Structure–Activity Studies of Melatonin Analogues in Prepubertal Male Rats

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Abstract
Comparison has been made between the activity of the pineal hormone melatonin, and several analogues and metabolites in inhibiting sexual development in a protein-restricted prepubertal rat model. Eleven melatonin analogues or metabolites were tested with the aim of evaluating the model as a test of the hypothesis that melatonin acts as a prohormone and that the ring schism metabolites (kynurenamines) mediate many of the effects attributable to melatonin. Although the hypothesis could not be confirmed, modification of the melatonin structure by lengthening the acylamide side chain or by replacing the 5 methoxy function with fluorine resulted in loss of biological potency. Modification of the melatonin structure to block the two known points of metabolism resulted in no significant alteration in biological activity. Thus 6-chloromelatonin (blocking 6-hydroxylation) and 2,3-dihydromelatonin (blocking oxidative cleavage of the C₂–C₃ bond) and 6-chloro-2,3-dihydromelatonin remained biologically active. The metabolic products of brain indoleamine-2,3-dioxygenase, N-acetyl-N²-formyl-5-methoxy kynurenamine (aFoMK) and N-acetyl-5-methoxy kynurenamine (aMK), paradoxically were also biologically active.

Introduction
Melatonin has an important role in the timing of seasonal breeding activity in many species. In animals that mate during the increasing daylength of spring [e.g. hamsters and other, native, rodents including rats (Irby et al. 1984)] the pineal gland is thought to be of importance for terminating breeding activity prior to winter. In other species such as sheep, goats, deer, etc., which breed generally in autumn (shortening daylength), apparently the same pattern of secretion of melatonin initiates reproductive activity (Kennaway 1984). While there is now compelling evidence to show that the pineal hormone initiates or terminates reproductive activity by interactions with steroids at the hypothalamic/pituitary level (Karsch et al. 1984) the molecular basis of the interaction is unknown.

As part of a study on the mechanism of action of melatonin we have investigated some of the structural requirements for melatonin-like activity. Analogues that were predicted to be more resistant to metabolism or had functional-group changes likely to alter biological potency were prepared. Other compounds examined were intended to test the hypothesis that melatonin is a prohormone and that the brain metabolites of melatonin, N-acetyl-N²-formyl-5-methoxy kynurenamine (aFoMK) and N-acetyl-5-methoxy kynurenamine (aMK) (Hirata et al. 1974), may mediate many of the effects ascribed to melatonin itself. In a previous communication we described the use of the prolactin-inhibiting activity of melatonin in sheep as our bioassay (Kennaway et al. 1986). In the work discussed in this report we
have exploited the potent puberty-delaying effect of melatonin in male rats (Lang et al. 1983). The sensitivity of this assay has been enhanced by using protein-deprived prepubertal rats (Blask et al. 1981), thus establishing a simple, economical and reliable method for investigating some aspects of melatonin-like activity.

Methods
Male rats of the Porton strain were obtained from the University of Adelaide Central Animal House at 21 days of age in groups of 30. On arrival the rats were weighed and placed five to a cage in a light-controlled box (14 h light: 10 h dark; lights off 1200 h). The animals were fed ad lib. a low-protein diet (500 g powdered Kelloggs Rice Bubbles, 10 g glucose, 5 g dicalcium phosphate and 10 ml cod liver oil; Irby et al. 1984). This was supplemented with approximately 8 g commercial rat chow per box per day. Water was available ad lib. In previous experiments (Kennaway et al. 1988) it was found that the body weights of Porton rats maintained under these conditions were 70% of peers fed rat chow ad lib.

In all of the experiments, groups of five rats were injected subcutaneously with vehicle [50–100 µl ethanolic saline (10%)] or test compound in vehicle 2–3 hours before lights out between Monday and Friday. (A preliminary experiment had indicated that there was no difference in the gonadal response to melatonin when animals were injected each day of the week or simply on the 5 working days.) After 21 days (42 days old) rats were killed and seminal vesicles, ventral prostate and right testis were dissected and weighed. In a preliminary experiment to establish the assay, the effect of different doses of melatonin (1 µg, 5 µg, 10 µg, 50 µg and 100 µg per day) and the duration of treatment on the gonadal response was examined.

In Experiment 1 a comparison was made between effects of saline and comparable dosages (50 µg/day) of melatonin (aMT) and the following compounds: 6-chloromelatonin (aMT.6Cl) 2,3-dihydromelatonin (DH.aMT), 6-chloro-2,3-dihydromelatonin (DH.aMT.6Cl) and 1-methyl melatonin (aMT.1Me).

Experiment 2 examined N-acetyl-5-fluorotryptamine (aFT), N-propionyl-5-methoxytryptamine (pMT), N-butryl-5-methoxytrypatmine (bMT) and N-propionyl-5-fluorotryptamine (pFT).

Experiment 3 examined N-acetyl-N²-formyl-5-methoxy kynurenamine (aFoMK; 1 mg) and N-acetyl-5-methoxykynurenamine (aMK; 1 mg).

Experiment 4 examined N-acetyl-5-bromotryptamine (aBT), aFT (300 µg), aFoMK (100 µg) and aMK (100 µg).

Determination of a significant delay in puberty (low sex and accessory gland weight) was made in each experiment by a one-way analysis of variance. Differences between the treatment groups and vehicle-treated groups were determined by the Student–Neuman–Keuls method.

Sources and Syntheses of Analogues
N-acetyl-N²-formyl-5-methoxykynurenamine
To a stirred solution of melatonin (0·04 mol, 9·3 g) in dichloromethane (500 ml) at 4°C was added m-chloroperbenzoic acid (0·174 mol, 30·0 g). The mixture was stirred at 4°C for 16 h and washed with saturated aqueous sodium bicarbonate (3 × 500 ml). The solution was dried (Na₂SO₄) and evaporated under reduced pressure to afford a red oil (6·0 g), which was absorbed on neutral alumina grade I (150 g) with ethyl acetate. The alumina column was eluted with ethyl acetate, ethyl acetate–chloroform (1 : 1) and chloroform, and the eluant discarded. Elution with chloroform–methanol (19 : 1) afforded N-acetyl-N²-formyl-5-methoxykynurenamine (2·6 g, 25%) as a yellow solid after evaporation of the solvent and washing with diethyl ether. The infra-red spectrum and the Rf value on thin layer chromatography were identical with those of a sample of the compound prepared by the method of Hirata et al. (1974). [Infra-red spectrum (Nujol) cm⁻¹, 1660, 1680 (C=O).]

N-acetyl-5-methoxykynurenamine
This compound was prepared by the method of Hirata et al. (1974) in 59% yield after recrystallization from isopropanol. [Infra-red spectrum (Nujol) cm⁻¹, 1640, 1665 (C=O). Mass spectrum m/z 236 (M+ , 40%), 177 (M–NH₂COCH₃)⁺, 63%), 176 (100%).]
N-acetyl-5-bromotryptamine

Oxalyl chloride (0.023 mol, 2.9 g) was slowly added to a well stirred solution of 5-bromo indole (0.02 mol, 3.9 g) in dry diethyl ether (100 ml) kept at 4°C. Following the development of an orange solution, the mixture was stirred for an additional 30 min before being filtered and washed with cold diethyl ether (80 ml). The acid chloride (yellow powder), which is stable at 0°C for several months, was suspended in toluene (100 ml) and dry ammonia gas was bubbled through the stirred mixture for 1 h. The yellow solid was filtered, washed with distilled water and dried under vacuum for 24 h to give the amide (4.7 g, 88% yield). Reduction of the amide was carried out in flame-dried glassware and in a dry nitrogen gas atmosphere in a well ventilated fume hood. The amide (0.05 mol, 1.34 g) was partially dissolved in dimethoxy ethane (50 ml) and magnetically stirred while being heated to reflux temperature for 1 h in a 100 ml three-necked flask fitted with a double-surface reflux condenser and nitrogen bubbler. Borane dimethyl sulfide (0.06 mol, 6.8 ml) was added via a syringe dropwise over 10 min into the reaction mixture. The solution was heated for 2 h and then cooled to room temperature. Methanol (20 ml) was carefully added, followed by a freshly prepared saturated dry hydrogen chloride-diethyl ether solution (5 ml). The solution was then stirred overnight at 0°C. Filtration of the solution gave 5-bromotryptamine hydrochloride [1.1 g, 79.7% yield; m.p. 288–289°C; Ho et al. (1969), m.p. 286–287°C]. The white amine hydrochloride (0.002 mol, 0.052 g) was partially dissolved in dimethoxy ethane–ethyl acetate (30 ml–30 ml), acetic anhydride (1 ml) and pyridine (3 drops). The solution was stirred at room temperature for 7 days under a nitrogen atmosphere. The mixture was concentrated to dryness, extracted with hot ethyl acetate (3 × 40 ml) and the extract subsequently washed with sodium hydrogen carbonate solution (2 × 30 ml), saturated sodium chloride (2 × 30 ml) and dried with anhydrous magnesium sulfate. The crude brown product was recrystallized from ethyl acetate and then chromatographed on a chromatotron using ethyl acetate–methanol (3 : 1), giving pure N-acetyl-5-bromotryptamine (0.31 g, 56% yield; m.p. 153–153.8°C). [Infra-red spectrum (KBr) cm\(^{-1}\), 3300–3060 (N–H), 2890 (C–H), 1640 (C=O, amide), 1560, 1540, 1470, 1440, 1360, 1320, 1220, 1205, 1110, 1095, 1030, 880, 860, 790. Mass spectrum \(C_{17}H_{15}N_{2}OBr\); m/z 282 (M+, 81Br, 32%), 280 (M+, 79Br, 32%), 210 [(M(81Br)-CH3NHOCH3)\(^+\), 53%], 208 [(M(79Br)-CH3NHOCH3)\(^+\), 59%].

Other compounds

N-acetyl 5-fluorotryptamine (aFT), N-propionyl 5-fluorotryptamine (pFT), N-propionyl 5-methoxytryptamine (pMT), and N-butryl 5-methoxytryptamine (bMT) were prepared by reaction of the respective acid chloride with the corresponding 5-substituted tryptamine as follows. The tryptamine was dissolved in either DMF (5-fluorotryptamine) or ethyl acetate with a few drops of DMF (5-methoxytryptamine), with a little warming. The solution was magnetically stirred, cooled in an ice bath, and 1·1 equivalents of the acid chloride slowly added. After 15 min at 0°C, 20 μl 2m HCl was added and the solution concentrated to dryness on a rotary evaporator. The residue was extracted into acetone and worked up to yield the hydrochloride salts, which were dried under vacuum. [Infra-red spectra (KBr), and m.p.; aFT cm\(^{-1}\), 3280 (N–H), 2600 (HCl salt), 1630 (C=O, amide), m.p. 279–281°C; pFT cm\(^{-1}\), 3280 (N–H), 2590 (HCl salt), 1630 (C=O, amide), m.p. 277.5–279.5°C; pMT cm\(^{-1}\), 3320 (N–H), 2590 (HCl salt), 1620 (C=O, amide), m.p. 248–250°C; bMT cm\(^{-1}\), 3300 (N–H), 2580 (HCl salt), 1630 (C=O, amide) m.p. 233–235°C.]

6-Chloro melatonin was synthesized according to Hugel (1983), 2,3-dihydromelatonin and 6-chloro-2,3-dihydromelatonin were prepared according to Kennaway et al. (1986). 1-Methyl melatonin was a gift from Dr. Clements, Eli Lilly and Company, and melatonin was purchased from Sigma Chemical Company.

Results

When 21-day-old male rats were maintained on a protein-restricted diet and injected with various doses of melatonin 5 days per week for 3 weeks, seminal vesicle, ventral prostate and testis weights were reduced in an apparent dose-related manner (Fig. 1). A dose of 50 μg/day for all further treatments was chosen on the basis that it was the lowest dose to give maximal reduction in the weight of all three organs. An investigation of the time course of the response confirmed that a 3-week treatment with 50 μg melatonin produced a significant response (Fig. 2).
Fig. 1. Weights of seminal vesicles, ventral prostate and right testis of rats injected 5 days per week with saline (S) and various doses of melatonin between 21 and 42 days of age. Values shown are the mean s.e.m. for five animals per group. A significant decrease in organ weight is indicated by *. $P < 0.05$ following analysis of covariance with body weight as the covariate.

Fig. 2. Body weight, right testis, seminal vesicle and ventral prostate weights of rats injected 5 days per week with 50 $\mu$g melatonin and killed after 1, 2 or 3 weeks treatment. Values are the mean ± s.e.m. for five animals per group. Solid symbols joined by solid lines represent the saline-injected control and the open symbols joined by broken lines represent melatonin-injected group. Solid symbols joined by broken lines represents body-weight increase of both saline-treated and melatonin-treated animals, there being no significant difference between them at any age. A significant difference between control and treatment groups ($P < 0.05$) indicated by *.

Table 1. Weights of accessory organs and testes (mg/100 g body weight mean ± s.d.) in male rats treated with melatonin (50 $\mu$g/day) between 21 and 42 days of age

| Experiment | Seminal vesicles | Ventral prostate | Right testis |
|------------|------------------|-----------------|--------------|
|            | Vehicle | Melatonin | Vehicle | Melatonin | Vehicle | Melatonin |
| 1          | 46 ± 4  | 25 ± 4  | 45 ± 2  | 26 ± 4  | 778 ± 59 | 451 ± 68  |
| 2          | 29 ± 3  | 17 ± 5  | 27 ± 7  | 21 ± 13 | 601 ± 73 | 323 ± 169 |
| 3          | 36 ± 5  | 18 ± 2  | 43 ± 3  | 22 ± 2  | 725 ± 63 | 376 ± 72  |
| 4          | 37 ± 3  | 24 ± 1  | 42 ± 4  | 23 ± 2  | 790 ± 55 | 490 ± 40  |
| 5          | 35 ± 2  | 22 ± 2  | 50 ± 6  | 39 ± 5  | 580 ± 30 | 350 ± 50  |
| 6          | 38 ± 2  | 21 ± 2  | 44 ± 5  | 21 ± 4  | 744 ± 45 | 360 ± 77  |
| Mean       | 37 ± 5  | 21 ± 2  | 42 ± 8  | 25 ± 7  | 703 ± 90 | 391 ± 64  |
| CV (%)     | 15      | 15      | 19       | 27       | 13       | 16        |
| % Inhibition | 57 ± 6 | 61 ± 13 | 56 ± 5  | 9        |
Table 1 shows the weights of accessory organs and testes (mg/100 g body weight) from all six experiments in which 50 µg melatonin was injected over a 3-week period. The reduced organ weight following this treatment is very reproducible, with inter-experiment coefficients of variation less than 16% in seminal vesicle and testis weight. While the ventral prostates were always significantly lower in melatonin-treated than in vehicle-treated rats, the between-experiment variation in weight was greater. If data are presented as the percentage reduction in organ weight the between-experiment variation as shown by the coefficient of variation is greatly reduced, especially in the seminal vesicle and testis weights. Thus for ease of comparison of the activity of analogues the percentage reduction in organ weights of treated rats compared with those of control groups has been used.

Table 2. Effects of analogues of melatonin on weights of accessory organs and testes in male rats between 21 and 42 days of age

| R1 | R2 | R3 | R4   | R5 | R6 | Dose (µg) | Seminal vesicles (% of control) | Ventral prostate | Testis (% of control) | Significance (*P < 0.05) |
|----|----|----|------|----|----|-----------|-------------------------------|-----------------|----------------------|----------------------|
|    |    |    |      |    |    |           |                               |                 |                      |                      |
|    |    |    |      |    |    | 100       | 100                           | 100             |                      |                      |
|    |    |    |      |    |    |           |                               |                 |                      |                      |
|    |    |    |      |    |    |           |                               |                 |                      |                      |
| aMT | CH₃O | H | H | CH₃ |      | 50 | 57        | 61                           | 56              | *                    |
| aFT | F   | H | H | CH₃ |      | 50 | 87        | 110                          | 94              | *                    |
|     |     |    |    | R₁H₂ |      | 300| 69        | 76                           | 83              | *                    |
| aBT | Br  | H | H | CH₃ |      | 50 | 61        | 76                           | 74              | *                    |
| pMT | CH₃O | H | H | CH₂CH₃ |      | 50 | 82        | 108                          | 90              |                      |
| bMT | CH₃O | H | H | (CH₂)₂CH₃ |      | 50 | 89        | 110                          | 106             |                      |
| pFT | F   | H | H | CH₂CH₃ |      | 50 | 105       | 109                          | 95              |                      |
| aMT.6Cl | CH₃O | Cl | H | CH₃ |      | 50 | 69        | 70                           | 52              | *                    |
| aMT.1Me | CH₃O | H | CH₃ | CH₃ |      | 50 | 63        | 60                           | 67              | *                    |
| DH.aMT | CH₃O | H | H | CH₃ |      | 50 | 60        | 65                           | 59              | *                    |
| DH.aMT.6Cl | CH₃O | Cl | H | CH₃ |      | 50 | 46        | 40                           | 50              | *                    |
| aFoMK | CH₃O | H | CHO | CH₃ |      | 100| 67        | 73                           | 72              | *                    |
|     |     |    |    |      |      | 1000| 53       | 62                           | 68              | *                    |
| aMK  | CH₃O | H | H | CH₃ |      | 100| 78        | 89                           | 93              | *                    |
|     |     |    |    |      |      | 1000| 63       | 71                           | 57              | *                    |

Table 2 summarizes the results of the four experiments in which analogues were tested. Substitution of the 5-methoxy group with fluorine or elongation of the ethylamide side chain resulted in lower biological potency. In contrast, the 5-bromo derivative was active at a dose of 50 µg/rat. Methylation of the ring nitrogen apparently failed to alter biological activity. Chlorine substitution at position 6 of the indole nucleus similarly resulted in the retention of biological activity even when accompanied by 2,3 saturation. The open-ring metabolites of melatonin (aFoMK and aMK) retained biological activity although aMK caused significant gonadal inhibition only at the high dose (1 mg).
Discussion

Daily injection of melatonin into prepubertal rats results in a time- and dose-dependent retardation of growth of the testes and accessory organs. While it is likely that this effect of melatonin is common among rodents there are strain differences; Sprague-Dawley-derived rats require some degree of food deprivation to become sensitive to melatonin (Blask et al. 1981) whereas Wistar rats apparently do not require this additional treatment (Lang et al. 1983; Kennaway et al. 1988). Under the conditions we employed, albino Porton rats maintained on a low-protein diet between 21 and 42 days of age had a remarkably consistent response to melatonin injection, with inter-experiment coefficients of variation being less than 16%. On this basis we considered the treatment procedure was likely to be of value for determining the structural requirements for melatonin-like activity.

The melatonin molecule was altered in four ways: (1) halogenation in the 5 position, (2) lengthening of the amide side chain, (3) halogenation in the 6 position and/or saturation of the 2,3 double bond, and (4) oxidative cleavage of the 2,3 bond.

Substitution of the 5-methoxy group with a fluorine resulted in a total loss of biological activity at 50 µg/day and partial activity at 300 µg/day. A bromine in position 5, however, resulted in almost total retention of activity when injected at a dose of 50 µg/rat. The contrasting effects of 5-fluorination and 5-bromination are not readily explained at this time. Comparison of the electronegativity of fluorine and bromine, F = 4·0, Br = 2·8 (Pauling 1960), indicates that fluorine would be more effective in the withdrawal of electrons from the aromatic ring, with resultant differences in chemical reactivity. However, the magnitude of the differences cannot be expected alone to account for the observed contrasting chemical/biological reactivities. It may be significant that the electronegative character of fluorine results in a shorter bond length, Csp2-F, 1·35 Å (Lide 1962) than is the case for bromine (Csp2-Br, 1·85 Å) and this coupled with the smaller size of the former (covalent radius, F = 0·71 Å, Br = 1·14 Å) could provide an explanation for their different reactivities through inductive electron withdrawal and resonance interactions with the conjugated orbitals of benzene. The possibility of in vivo chemoselective transformation of the 5-bromo compound cannot be ruled out.

In a previous study of the ovulation-blocking activity of melatonin analogues, acetyl, ethyl and phenyl substitutions in the 5 position resulted in total loss of activity (Flaugh et al. 1979). Similarly, the pencil fish pigmentation response to 5-ethoxy and 5-propyloxy derivatives was poor (Frohn et al. 1980). Thus, the methoxy group occupying the 5 position has special biological significance as a result of either its effect on the electron distribution of the phenyl ring structure or its physical size.

When the amide portion of the molecule was altered to produce compounds such as N-propionyl-5-methoxy tryptamine and the N-butryl analogue (bMT), activity was lost at 50 µg/rat. This result contrasts with the very potent activity of bMT in the pencil fish assay (Frohn et al. 1980).

The third group of analogues represented attempts to prevent peripheral metabolism of melatonin to 6-hydroxymelatonin (Kopin et al. 1961) and oxidative ring cleavage in the brain to kynurenamines (Hirata et al. 1974). Three compounds, aMT.6 CI, DH.aMT and DH.aMT.6 CI, were synthesized in this group. In our assay system all three analogues were very potent blockers of puberty. We have previously shown these compounds shared with melatonin potent prolactin-lowering activity in sheep (Kennaway et al. 1986). Other groups have also shown that 6-chloro- and 6-fluoro-melatonin derivatives possess full biological potency in various assay systems (Flaugh et al. 1979; Martin et al. 1980; Vaughn et al. 1984). In the only other reported assay of 2,3-dihydromelatonin, it was found to be 10 times less potent than melatonin using the pencil fish assay (Frohn et al. 1980).

The fourth group of melatonin-like compounds tested were the brain metabolites of melatonin, N-acetyl-N2-formyl-5-methoxykynurenamine (aFoMK) and N-acetyl-5-methoxykynurenamine (aMK). When injected subcutaneously at a dose of 100 µg/rat, aFoMK
caused testis and accessory sex organ weights to be depressed whereas aMK did not produce significant effects. When injected at a 10-fold higher dose both aFoMK and aMK significantly retarded reproductive maturation. This activity is in contrast to the lack of any prolactin-inhibiting activity of aFoMK following intravenous injection of 100 μg into sheep (Kennaway et al. 1986) and the absence of melanophore activity in cultured melanocytes of both aFoMK and aMK (Messenger and Warner 1977).

The results of this study have shown how much the melatonin 'nucleus' can be altered while still retaining various activities. 13C nuclear magnetic resonance spectroscopic studies of serotonin, melatonin and analogues (Hugel, unpublished results) have shown that all these compounds have similar electron density distribution. It appears that biological activity is related to the shape and size of the molecules rather than to chemical reactivity.

In view of the hypothesis that melatonin acts as a prohormone the results obtained with dihydromelatonin and the kynurenamines do, however, pose an apparent contradiction. One possible explanation is that melatonin may have two mechanisms of action, one on the pituitary and gonadal axis that requires an intact indole structure and another central action within the hypothalamus via the 2,3 dioxygenase metabolic products. The notion that melatonin might act peripherally has had some support over recent years. The report of Peat and Kinson (1971) is of particular interest since they found a significant supression of testosterone production by melatonin in vitro. Such an effect in vivo might also partly explain melatonin-induced delayed puberty. Similarly, the melatonin inhibition of LHRH-stimulated LH secretion by neonatal rat pituitaries in vitro (Martin and Klein 1976) and direct inhibition of FSH and LH secretion by adult hamster pituitaries (Wun et al. 1986) are evidence of a peripheral effect and may contribute to the retarded growth of testicular tissue.

With respect to the central actions of melatonin, numerous interactions with neurotransmitter systems have been proposed (Cardinali et al. 1985). Some of these effects of melatonin may be due to aFoMK and aMK. The latter metabolite has, for instance, been shown to inhibit prostaglandin synthesis (Kelly et al. 1984). Melatonin and aMK also show in vitro activity on the benzodiazepine–GABA–chloride ionophore complex (Marangos et al. 1981). One endocrine effect of aMK at this level may be the potentiation of the effects of GABA in neuroendocrine target areas like the pre-optic area of the brain, which is known to be a steroid-sensitive site, or the suprachiasmatic nucleus where putative melatonin-binding sites have been identified (Vanecek et al. 1987). In the present study the higher biological potency of aFoMK compared with aMK may simply reflect its presumed greater accessibility to the brain following subcutaneous injection due to greater lipid solubility.

In summary, a series of compounds representing various modifications of the melatonin molecule have been tested for biological activity in protein-deprived male prepubertal rats. Modifications to the molecule in the 5 position of the aromatic indole ring or on the aliphatic ethyl amine side chain nitrogen indicate that biological activity of melatonin is sensitive to chemical changes. To date our substituent effects have not shown clear and consistent structure–activity relationships. For example, the 5-bromo substituent effects are anomalous and cannot readily be rationalized. Further compounds are under investigation to clarify structure–activity relationships.

Acknowledgment

This work was supported by a grant from the National Health and Medical Research Council of Australia.

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Manuscript received 19 October 1987, revised 16 May 1988, accepted 5 July 1988