Induction of blindness by formoguanamine hydrochloride in adult male roseringed parakeets (*Psittacula krameri*)

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Formoguanamine (2,4-diamino-*s*-triazine) was known to be an effective chemical agent in inducing blindness in poultry chicks, but not in adult birds. The present study was undertaken to demonstrate the influences, if any, of this chemical on the visual performance and retinal histology in an adult sub-tropical wild bird, the roseringed parakeet (*Psittacula krameri*). Formoguanamine (FG) hydrochloride was subcutaneously injected into adult parakeets at a dosage of 25 mg (dissolved in 0·75 ml physiological saline)/100 g body weight/day, for two consecutive days while the control birds were injected only with a placebo. The effects were studied after 10, 20 and 30 days of the last treatment of FG. Within 24 h of the treatment of FG, about 90% of the total birds exhibited lack of visual responses to any light stimulus and even absence of pupillary light reactions. The remaining birds became totally blind on the day following the last injection of FG and remained so till the end of investigation. At the microscopic level, conspicuous degenerative changes were noted in the outer pigmented epithelium and the photoreceptive layer of rods and cones in the retinas of FG treated birds. A significant reduction in the thickness of the outer nuclear layer was also found in the retinas of FG treated parakeets, compared to that in the control birds. However, the inner cell layers of the retina in the control and FG administered parakeets were almost identical. It deserves special mention that the effects of FG, noted after 30 days of last treatment, were not very different from those noted just after 10 days of treatment. Collectively, the results of the present investigation demonstrate that FG can be used as a potent pharmacological agent for inducing irreversible blindness through selective damage in retinal tissue even in the adult wild bird, thereby making FG treatment an alternative euthanasic device to a cumbersome, stressful, surgical method of enucleation of the ocular system for laboratory studies.

[Sengupta A, Obara Y, Banerji T K and Maitra S K 2002 Induction of blindness by formoguanamine hydrochloride in adult male roseringed parakeets (*Psittacula krameri*); *J. Biosci*. **27** 687–693]

1. Introduction

It has become abundantly apparent that light has a major impact on the physiological status of any animal and that the light/dark ratio influences, either directly or indirectly, almost every organ system in the body, especially reproduction in seasonally breeding animals, including birds (Lang and Sizonenko 1988; Vriend and Steiner 1988; Arendt 1995). The endogenous circadian oscillator that synchronises the annual gonadal cycle with the annual photoperiodic cycle among avian species is known to be located in the pineal organ, the hypothalamus [called the suprachiasmatic nucleus (vSCN)], and the eyes (Takahashi *et al* 1989; Bernard *et al* 1997). The presence of photoreceptors and visual pigments like rhodopsin and pinopsin in the pineal organ and retina

Keywords. Blindness; formoguanamine; retinopathy; roseringed parakeet

strongly argues for the participation of both in photoreception (Ebadi *et al* 1989). Thus, the demonstration of the photo-neuroendocrine pathway in any species is obligatory to the study of the relative roles of the pineal organ and retina.

The adult roseringed parakeet is a sub-tropical seasonally breeding bird that uses part of the annual photoperiodic cycle in the temporal organization of reproduction (Maitra and Dey 1992, 1993). A recent study has shown that surgical pinealectomy in this psittacine bird evokes variable testicular response to altered photoperiods during the different reproductive phases of an annual cycle (Maitra *et al* 2001). However, it remains to be seen whether processing of photoperiodic information in the regulation of reproduction in this bird is performed by the pineal organ alone, or in association with the retina.

Generally, surgical removal of the eyes or ocular enucleation is considered as a tool of investigation for the purpose of demonstration of any physiological role of extra-retinal photoreceptors, and such a method has been adopted by several workers for the study of different birds (*Japanese quail*: Saylor and Wolfson 1968; Siopes and Wilson 1980; Bayle *et al* 1983; Konishi *et al* 1988; *Chicken*: Harrison 1972; *Ducks*: Cuello *et al* 1972; *White-crowned sparrows*: Yokoyama and Farner 1976; *Turkey*: Siopes and El Halawani 1989; *Tree sparrows*: Wilson 1991). However, it is widely proclaimed that the surgical method of enucleation is immensely stressful to the subject, and considering the extent of damage in the entire optic system, this method is not very specific for studying the exclusive functions of the retina. It was thus a challenge, searching for a chemical agent that would induce blindness by the selective degeneration of the retina without causing any other hazards generally associated with surgery.

A heterocyclic compound, ammeline (2,4-diamino-6-hydroxy-*s*-triazine), isolated from chicken feed in the western part of Japan's Honshu Island, was reported to be a blinding agent, the first of its kind in newly hatched chicks (Kuba *et al* 1970). Subsequently, about 100 *s*-triazine compounds were synthesized, but leaving ammeline, only three compounds namely formoguanamine (2,4-diamino-*s*-triazine), 2-amino-*s*-triazine, and 2-amino-4-hydroxy-*s*-triazine, were found to be capable of causing blindness (Matsubara *et al* 1975). Even among these compounds, formoguanamine (FG) was proved to be the most potent (Goodman and Gilman 1975).

Earlier studies demonstrated that FG hydrochloride was effective in causing blindness by disruption of the pigmented epithelium and the retinal photoreceptor cells in newly hatched chicks (Obara *et al* 1985) and Japanese quail (Oishi and Obara 1994), but not in adult birds. However, a study on the efficacy of FG as a pharmacological agent for inducing blindness in any wild adult bird was not undertaken. Accordingly, an attempt has been made to study the effects of FG treatment on the morphology of the retina in a subtropical wild psittacine adult bird *Psittacula krameri*. This was to demonstrate its usefulness as a pharmacological agent in inducing blindness in adult birds, replacing the conventional surgical method of ocular enucleation, which is cumbersome as well as stressful to the subjects. The present study also addressed the question of whether the effects of FG treatment were irreversible.

2. Materials and methods

The roseringed parakeet (*Psittacula krameri borealis*, Neumann; Aves; Psittaciformes) is a resident, subtropical wild bird and is unique in showing sexual dimorphism in plumage colours only during adulthood. The males acquire a rose ring in the neck only after attaining sexual maturity in the post-hatch second year, and once this plumage characteristic appears, it remains throughout their lives (Ali and Ripley 1969). Accordingly, only adult males were selected for the purpose of the present investigation.

Sixty adult male roseringed parakeets (weighing in between 110–120 g) were captured from the vicinity of Burdwan (lat. 23°14′N long 81°51′E) in India by professional catchers during the month of November, corresponding to the progressive phase in an annual testicular cycle (Dey and Maitra 1992). They were brought to the laboratory and maintained in an open-air aviary with food (paddy *Oryza* sp.) and water *ad libitum* for about a week prior to their use. Subsequently, they were divided into two groups, each containing 30 birds. The first group of birds was considered as the control and the other group for FG treatment. The control birds were injected subcutaneously with 0·75 ml of physiological saline at a time, for two consecutive days. Each bird belonging to the treatment group was injected identically with an equal volume (0·75 ml) of physiological saline containing 25 mg of FG hydrochloride per day, for two consecutive days. FG hydrochloride was synthesized chemically in the Department of Applied Biological Chemistry, Meijo University, Japan and transported to India for the current purpose. FG was of 99% purity. The experimental schedule was part of the programme included in a research project financed by the Department of Science and Technology, New Delhi, and was duly approved by the Board of Research Studies, corresponding to the animal ethical study group, in the University of Burdwan, Burdwan.

All the birds, irrespective of their treatment, were maintained under stress-free laboratory conditions (daily photoperiods: about 11 h; ambient temperature between 22–28°C; humidity between 60–70%) with food and

water *ad libitum*, and the behaviour of individual birds was observed carefully throughout the investigation. Effects were studied following 10–, 20–, and 30 days of the last treatment. At each point, the body weight of each bird was recorded and 10 birds each from the control and FG-treated groups were sacrificed by cervical dislocation. Quick dissection followed for the removal of retinal tissue from each eye. The retinal tissues of both the control and FG treated birds were fixed in aqueous Bouin's fixative, dehydrated in graded alcohols, followed by routine microtomy for procuring 4 μm thick paraffin sections which were subsequently stained, following Masson's trichrome technique. The stained sections of the retina were carefully studied with the use of a PRIOR (UK) microscope. The thickness of different histological layers of retina in the control and FG treated birds was measured under this microscope, with the help of a calibrated eye-piece ocular, and the values that were obtained thereof were statistically analysed, following Student's '*t*'-test (Zar 1974).

3. Results

3.1 *Behaviour of the birds*

Most of the birds exhibited remarkable changes in their behaviour, like sluggishness, abnormal neck movement and a sad peep, within 5–6 h of the last treatment of FG. Within the next 24 h, nearly 90% of the total FG-treated birds became sightless, as seen from their lack of responsiveness to a moving object in front of them, or to a light stimulus evidenced by a lack of pupillary light reactions. The remaining birds became totally blind following injection of an additional dose of FG (25 mg/bird) the next day. There were no significant changes in the values of body weight between the control and FG-treated groups of parakeets studied 10, or 20, or 30 days after the last treatment.

3.2 *Histology of the retina*

Microscopic study revealed that following the general avian pattern (Sturkie 1986), the retina in parakeets has two main components: a pigmented retina next to the choroid layer and a neural retina next to the vitreous body. The pigmented retina is a simple layer of cells rich in melanin granules. The neural retina consists of photoreceptor neurons called rods and cones, and seven other layers, namely the outer nuclear layer, the outer plexiform layer, the inner nuclear layer, the inner plexiform layer, the ganglion cell layer, the optic nerve fibre layer, and the inner limiting membrane (figure 1). The microscopic features of the retina of parakeets, noted after 10–, 20–, and 30 days after FG treatment, were almost alike. At each point, conspicuous degenerative changes occurred in the outer pigmented epithelium, and the photoreceptive layer of rods and cones in the retina of FG-treated birds (figure 2). Moreover, a significant reduction in the thickness of the outer nuclear layer was found in the retina of FG treated parakeets, compared to those in the control group (table 1). The remaining inner cell layers of the retina of FG treated birds, however, were almost identical to those studied with the respective groups of control parakeets.

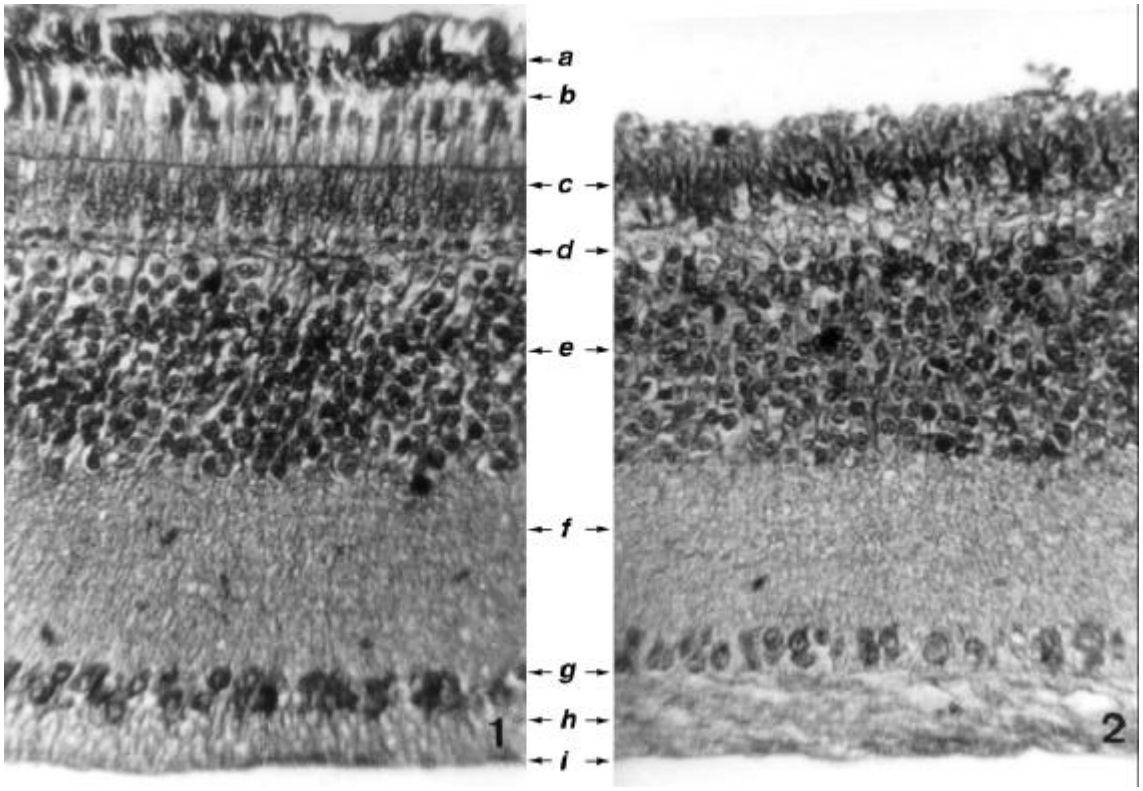
4. Discussion

The presence of FG, an uracil analogue, was detected accidentally by epidemiologists in Japan as a contaminant in commercial chicken feed; consumption of this chicken feed was found to be associated with impaired vision among the chicks (Kuba *et al* 1970). However, subsequent studies demonstrated that FG could be used in the laboratory to induce blindness in newly hatched male chicks of domestic fowl and Japanese quail, but not in the case of adult poultry birds (Obara *et al* 1985; Oishi and Obara 1994). The present communication reports for the first time, that FG is equally effective in inducing blindness in an adult non-poultry avian species. However, it is notable that the dosage regimen followed in this study for administration of FG (25 mg/bird/day for two days) was not identical to those employed earlier (Obara *et al* 1985; Oishi and Obara 1994). In the case of male chicks of the domestic fowl (*Gallus domesticus*), 50 mg ml^{-1} FG, 0·2 ml, solution was injected subcutaneously four times in the leg at 12, 24, 36 and 48 h after hatching, so that each chick (average body weight was approximately 40 g) received a total of about 40 mg (Obara *et al* 1985). On the other hand, a post-hatch two-day old male Japanese quail (*Coturnix coturnix japonica*) became completely blind following a single subcutaneous injection of 1·5 mg FG, dissolved in 0·1 ml of saline (Oishi and Obara 1994). Thus, it becomes quite apparent that FG may be used as a pharmacological agent to induce blindness in both domestic as well as in wild birds, but the optimum dose may vary with the species and/or the age of the concerned birds. Since all the studies, including the present one, have dealt only with males, the sex discrepancy (if any), in response to FG remains unknown.

Histological examination of the retina in parakeets indicated that FG-induced blindness resulted from degeneration of the outer cell layers. It is notable that the outermost pigmented epithelial layer of the retina lying next to the choroids degenerated completely when studied after 10 days of FG treatment; the results show no signs of regeneration in the concerned retinal layer, even after 30 days of the last treatment, which clearly indicates that the

effects of FG are irreversible. The outermost pigmented layer of the retina is richly packed with melanin pigment granules, and prevents entrance of light from the stimulated retina. It is known that the photoreceptive cell layer of the rods and cones is packed with stacked discs containing photoreceptive pigments namely, rhodopsin (in rods) and iodopsin (cones), which absorb photons of light, stimulating the visual cycle in the eye under low and high intensity illuminations respectively. Thus, degeneration of this layer, along with the changes noted in the outer nuclear layer containing cell bodies and nuclei of the rods and cones, may be the cause of sightlessness induced by FG in adult parakeets. A study of the FG-induced changes in the retina of the currently considered wild psittacine bird confirms the earlier observations (Obara *et al* 1985; Oishi and Obara 1994) that this uracil analogue has a degenerative effect on the outer photoreceptive cell layers of the retina, but has the least effect upon the inner retinal layers.

The mechanism of FG-induced changes in the retina was studied in newly hatched chicks (Obara *et al* 1985). It was noted that the specific activity of retinal ornithine aminotransferase was localised exclusively in the mitochondria of the retinal pigment epithelium, and the retinal vitamin B_6 content decreased rapidly after FG was administered. An absorption spectrum of retinal extract showed reduced absorbance at 400 nm in the FG-treated chicks, but Δ^1-pyrroline-5-carboxylate reductase, an enzyme in the retinal cytosol, did not change significantly. These findings suggest that FG-induced blindness correlates with a decrease in retinal vitamin B_6 content. This, in turn, may be responsible for the decrease in retinal ornithine aminotransferase that may ultimately lead to the impaired physiological functions of retinal pigment and photoreceptors in newly hatched chicks (Obara *et al* 1985). As efficacy of FG as an agent for induction of blindness was not known for adult birds, elucidation of the mechanism of action of FG in the retina of adult



Figures 1–2. Masson-trichrome stained 4 µm thick paraffin sections of retina showing influences of FG treatment in roseringed parakeets (× 450). **(1)** Section of the retina of a control bird showing different layers, namely (a) the pigmented epithelium, (b) the photoreceptor cell layer, (c) the outer nuclear layer, (d) the outer plexiform layer, (e) the inner nuclear layer, (f) the inner plexiform layer, (g) the ganglionic cell layer, (h) the optic nerve fibre layer, and (i) the inner limiting membrane. **(2)** Section of the retina of a FG-treated parakeet showing complete loss of the pigmented epithelium, and the photoreceptor cell layer. Moreover, desquamation of cells can be noted in the outer nuclear layer (c). Remaining layers (d–i) of neural retina do not show marked differences from those in the retina of control birds.

Table 1. Mean values (± SE) of the thickness (μm) of different cell layers of retina in control and formoguanamine (FG) hydrochloride treated adult male roseringed parakeets.

	Thickness (μm) of different cell layers of retina					
	At day-10		At day-20		At day-30	
Retinal cell layer	Control (μm)	FG-treated (μm)	Control (μm)	FG-treated (μm)	Control (μm)	FG-treated (μm)
Pigmented epithelium	14·82 ± 2·05	Absent	14·66 ± 1·94	Absent	14·25 ± 2·59	Absent
Photoreceptor cell layer	20·92 ± 1·65	Absent	21·28 ± 1·86	Absent	21·50 ± 1·32	Absent
Outer nuclear layer	17·15 ± 0·63	12·14 ± 1·22*	16·86 ± 0·96	11·08 ± 1·14*	16·75 ± 0·63	10·60 ± 1·03*
Outer plexiform layer	4·58 ± 0·42	4·42 ± 1·06	4·81 ± 0·53	4·26 ± 0·92	4·75 ± 0·33	4·67 ± 0·75
Inner nuclear layer	56·42 ± 1·69	54·65 ± 2·39	57·21 ± 1·82	56·21 ± 2·12	55·72 ± 1·33	51·33 ± 2·39
Inner plexiform layer	55·73 ± 1·55	52·96 ± 2·84	56·08 ± 1·62	51·87 ± 2·66	54·25 ± 1·33	51·33 ± 2·25
Ganglionic cell layer	12·11 ± 1·47	10·88 ± 1·26	11·82 ± 1·54	11·21 ± 1·07	11·25 ± 1·18	10·32 ± 0·88
Optic nerve fibre layer	13·94 ± 1·46	14·16 ± 2·63	14·12 ± 1·36	14·02 ± 1·86	14·25 ± 1·15	15·00 ± 2·17
Inner limiting membrane	2·08 ± 0·21	1·88 ± 0·62	2·11 ± 0·16	1·72 ± 0·81	2·02 ± 0·17	1·83 ± 0·41

*Statistically significant at 5% level.

parakeets was far from the objective of this current investigation. However, it would be worthwhile studying whether the mechanism of retinopathic actions of FG in adult parakeets is different from that noted in newly hatched chicks. Since the functional organisation of the retina is known to vary in relation to the age and habit of different birds (Sturkie 1986), it may not be wise to rule out the possibility of a different mechanism of action of FG in the induction of blindness in chicks and parakeets until further research.

Apart from vision, in birds, the retina is thought to be associated with the circadian functions of the body, and this role is played mostly by the rhythmic production of melatonin – a putative physiological time-keeping hormone which acts as a local modulator of the neuronal activities and photoreceptive metabolism (Besharse and Dunis 1983; Hastings 1997; Iuvone 1995). Though the pineal organ is the major source of this hormone, the retina is known to contribute about 33% of the melatonin that is circulating in certain avian species (Underwood *et al* 1984). Obviously, interest has grown in searching the site of intra-retinal synthesis of this hormone. Melatonin-like immunoreactivity has been noted in the outer nuclear layer of the retina (Bubenik *et al* 1976). Several lines of evidence indicate that a large part of the circulating melatonin in birds is contributed by the retinal photoreceptors (Weichmann 1986; Cahill *et al* 1991; Cahill and Besharse 1992). Disruption of the inner retina of the chick by kianate treatment did not affect the rhythms of melatonin synthesis *in vivo*, indicating further that it is the outer photoreceptors, that are involved in the circadian rhythmicity of melatonin within the retina (Zawilska and Iuvone 1992; Thomas and Iuvone 1991; Chong *et al* 1998). It is notable, further, that treatment of FG in parakeets resulted in complete disruption of the pigmented epithelium, which in normal subjects does not produce melatonin, but plays a permissible role in enabling maximal melatonin synthesis by photoreceptors (Cahill and Besharse 1993). Moreover, the circadian oscillator of the avian retina has been reported to be localized within the photoreceptors (Zawilska and Iuvone 1992; Pierce *et al* 1993). Collectively, these reports suggest that the outer retinal layers are the seats of ocular circadian oscillators as well as melatonin biosynthesis. In this context, the results of the present investigation showing selective but irreversible degeneration of only the outer retinal cell layers in the FG treated birds, support the contention that it may be used as a potent pharmacological tool in the laboratory. It can be used to analyse the role of retinal melatonin, as well as of retinal circadian oscillator in the regulation of various physiological functions in adult birds in general, and in adult male roseringed parakeets in particular.

Acknowledgements

Financial assistance from the Department of Science and Technology, New Delhi through a research project (No. SP/SO/C-18/96) sanctioned to SKM is thankfully acknowledged.

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MS received 25 April 2002; accepted 31 October 2002

Corresponding editor: SAMIR BHATTACHARYA