A Diurnal Rhythm in Pineal Protein 1 Content Mediated by β-Adrenergic Neurotransmission

Abstract. *A diurnal rhythm was found in the total amount of the neuron-specific phosphoprotein protein 1 in rat pinealocytes. β-Adrenergic neurotransmission appears to be the mechanism regulating the amount of pineal protein 1 in vivo.*

Protein 1, a neuron-specific phosphoprotein concentrated at synapses, is present in presynaptic nerve terminals primarily in association with neurotransmitter vesicles (*1–4*). It is present throughout the central (*3, 5*) and peripheral (*2*) nervous systems, including the pineal gland (*5*), and it may play an important role in the functioning of neurotransmitter vesicles (*6*). Protein 1 is an endogenous substrate for both cyclic AMP (adenosine 3′,5′-monophosphate)–dependent (*1*) and calcium-plus-calmodulin-dependent (*7*) protein kinases. In intact preparations of neuronal tissue, phosphorylation of protein 1 is increased by impulse conduction (*8*), by the neurotransmitters serotonin (*9*) and dopamine (*10*), and by depolarizing agents (*9–11*). We report that β-adrenergic stimulation, under both physiological and pharmacological conditions, can increase the total amount of protein 1 in pinealocytes, indicating that the state of phosphorylation as well as the total amount of protein 1 may be regulated by neurotransmitters.

The rat pineal gland was used to investigate the regulation of protein 1 for several reasons. It contains only one type of presynaptic element—postganglionic sympathetic nerve terminals arising from the superior cervical ganglion (*12, 13*)—and one type of postsynaptic element—the pinealocyte (*12, 13*). The sympathetic input to the gland shows a circadian variation in activity, which is regulated by environmental lighting, with adrenergic stimulation increased at night and decreased during the day (*14*). The gland can be isolated readily and studied in culture (*15*). Finally, the role and consequences of activating β-adrenergic receptors on pinealocytes have been extensively investigated (*14*).

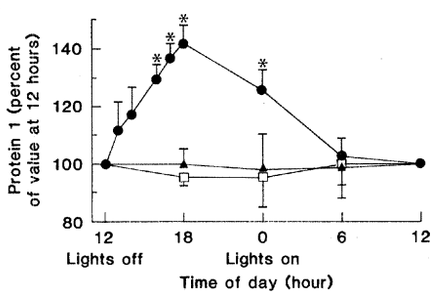

Fig. 1. Protein 1 content, determined by radioimmunoassay, of intact (●), denervated (▲), and decentralized (□) pineal glands as a function of time of day. Data represent the percentage of protein 1 present in pineal glands of rats killed at 12 hours; values are means ± S.E.M. The number of glands varied from 5 to 20. Typical amount of protein 1 in intact, denervated, and decentralized pineal glands of animals killed at 12 hours are shown in Table 1. The asterisk indicates values that showed a statistically significant difference from 12 hours (χ^2 test, $P < .05$).

The total amount of protein 1 was measured by radioimmunoassay (*16*) in pineal glands from rats killed at the end of the light period of a light-dark cycle of 12 hours of light and 12 hours of dark. Denervation of the gland (achieved by bilateral superior cervical ganglionectomy) reduced the amount of protein 1 by about 25 percent (Table 1). Since light abolishes adrenergic stimulation of the pineal gland (*14*), it seemed likely that the lower amount of protein 1 observed in the denervated glands at the end of the light period was secondary to the loss of presynaptic nerve terminals rather than to a loss of adrenergic stimulation. This interpretation was supported by experiments in which the amount of protein 1 was measured in decentralized glands (that is, glands in which the preganglionic nerve to the superior cervical ganglion was transectioned bilaterally). Decentralized pineal glands, unlike denervated pineals, contain presynaptic nerve terminals but, like denervated pineals, receive no adrenergic stimulation. There was no reduction in the amount of protein 1 in decentralized glands (Table 1). Thus, about 25 percent of the protein 1 in the pineal gland is present in presynaptic nerve terminals and the rest in pinealocytes (*13*).

Because of the prominent circadian rhythm in indoleamine metabolism displayed by the rat pineal (*14*), we measured protein 1 at different times of day. The amount of protein 1 in intact glands increased steadily during the first half of the night, peaked at midnight, and then declined, reaching a nadir by midday (Fig. 1). The diurnal rhythm in the protein 1 content of the intact pineal glands parallels the circadian oscillation in indoleamine metabolism (*14*). In denervated and decentralized glands the amount of protein 1 did not change during the light-dark cycle (Fig. 1). Thus, the nocturnal rise in protein 1 content in intact pineal glands, like the nocturnal rise in indoleamine metabolism (*17*), depends on the gland's sympathetic innervation.

The rhythms in pineal indoleamine metabolism are true circadian rhythms, not merely responses to light and darkness (*14*). Thus, in continuous darkness, a circadian rhythm in pineal indoleamine metabolism is still observed, which is driven by a circadian oscillator in the central nervous system. The suprachiasmatic nucleus has been implicated in the regulation and generation of circadian rhythms in the pineal and elsewhere (*18*). It is characteristic of the circadian rhythm in the pineal that light can block the nocturnal rise in indoleamine metabolism and that darkness is ineffective in permitting a rise during the day (*14*). The diurnal change in protein 1 in the rat pineal gland may also reflect a true circadian rhythm. Thus, exposure of animals to light between 12 and 18 hours blocked the nocturnal rise in pineal protein 1 (*19*), and exposure of animals to darkness between 0 and 6 hours failed to increase pineal protein 1 (*20*).

The nocturnal rise in pineal indoleamine metabolism is mediated by increased sympathetic stimulation from the superior cervical ganglion which in turn is driven by the central nervous system (*14, 21*). The sympathetic nerve endings in the gland release norepinephrine (*22*), which acts on β-adrenergic

Table 1. Amount of protein 1 in intact, denervated, and decentralized pineal glands. Rats were killed at 12 hours. Values are means ± S.E.M.; numbers in parentheses represent the number of glands.

Condition	Protein 1 (fmole per gland)
Intact	237 ± 10 (11)
Denervated	181 ± 9 (10)*
Decentralized	240 ± 13 (11)

*Difference from intact and decentralized glands is statistically significant (paired *t*-test, $P < .05$).

Table 2. Adrenergic regulation of protein 1 content of pineal glands in vivo. Animals were killed at 12 or 17 hours, and lights were either turned off between 12 and 17 hours (dark) or left on (light). Propranolol (20 mg/kg) and isoproterenol (5 mg/kg) were administered subcutaneously at 12 hours where indicated. Values are means ± S.E.M.; numbers in parentheses are the number of glands.

Condition (hours)	Protein 1 (fmole per gland)
12	219 ± 12 (8)
17 (dark)	285 ± 6 (8)*
17 (dark) + propanolol	218 ± 7 (10)
17 (light)	226 ± 17 (9)
17 (light) + isoproterenol	304 ± 22 (7)*

*Difference from 12 hours is statistically significant (paired *t*-test, $P < .05$).

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Table 3. Adrenergic regulation of protein 1 content of pineal glands in vitro. Animals were killed at 12 hours and the pineal glands were maintained in culture for 3 hours in the presence or absence of the following compounds: isoproterenol ($10^{-6}M$); propranolol ($5 \times 10^{-6}M$); 8-bromo cyclic AMP ($10^{-3}M$); 8-bromo cyclic GMP ($10^{-3}M$). Values are means ± S.E.M.; numbers in parentheses represent the number of glands.

Condition	Protein 1 (fmole per gland)	
	Intact	Denervated
Control	217 ± 14 (12)	171 ± 11 (17)
Isoproterenol	268 ± 7 (12)*	229 ± 13 (18)*
Isoproterenol + propranolol	222 ± 12 (11)	182 ± 26 (6)
8-Bromo cyclic AMP	264 ± 11 (13)*	
8-Bromo cyclic GMP	211 ± 11 (13)	

*Difference from control is statistically significant (paired t-test, $P < .05$).

receptors to activate adenylate cyclase and increase the synthesis of cyclic AMP (*23*). Cyclic AMP, in turn, stimulates a cyclic AMP–dependent protein kinase (*24*) and may be responsible for most, and possibly all, of the known consequences of the nocturnal stimulation of the pineal (*25*). Experiments designed to determine whether a similar mechanism is involved in the diurnal regulation of the amount of pineal protein 1 showed that pineal protein 1 increased by approximately 30 percent when control rats were exposed to darkness for 5 hours from hour 12 to hour 17 (Table 2), in agreement with the results shown in Fig. 1. This nocturnal rise was blocked by propranolol, a β-adrenergic receptor antagonist, and by light, both of which block the nocturnal activation of indoleamine metabolism (*23*). Moreover, in the presence of light, isoproterenol, a specific β-adrenergic agonist known to activate indoleamine metabolism when administered at 12 hours (*14, 23*), increased protein 1 content (Table 2). These data suggest that the nocturnal increase in pineal protein 1 is mediated by β-adrenergic stimulation.

The adrenergic regulation of protein 1 content of pineal glands explanted into culture was also studied (Table 3) (*26*). Culture times were short (3 hours) because protein 1 content decreased at longer incubation times (data not shown). Consistent with the results obtained in vivo, isoproterenol increased the protein 1 content of normal glands by about 25 percent (Table 3). Norepinephrine increased protein 1 by about the same amount (data not shown). The increase observed in response to isoproterenol was blocked by propranolol, indicating that isoproterenol increased the protein 1 content by activating β-adrenergic receptors. Furthermore, 8-bromo cyclic AMP, but not 8-bromo cyclic GMP (guanosine 3′, 5′-phosphate) mimicked the ability of isoproterenol to elevate pineal protein 1 content, suggesting that an increase in the concentration of cyclic AMP is a step in the sequence of events by which β-adrenergic stimulation increases the protein 1 content of pineal glands. The mechanism by which cyclic AMP increases protein 1 is not known, but cyclic AMP–induced increases in the activities of several pineal enzymes require RNA and protein synthesis (*15, 21, 23*).

Isoproterenol also increased protein 1 in denervated pineal glands in culture (Table 3), and this increase (58 fmole) was approximately the same as that in intact glands (51 fmole). Thus, most of the increase in intact glands appears to be located postsynaptically in pinealocytes.

Previously, protein 1 had been found solely in neurons (*1–3*). Pinealocytes are phylogenetically and developmentally related to neurons and have processes that contain dense core and clear vesicles (*27*). The norepinephrine-induced increase in protein 1 content of pinealocytes may contribute to functional changes during the circadian cycle of the pineal gland. Corticosterone, a representative of another class of compounds that affects neuronal function, has been shown to increase the total amount of protein 1 in brain regions rich in corticosterone receptors (*28*).

These results indicate that there is a diurnal rhythm in pineal protein 1 content that is mediated by β-adrenergic stimulation. Thus, neurotransmitters acting through cyclic AMP, appear to regulate both the total amount (present results) and the state of phosphorylation (*9, 10*) of protein 1.

ERIC J. NESTLER
Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510
MARTIN ZATZ
Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Maryland 20205
PAUL GREENGARD
Department of Pharmacology, Yale University School of Medicine

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16. Male rats (~ 200 g) were housed for at least 2 weeks in a light-dark cycle of 12 hours light and 12 hours dark. Times of day are defined with respect to the light-dark cycle with lights on at 0 hour and off at 12 hours. Rats with denervated or decentralized pineal glands (Zivic-Miller Laboratories, Allison Park, Pa.) were used 3 to 8 weeks after surgery (experimental results did not vary with the different times after surgery). Rats were killed at the indicated times, and the pineal glands were rapidly dissected, frozen on dry ice, homogenized or sonicated, and then boiled in 200 μl of 1 percent sodium dodecyl sulfate (SDS). Extracts were frozen and shipped on dry ice from Bethesda to New Haven. Within 1 week, protein 1 contents were measured in triplicate by a detergent-based radioimmunoassay specific for protein 1 (*5*) and showed a linear relation over a tenfold concentration range of pineal gland extract. Pineal gland extract competed in a manner parallel to purified rat and bovine brain protein 1, suggesting that the three are immunologically indistinguishable. As a further control, pineal gland extract was subjected to SDS–polyacrylamide gel electrophoresis, and the gel was then labeled for protein 1 with antiserum to protein 1 and ^{125}I-labeled protein A (*8*). Only one protein in pineal gland extract was labeled, and it had the same apparent molecular weight as pure brain protein 1. In addition, the immunoreactive material in pineal gland extract shared several biochemical properties with pure protein 1: (i) it is a substrate for cyclic AMP–dependent protein kinase, (ii) it yields a one-dimensional peptide map identical to that of pure protein 1, and (iii) it is soluble at *p*H 3. These findings indicate that the protein 1–like immunoreactivity detected in pineal gland extracts was indeed protein 1. For these reasons the protein 1–like immunoreactive material is referred to as protein 1.
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19. Mean values were 290 ± 32 fmole and 215 ± 23 fmole of protein 1 per gland ± standard error of the mean (S.E.M.) of five glands in the dark and light, respectively.
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26. For the experiments on cultured pineal glands, pineals were obtained from animals killed at 12 hours and were maintained for 3 hours in culture (*15*), either in control culture medium or in the presence of various compounds. Pineals were frozen on dry ice and analyzed for protein I content (*16*).
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Lung Fibrosis and Emphysema: Divergent Responses to a Common Injury?

Abstract. *Cadmium chloride, administered intratracheally to golden Syrian hamsters, causes an acute lung injury which evolves into a lesion with functional and morphological features of diffuse fibrosis. With simultaneous feeding of a lathyrogen, β-aminoproprionitrile, this same injury evolves into functional and morphological changes of bullous emphysema. These results suggest that the same lung injury might result in either fibrosis or emphysema, connective tissue synthesis during the healing phase being the critical determinant.*

Interstitial lung fibrosis and emphysema are generally considered to be separate disorders, each having distinguishing clinical, radiological, physiological, and pathological characteristics (*1*). Despite these differences, current concepts suggest common features in pathogenesis. Both disorders are thought to represent a late healing stage after lung injury. Both diseases in humans, as well as in animal-model counterparts, are at some stage associated with inflammation (*2*). It has been proposed that the products of the inflammatory reaction, particularly proteases and oxygen-centered radicals, may play a central role in mediating lung damage that precedes both fibrosis and emphysema (*3*). Even the repair phase after injury may have similarities. Post-injury accumulation of connective tissue proteins is a prominent feature of both emphysema and fibrosis in animal models (*4*).

What determines whether lung damage evolves into fibrosis or emphysema? One factor might be the nature of the initial injury; however, differences in the repair processes might be equally as important. The metabolism of collagen and elastin is of particular interest in the healing phase since these proteins are so important to lung structure and function. We present evidence to support the idea that alterations in the synthesis of these proteins might determine whether emphysema or fibrosis evolves after lung injury. We show that the intratracheal administration of cadmium chloride ($CdCl_2$) to hamsters causes functional and morphological abnormalities that are characteristic of lung fibrosis. With the simultaneous administration of β-aminoproprionitrile fumarate (BAPN), which interferes with normal collagen and elastin synthesis by inhibiting lysyl oxidase (*5*), this same injury results in morphological and functional changes of bullous emphysema.

Female golden Syrian hamsters (LVG outbred strain, Charles River Laboratories) weighing 90 to 120 g were fed either a regular diet (Ralston Purina Rodent Chow) or the same diet to which was added 0.5 percent (by weight) BAPN (Sigma). Seven days later, pentobarbital-anesthetized animals received by intratracheal instillation either 0.5 ml of normal saline containing 0.04 μmole of $CdCl_2$ per 100 g of animal weight or normal saline alone. After 5 weeks, the surviving animals were killed and pressure-volume relationships were measured in the excised lungs. The lungs were then fixed in Formalin at constant pressure (25 cm H_2O), and sections were processed for histopathological examination. To determine the extent of any airspace enlargement, the mean linear distance between alveolar intercepts (L_m) was measured on each lung section (*6*).

After 24 hours, hamsters receiving $CdCl_2$ and either the regular diet or the regular diet with BAPN exhibited signs of respiratory distress, which subsided within a few days. Histopathological examination of the lungs at this stage revealed edema, hemorrhage, and an intense, predominantly neutrophilic, inflammatory reaction.

Nine out of 16 animals receiving $CdCl_2$ and BAPN died between 2 and 5 weeks after instillation of the $CdCl_2$. At autopsy, the lungs from each of these animals contained multiple, large (up to 2 cm in diameter), thin-walled subpleural bullae (Fig. 1a). In some animals a single bulla occupied nearly an entire hemithorax.

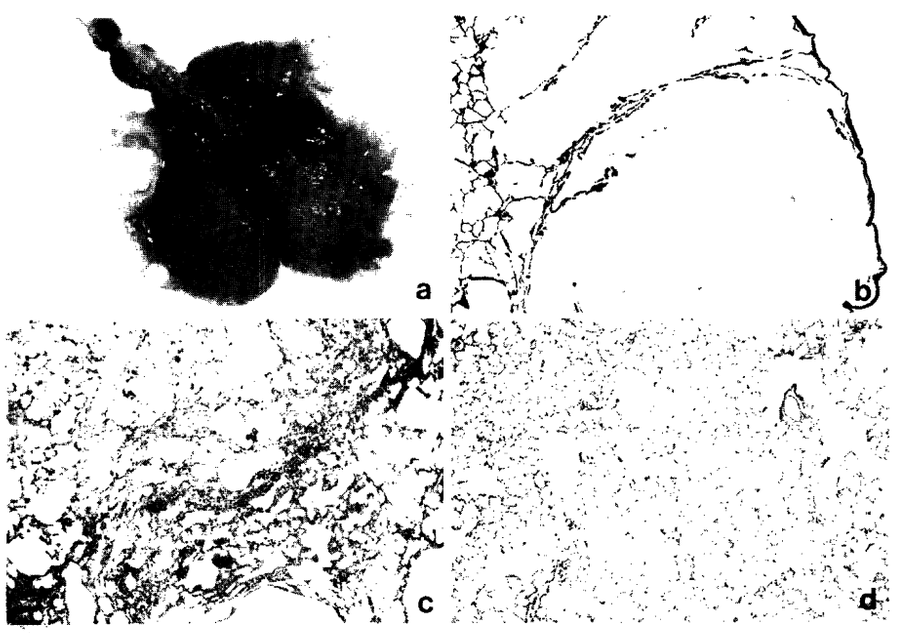

Fig. 1. (a) Air-filled lung from a hamster that had received $CdCl_2$ 5 weeks before and was also fed BAPN. The multiple subpleural bullae occurred only in this treatment group. (b) Histologic section showing a bulla and adjacent emphysematous changes in the same lung. (c) Diffuse lung fibrosis in an animal that received $CdCl_2$ but no BAPN. (d) Normal hamster lung. (All photomicrographs are ×22.)

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