein phosphatase activity because vanadate, a potent phosphatase inhibitor (Lopez et al., 1976) is present in the assay buffer.

In contrast to the daily pattern in pineal RK, retinal RK was higher at the onset of the light period (0500) as compared to midnight and midday. It is interesting to note that in rats, a burst of rod outer segment disk shedding also occurs in the retina soon after onset of the light period (LaVail, 1976), which is thought to be a direct response to light. Whether these two events are related remains to be determined. It is also possible that another direct effect of light might explain the decrease in retinal RK in LL. Long-term exposure to light causes severe photoreceptor damage and degeneration in the retina of albino rats (Kuwabara and Gorn, 1968; O'Steen et al., 1972); this would be expected to decrease RK.

The high level of RK in the mature rat pineal gland is especially intriguing in view of the report that the pineal gland of the mature rat does not contain rhodopsin-like immunoreactivity (Vigh and Vigh-Teichmann, 1981). The question of the identity of natural substrate and the function of RK in the pineal gland remains to be determined. In the

retina, RK, which binding to the rhodopsin is light-dependent (Kuhn, 1978), is thought to play a critical adaptive role in photochemical transduction in the retina by reducing the potency of photoactivated rhodopsin to stimulate guanosine 3',5'-monophosphate phosphodiesterase (Sitaramyya et al., 1977; Sitaramyya and Liebman, 1983). The evidence of neural regulation of pineal RK may reflect an important function. It has been proposed that RK may be involved in neurochemical transduction and phosphorylate an integral membrane receptor for norepinephrine in the pineal (Somers and Klein, 1984). The validity of this proposal remains to be tested.

Finally, it is interesting to speculate on the observation that the response of pineal RK to environmental lighting and SCGX is similar to that of HIOMT. Neither enzyme has a large circadian rhythm and both decline gradually after SCGX or exposure to constant light. Retinal RK appears to be involved in attenuating the level of cyclic GMP; cyclic GMP has been suggested as a regulator of pineal HIOMT activity (Sugden and Klein, 1983). Perhaps RK is involved in the regulation of pineal HIOMT activity via a cyclic GMP mechanism.

REFERENCES

- Axelrod J., Wurtman R. J., and Snyder S. (1965) Control of hydroxyindole-*O*-methyltransferase activity in the rat pineal gland by environmental lighting. *J. Biol. Chem.* **204**, 949-954.
- Bownds D., Dawes J., Miller J., and Stahlman M. (1972) Phosphorylation of frog photoreceptor membranes induced by light. *Nature* **237**, 125-127.
- Bradford M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
- Cardinali D. P. and Wurtman R. J. (1972) Hydroxyindole-*O*-methyltransferase in rat pineal, retina and harderian gland. *Endocrinology* **91**, 247-252.
- Clabough J. W. (1973) Cytological aspects of pineal development in rats and hamsters. *Am. J. Anat.* **137**, 215-230.
- Collin J. P. (1971) Differentiation and regression of the cells of the sensory line in the epiphysis cerebri, in *The Pineal Gland* (Wolstenholme G. E. W. and Knight J., eds.), pp. 79-120. J. & A. Churchill, London.
- Duncan D. B. (1955) Multiple range and multiple F-tests. *Biometrics* **11**, 1-42.
- Kappers J. A. (1969) The mammalian pineal organ. *J. Neurovisc. Relat.* (Suppl.) **9**, 140-184.
- Kelly D. E. (1962) Pineal organs: photoreception, secretion and development. *Am. Sci.* **50**, 597-625.
- Klein D. C. and Moore R. Y. (1979) Pineal *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase: control by the retinohypothalamic tract and the suprachiasmatic nucleus. *Brain Res.* **174**, 245-262.
- Klein D. C. and Weller J. L. (1970) Indole metabolism in the pineal gland: A circadian rhythm in *N*-acetyltransferase. *Science* **169**, 1093-1095.
- Kuhn H. (1978) Light-regulated binding of rhodopsin kinase and other proteins to cattle photoreceptor membranes. *Biochemistry* **17**, 4389-4395.
- Kuhn H. and Dreyer W. J. (1972) Light dependent phosphorylation of rhodopsin by ATP. *FEBS Lett.* **20**, 1-6.
- Kuwabara T. and Gorn R. A. (1968) Retinal damage by visible light. An electron microscopic study. *Arch. Ophthalm. (Chic)* **79**, 69-78.
- Laemmli U. K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- LeVail M. M. (1976) Rod outer segment disk shedding in rat retina; relationship to cyclic lighting. *Science* **194**, 1071-1073.
- Lopez V., Stevens T., and Lindquist R. N. (1976) Vanadium ion inhibition of alkaline phosphatase catalyzed phosphate ester hydrolysis. *Arch Biochem. Biophys.* **175**, 31-38.
- Moore R. Y. (1978) The innervation of the mammalian pineal gland, in *The Pineal Gland and Reproduction* (Reiter R. J., ed), pp. 1-29. Karger, Basel.
- Moore R. Y. and Rapport R. L. (1971) Pineal and gonadal function in the rat following cervical sympathectomy. *Neuroendocrinology* **7**, 361-374.
- Oksche A. (1984) Evolution of pineal complex: correlation of structure and function. *Ophthalm. Res.* **16**, 88-95.
- O'Steen W. K., Shear C. R., and Anderson K. V. (1972) Retinal damage after prolonged exposure to visible light. A light and electron microscopic study. *Am. J. Anat.* **134**, 5-22.
- Quay W. B. (1965) Retinal and pineal hydroxyindole-*O*-methyltransferase activity in vertebrates. *Life Sci.* **4**, 983-991.
- Shichi H. and Somers R. L. (1978) Light dependent phosphorylation of rhodopsin. *J. Biol. Chem.* **253**, 7040-7046.
- Shichi H., Somers R. L., and Yamamoto K. (1983) Rhodopsin kinase. *Methods Enzymol.* **99**, 362-366.
- Sitaramyya A. and Liebman P. A. (1983) Phosphorylation of rhodopsin and quenching of cyclic GMP phosphodiesterase activation by ATP at weak bleaches. *J. Biol. Chem.* **258**, 12106-12109.
- Sitaramyya A., Virmaux N., and Mandel P. (1977) On a soluble system for studying light activation of rod outer segment cyclic GMP phosphodiesterase. *Neurochem. Res.* **2**, 1-10.
- Somers R. L. and Klein D. C. (1984) Rhodopsin kinase activity in the mammalian pineal gland and other tissues. *Science* **226**, 182-184.
- Sugden D. and Klein D. C. (1983) Regulation of rat pineal hydroxyindole-*O*-methyltransferase in neonatal and adult rats. *J. Neurochem.* **40**, 1647-1653.
- van Veen T., Estrom P., Niberg L., Borg B., Vigh-Teichmann I., and Vigh B. (1984) Serotonin and opsin immunoreactivities in the developing pineal organ of 3 spined Stickle back, *Gasterostaus aculeatus*. *L. Cell. Tissue Res.* **237**, 559-564.
- Vigh B. and Vigh-Teichmann I. (1981) Light- and electron-microscopic demonstration of immunoreactive opsin in the pinealocytes of various vertebrates. *Cell. Tissue Res.* **221**, 451-463.
- Wurtman R. J., Axelrod J., and Phillips L. S. (1963) Melatonin synthesis in the pineal gland: control by light. *Science* **142**, 1071-1073.
- Zimmerman B. L. and Tso M. O. M. (1975) Morphologic evidence of photoreceptor differentiation of pinealocytes in the neonatal rat. *J. Cell. Biol.* **66**, 60-75.