J. exp. Biol. 143, 235–243 (1989)
Printed in Great Britain © The Company of Biologists Limited 1989

PARACHLOROPHENYLALANINE RETARDS TAIL REGENERATION IN THE GEKKONID LIZARD *HEMIDACTYLUS FLAVIVIRIDIS* EXPOSED TO CONTINUOUS LIGHT

BY A. V. RAMACHANDRAN AND PATRICK I. NDUKUBA

Division of Developmental Physiology and Endocrinology, Department of Zoology, MS University of Baroda, Baroda 390 002, Gujarat State, India

Accepted 13 January 1989

Summary

Parachlorophenylalanine (*p*-CPA) was used for chemical pinealectomy in a study of tail regeneration in the gekkonid lizard, *Hemidactylus flaviviridis*. Two doses of *p*-CPA (200 or 400 $\mu g\,kg^{-1}$ body mass) were injected into two groups of lizards (5 days prior to and 30 days after caudal autotomy) exposed to continuous light of 2500 lx intensity during the summer season (March–May). Our observations show that the initiation of regeneration, the daily growth rate, the total length of new growth (regenerate) produced, and the total percentage replacement of the lost (autotomized) tails 30 days after autotomy were all significantly less with 400 $\mu g\,kg^{-1}$ and insignificantly less with 200 $\mu g\,kg^{-1}$ of *p*-CPA than in the control group of animals. The results may indicate that the effect of the drug *p*-CPA, an agent employed for chemical pinealectomy, on tail regeneration in *H. flaviviridis* is dose-dependent and that *p*-CPA at the high dose of 400 $\mu g\,kg^{-1}$ has a similar retardation effect to that of complete pineal ablation. The role of the pineal in photoperiodic photoreception, and the effect of *p*-CPA on serotonin–melatonin biosynthesis and the consequent effects on tail regeneration, are discussed.

Introduction

A physiological role for serotonin (5-HT) in the regulation of gonadotrophin secretion in vertebrates has frequently been suggested (see Vitale *et al.* 1986). The distribution of serotonergic fibres in the median eminence (Villar *et al.* 1984) and their spatial relationship to luteinizing hormone-releasing hormone (LHRH) fibres (Jennes *et al.* 1982) provide neuroanatomical support for the conclusion that 5-HT can be involved physiologically in the release of LHRH from the median eminence through an action on axon terminals (Vitale *et al.* 1984).

The large number of studies supporting a neurohormonal role for 5-HT in the central nervous system accounts for the continuing interest in drugs capable of selectively depleting brain 5-HT, either by a selective release mechanism or by

Key words: light, lizard, *p*-CPA, regeneration, tail.

inhibiting 5-HT biosynthesis (see Costa *et al.* 1962*a*,*b*). *p*-CPA is reported to deplete the 5-HT stores in the brain, peripheral tissues and blood in rats and dogs. The 5-HT content of the brain, in particular, is reduced to very low levels, although brain norepinephrine and dopamine concentrations are only slightly decreased (Sloviter *et al.* 1978). The injection of *p*-CPA, an inhibitor of tryptophan hydroxylase (Koe & Weisman, 1966; Walker, 1982), is reported to increase luteinizing hormone (LH) levels and suppress prolactin (PRL) levels of broody turkeys, resulting in ovarian growth (El Halawani *et al.* 1983). Blockage of 5-HT synthesis by *p*-CPA completely inhibits the rise in PRL that is normally associated with the return of broody turkeys from cages to the nest (El Halawani *et al.* 1980). *p*-CPA, as well as the 5-HT antagonists methysergide, SQ10631 and cyptoheptadine, have been shown to decrease basal PRL levels in male chickens (Rabii *et al.* 1981).

There are reports indicating the influence of the pineal and PRL in the regeneration of amphibian appendages (see Maier & Singer, 1981). Our recent observations have shown that exogenous PRL improves tail regeneration in lizards exposed to continuous darkness (P. I. Ndukuba & A. V. Ramachandran, in preparation). The aim of the present investigation was to determine the effect, if any, on the regenerative performance of lizards exposed to continuous light with physically intact pineals, but deprived of their ability to synthesize 5-HT by the injection of *p*-CPA.

Materials and methods

Experimental animals

Adult *Hemidactylus flaviviridis* of both sexes weighing 10 ± 1 g ($\pm$ S.D.) and measuring 80 ± 5 mm ($\pm$S.D., snout–vent length) were obtained from a commercial supplier (M/S Zoophyton, Baroda, India) and maintained on a diet of cockroaches *ad libitum* for a period 7 days prior to experimentation, for acclimation to the laboratory conditions. 30 lizards were used for the investigation, and they were divided into three groups of 10 lizards each and exposed to continuous light (24 h:0 h L:D) of 2500 lx intensity.

Experimental methods

Group 1. p-CPA treated (200 μg kg^{-1} body mass)

The first group of 10 lizards received a daily intraperitoneal injection of 200 μg kg^{-1} *p*-CPA (low dose) 5 days before and 30 days after tail autotomy. Food and water were provided *ad libitum*.

Group 2. p-CPA treated (400 μg kg^{-1} body mass)

A second group of 10 lizards received daily intraperitoneal injection of 400 μg kg^{-1} *p*-CPA (high dose) 5 days before and 30 days after tail autotomy. Food and water were provided *ad libitum*.

Group 3. Saline-treated (0·6 % sterile saline)

The third group of 10 lizards, which served as the control, received a daily intraperitoneal injection of 0·6 % sterile saline 5 days before and 30 days after tail autotomy. Food and water were provided *ad libitum*.

Preparation of solutions

Parachlorophenylalanine, *p*-CPA (Sigma chemical company, St Louis, USA), was dissolved in 0·6% (w/v) NaCl and brought to pH 6·0 by the addition of 5 mol l^{-1} Na_2HPO_4 · 0·6 g of reagent grade sodium chloride (NaCl) was dissolved in 100 ml of redistilled water and stored in a refrigerator for daily use.

Experimental set-up

All the experimental animals were exposed to continuous light of 2500 lx intensity. The cages housing the animals measured 46 cm × 38 cm × 25 cm with one side made of transparent glass and ventilation on three sides. Each cage housed 10 lizards, five males and five females, and the animals selected were of similar size to eliminate any possible error in the comparative analysis of the regeneration process due to sex and size differences. The three cages housing the animals were placed (glass surface up) under suspended 40 W fluorescent lamps, facing the source of illumination. The inside of the wooden cage was lined with aluminium foil so that lighting was direct as well as reflected. The distance from the fluorescent lamp to the glass surface of the cage was 38 cm and to the floor level 63 cm. The light intensity was measured at the floor level using a luxmeter (Weston Electrical Instrument Corporation, New Jersey, USA). To obtain the high light intensity of 2500 lx needed for the experiment, four fluorescent lamps were fixed and beamed together. We employed a high light intensity in this investigation because we have earlier demonstrated that the regeneration process is markedly enhanced by the length of photoillumination as well as its intensity (P. I. Ndukuba & A. V. Ramachandran, in preparation).

Tail autotomy was performed by pinching off the tail at the third segment from the vent. The length of tail removed from the animals varied from 50 to 60 mm. The length of new growth (regenerate), in mm, was measured daily with a meter rule and recorded at fixed intervals of 10, 15, 20, 25 and 30 days after caudal autotomy. The recorded readings were used later for morphometric calculations and Student's *t*-tests were used in determining the statistical significance. This investigation was conducted during the summer month of May and the average daily temperature at the level of the animals was 30°C. Differences at the $P < 0{\cdot}05$ level were considered to be statistically significant.

Results

Growth rate, total length of tail regenerated and total percentage replacement

The regeneration blastema appeared in saline-treated animals and those treated

Table 1. *Approximate number of days taken to reach the various arbitrary stages of tail regeneration in p-CPA-treated and control lizards,* Hemidactylus flaviviridis, *exposed to continuous light during the summer*

	Days after tail autotomy					
Experimental animals ($N = 10$)	Wound healing	Blastema	Early differentiation	Mid differentiation	Late differentiation	Growth
Controls	1	3–5	5–7	8	14	20
200 $\mu g\,kg^{-1}$ *p*-CPA	1	3–5	5–7	8	14	20
400 $\mu g\,kg^{-1}$ *p*-CPA	5	8–10	12–14	16	18	24

p-CPA, parachlorophenylalanine.

with 200 $\mu g\,kg^{-1}$ *p*-CPA by day 5 and in those injected with 400 $\mu g\,kg^{-1}$ *p*-CPA by the tenth day after tail autotomy (Table 1). The high dose of *p*-CPA retarded the regeneration process more than the low dose. The total lengths of tail regenerated by day 30 in control lizards and lizards injected with 200 and 400 $\mu g\,kg^{-1}$ *p*-CPA were 27·7 mm, 26·3 mm and 13·2 mm, respectively, which corresponded to a replacement of 52·8 %, 50·5 % and 25·7 % (Figs 1, 3). The pattern of growth rate (Fig. 2) indicates a linear increase up to 15–20 days in animals treated with 400 $\mu g\,kg^{-1}$ *p*-CPA. The saline-injected lizards showed a biphasic growth rate curve, with the first phase lasting up to 10 days and the second occurring between

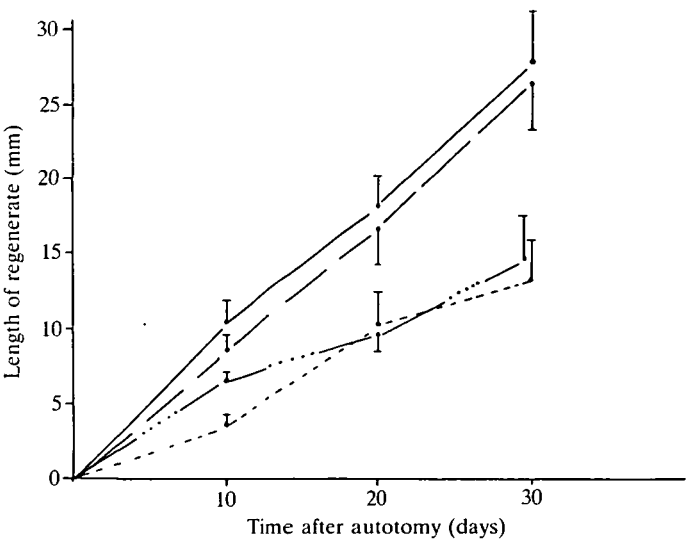

Fig. 1. Length of tail regenerated at the end of 30 days in control (●——●) and *p*-CPA-treated (●— —● 200 $\mu g\,kg^{-1}$, ●---● 400 $\mu g\,kg^{-1}$) lizards exposed to continuous light. Vertical lines are ± S.D. $N = 10$. ●–·–●, pinealectomized and exposed to continuous light (data from Ramachandran & Ndukuba, 1988).

20 and 30 days, whereas the lizards treated with 200 $\mu g\,kg^{-1}$ *p*-CPA did not show the second phase.

Comparisons (total length of tail regenerated and total percentage replacement) between the three groups of animals (Student's *t*-test) revealed no statistically

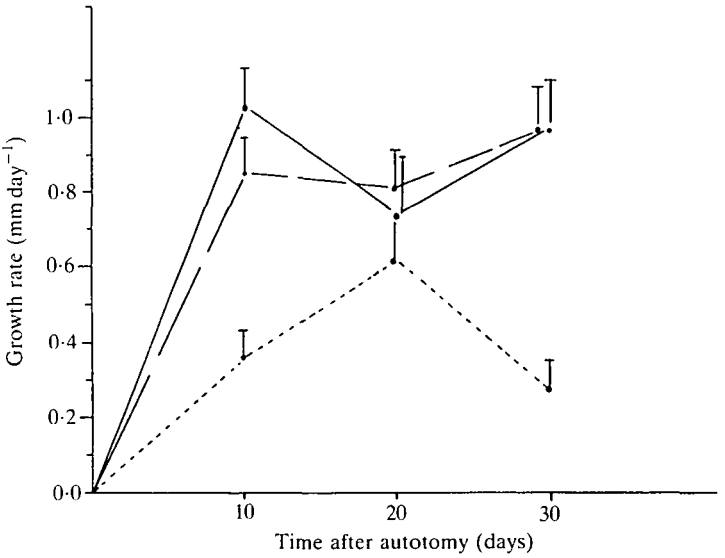

Fig. 2. Growth rate in blocks of 10 days in control (●——●) and *p*-CPA-treated (●— —●, 200 $\mu g\,kg^{-1}$, ●---●, 400 $\mu g\,kg^{-1}$) lizards exposed to continuous light. Mean ± S.D. ($N = 10$).

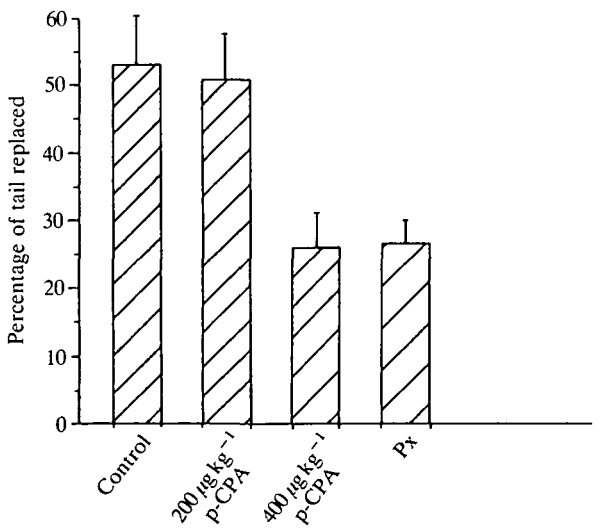

Fig. 3. Percentage of tail replaced at the end of 30 days in control and *p*-CPA-treated lizards exposed to continuous light. Px, pinealectomized and exposed to continuous light (taken from Ramachandran & Ndukuba, 1988).

significant difference between the saline and 200 $\mu g\,kg^{-1}$ *p*-CPA groups. However, comparisons between the control and 400 $\mu g\,kg^{-1}$ *p*-CPA groups and between 200 $\mu g\,kg^{-1}$ *p*-CPA and 400 $\mu g\,kg^{-1}$ *p*-CPA groups were statistically significant at the 5% level (Student's *t*-test).

Discussion

These results show that tail regeneration in the gekkonid lizard *Hemidactylus flaviviridis* was significantly retarded with daily intraperitoneal injection of 400 $\mu g\,kg^{-1}$ *p*-CPA (high dose) but only insignificantly so with a low dose (200 $\mu g\,kg^{-1}$) of *p*-CPA (Table 1, Figs 1, 3). This finding demonstrates that in *Hemidactylus* the retardation effect of *p*-CPA is dose-dependent, with the high dose producing a marked effect. The mechanism of action of *p*-CPA in higher vertebrates has been demonstrated previously. *p*-CPA is a neutral amino acid and can compete with tyrosine for uptake into catecholamine neurones (Wurtman, 1975). It has been shown that *p*-CPA selectively decreases the concentration of 5-HT in the brain without altering the concentration of noradrenaline or dopamine. This selective action is probably effected by inhibition of the enzyme tryptophan hydroxylase (Koe & Weisman, 1966; Walker, 1982).

The perception of light provides important information for the organism about its environment. For this purpose, most animals possess well-developed photoreceptors and neuronal networks in the retina of the lateral eyes. Interestingly, even in species with highly organized ocular photoreceptors, additional photoreceptive structures – extraocular photoreceptors – are utilized in the transmission of photic information about the day–night schedule and seasonal photoperiodic changes. Considerable evidence supports the view that the pineal organ is the principal site of extraocular photoreception in lower vertebrates (see Meissl & Dodt, 1981). The pineal system (pineal organ and parietal eye) has been shown to be light-sensitive on the basis of neurophysiological and cytological evidence (Wurtman *et al.* 1968). Recent studies from our laboratory have demonstrated that continuous light stimulates tail regeneration in the lizard, *H. flaviviridis*, whereas continuous darkness depresses it (P. I. Ndukuba & A. V. Ramachandran, in preparation) and, further, that the lateral eyes, or retinae, do not participate in this photoperiodic response as blinded lizards regenerated their lost (automized) tails as effectively as did their sighted counterparts exposed to the same experimental photoperiodic conditions (Ndukuba & Ramachandran, 1988). It has been shown that the pineal organ is the principal site of extraretinal photoreception in *Hemidactylus*, since pinealectomy, as well as light deprivation to the pineal, abolished the stimulatory influence of continuous illumination and significantly retarded the regeneration process (Ramachandran & Ndukuba, 1988), and also tail regeneration was stimulated by exogenous PRL in lizards kept in continuous darkness (P. I. Ndukuba & A. V. Ramachandran, in preparation). The present report shows that the initiation of regeneration, the daily growth rate, the total length of new growth (regenerate) produced at the end of regeneration, and the

total percentage replacement of the lost (autotomized) tails in lizards exposed to continuous light were all significantly retarded by a daily intraperitoneal injection of 400 $\mu g\,kg^{-1}$ *p*-CPA. The results obtained here were similar to those obtained earlier with pinealectomized lizards exposed to continuous illumination (see Figs 1, 3; Ramachandran & Ndukuba, 1988).

PRL has been established as a growth promoter in developing organisms (Crim, 1975) and in regenerating systems (Maier & Singer, 1981; P. I. Ndukuba & A. V. Ramachandran, in preparation) and has been shown to stimulate protein synthesis in developing tadpoles (Yamaguchi & Yasumasu, 1977). Depletion of hypothalamic catecholamines by compounds that inhibit their synthesis resulted in a rise in serum PRL level (Donoso *et al.* 1971). In contrast, pharmacological procedures that enhance the amine levels in brain, the injection of monoamine oxidase inhibitors or L-dopamine, inhibit PRL release (Lu & Meites, 1971). In addition to the vast literature implicating dopamine in the control of PRL secretion, some studies suggest that 5-HT is a neurotransmitter involved in the stimulation of PRL release. Kamberi *et al.* (1971) induced PRL release by injecting 5-HT into the third ventricle, and Lawson & Gala (1976) stimulated PRL release by systemic administration of 5-HT. The 5-HT precursor 5-hydroxytryptophan (5-HTP) has been shown to induce PRL release in rats (Chen & Meites, 1975). The above reports are consistent with a stimulatory role for 5-HT in the control of PRL secretion. In the present investigation, the marked retardation in tail regeneration in lizards treated with *p*-CPA indicates that 5-HT neurones may be mediating the stimulatory effect of continuous illumination by way of PRL secretion during tail regeneration in lacertilians (P. I. Ndukuba & A. V. Ramachandran, in preparation).

Our recent observations have shown that half the tail is replaced, irrespective of the light factor, since lizards exposed to continuous darkness regenerated 50 % of their lost tails (P. I. Ndukuba & A. V. Ramachandran, in preparation). This study, together with that of Ramachandran & Ndukuba (1988), demonstrated that continuous light can increase both the rate and the extent of tail regeneration and that pinealectomy can totally abolish these light-induced effects. Apparently, the intact pineal is the photoreceptor which mediates the favourable influence of light on tail regeneration in *H. flaviviridis*. The present study further reveals that lizards with physically intact pineals, but deprived of their ability to synthesize 5-HT by the injection of *p*-CPA, failed to show the positive influences of continuous light on tail regeneration. This sequence of observations leads to the conclusion that the pineal is not only the photoreceptor but also the essential synchronizer which transduces and translates the photic information into favourable regenerative growth in lacertilians. Hence, it may be tentatively surmised that the purported serotonergic mechanism of PRL release (Clemens *et al.* 1977) may be the operative mechanism in lizards, triggered by continuous light, and that such a release of PRL can be blocked at the level of the enzyme tryptophan hydroxylase by its inhibitor, *p*-CPA, leading to the depletion of 5-HT from the brain. However, since *p*-CPA inhibits only the first step in the synthesis of 5-HT, it is possible to

bypass its blocking action, and thereby re-establish the concentration of 5-HT, by injecting the direct precursor of 5-HT following the injection of *p*-CPA. A study of this is now in progress in our laboratory, employing the direct precursor of 5-HT, 5-HTP, which readily crosses the blood–brain barrier. The observation that *p*-CPA did not completely inhibit tail regeneration in *Hemidactylus* (only 50% retardation was obtained) strengthens our earlier inference that 50% tail replacement is an innate ability which is independent of photoperiodism and associated neuroendrocrine mechanisms and, apparently, occurs under basal levels of PRL secretion (Ramachandran & Ndukuba, 1988).

The authors wish to acknowledge the facilities provided by the University Grants Commission of India sponsored Departmental Research Support in Developmental Physiology.

References

CHEN, H. J. & MEITES, J. (1975). Effects of biogenic amines and TRH on release of prolactin and TSH in the rat. *Endrocrinology* **96**, 10–14.

CLEMENS, J. A., SAWYER, B. D. & CERIMELE, B. (1977). Further evidence that serotonin is a neurotransmitter involved in the control of prolactin secretion. *Endrocrinology* **100**, 692–698.

COSTA, E., GESSA, C. L., HIRSCH, C., KUNTZMAN, R. & BRODIE, B. B. (1962*a*). On current status of serotonin as a brain neurohormone and in action of reserpine-like drugs. *Ann. N. Y. Acad. Sci.* **96**, 118–133.

COSTA, E., GESSA, G. L., HIRSCH, C., KUNTZMAN, R. & BRODIE, B. B. (1962*b*). A differential action of reserpine on brain dopamine stores in rabbit. *Life Sci.* **1**, 599–604.

CRIM, J. W. (1975). Prolactin–thyroxine antagonism and the metamorphosis of visual pigments in *Rana catesbeiana* tadpoles. *J. exp. Zool.* **192**, 355–362.

DONOSO, A. O., BISHOP, W., FAWCETT, C. P., KRULICH, L. & MCCANN, S. M. (1971). Effects of drugs that modify brain monoamine concentrations on plasma gonadotropin and prolactin levels in the rat. *Endocrinology* **89**, 774–784.

EL-HALAWANI, M. I., BURKE, W. H. & DENNISON, P. T. (1980). Effects of *p*-chlorophenylalanine on the rise in serum prolactin associated with nesting in broody turkey. *Gen. comp. Endocr.* **23**, 815–819.

EL-HALWANI, M. E., SILSBY, J. L., FEHRER, S. C. & BEHNKE, E. J. (1983). Reinitiation of ovulatory cycles in incubating female turkeys by an inhibitor of serotonin synthesis, *p*-chlorophenylalanine. *Biol. Reprod.* **28**, 221–228.

JENNES, L., BECKMAN, W. C., STUMPF, W. E. & GRZANNA, R. (1982). Anatomical relationship of serotoninergic and noradrenalinergic projections with the Gn-RH system in septum and hypothalamus. *Expl Brain Res.* **46**, 331–338.

KAMBERI, I. A., MICAL, R. S. & PORTER, J. C. (1971). Hypophysial portal vessel infusion: *in vivo* demonstration of LRF, FRF and PIF in pituitary stalk plasma. *Endrocrinology* **88**, 1012–1020.

KOE, B. K. & WEISMAN, A. (1966). *p*-Chlorophenylalanine: specific depletor of brain serotonin. *J. Pharmac. exp. Therap.* **1548**, 499–516.

LAWSON, D. M. & GALA, R. R. (1976). The interaction of dopaminergic and serotoninergic drugs on plasma prolactin in ovariectomized, estrogen-treated rats. *Endrocrinology* **98**, 42–47.

LU, K. H. & MEITES, J. (1971). Inhibition by L-Dopa and monoamine oxidase inhibitors of pituitary prolactin release; stimulation by methyldopa and d-amphetamine (35604). *Proc. Soc. exp. Biol. Med.* **137**, 480–483.

MAIER, C. E. & SINGER, M. (1981). The effect of prolactin on the rate of forelimb regeneration in newts exposed to photoperiod extremes. *J. exp. Zool.* **216**, 395–397.

MEISSL, H. & DODT, E. (1981). Comparative physiology of pineal photoreceptor organs. *Devl Endrocr.* **14**, 61–80.

NDUKUBA, P. I. & RAMACHANDRAN, A. V. (1988). Extraretinal photoreception in lacertilian tail regeneration: the lateral eyes are not involved in photoperiodic photoreception in the Gekkonid lizard *Hemidactylus flaviviridis*. *J. exp. Zool.* **248**, 73–80.

RABII, J., BUONOMO, F. & SCANES, C. G. (1981). Role of serotonin in the regulation of growth hormone and prolactin secretion in the domestic fowl. *J. Endrocr.* **90**, 355–358.

RAMACHANDRAN, A. V. & NDUKUBA, P. I. (1988). Preliminary evidence for pineal mediated extraretinal photoreception in relation to tail regeneration in the Gekkonid lizard, *Hemidactylus flaviviridis*. *J. Pineal Res.* (in press).

SLOVITER, R. S., DRUST, E. G. & CONNOR, J. D. (1978). Serotonin agonist: actions of *p*-chlorophenylalanine. *Neuropharmacology* **17**, 1029–1033.

VILLAR, M. J., CHIOCCHIO, S. R. & TRAMEZZANI, J. H. (1984). Origin and termination of dorsal raphe-median eminence projection. *Brain Res.* **324**, 165–170.

VITALE, M. L., PARISI, M. N., CHIOCCHIO, S. R. & TRAMEZZANI, J. H. (1984). Median eminence serotonin involved in the proestrus gonadotrophin release. *Neuroendocrinology* **39**, 136–141.

VITALE, M. L., PARISI, M. N., CHIOCCHIO, S. R. & TRAMEZZANI, J. H. (1986). Serotonin induces gonadotrophin release through stimulation of LH-releasing hormone release from the median eminence. *J. Endrocr.* **111**, 309–315.

WALKER, R. F. (1982). Serotonin-reinstatement of luteinizing hormone surges after loss of positive feedback in ovariectomized rats bearing subcutaneous capsules containing oestrogen. *J. Endrocr.* **98**, 7–17.

WURTMAN, R. J. (1975). The effects of light on man and other mammals. *A. Rev. Physiol.* **37**, 467–483.

WURTMAN, R. J., AXELROD, J. & KELLY, D. E. (1968). *The Pineal*. New York: Academic Press.

YAMAGUCHI, K. & YASUMASU, I. (1977). Effects of thyroxine and prolactin on the rate of protein synthesis in the thigh bones of *Rana catesbeiana*. *Dev. Growth Diff.* **19**, 161–169.