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RESEARCH PAPER

5-HT receptors as novel targets for optimizing pigmentary responses in dorsal skin melanophores of frog, *Hoplobatrachus tigerinus*

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BACKGROUND AND PURPOSE

Biochemical identification of 5-HT has revealed similar projection patterns across vertebrates. In CNS, 5-HT regulates major physiological functions but its peripheral functions are still emerging. The pharmacology of 5-HT is mediated by a diverse range of receptors that trigger different responses. Interestingly, 5-HT receptors have been detected in pigment cells indicating their role in skin pigmentation. Hence, we investigated the role of this monoaminergic system in amphibian pigment cells, melanophores, to further our understanding of its role in pigmentation biology together with its evolutionary significance.

EXPERIMENTAL APPROACH

Pharmacological profiling of 5-HT receptors was achieved using potent/selective agonists and antagonists. *In vitro* responses of melanophores were examined by Mean Melanophores Size Index assay. The melanophores of lower vertebrates are highly sensitive to external stimuli. The immediate cellular responses to drugs were defined in terms of pigment translocation within the cells.

KEY RESULTS

5-HT exerted strong concentration-dependent pigment dispersion at threshold dose of 1×10^{-6} g·mL^{-1}. Specific 5-HT$_1$ and 5-HT$_2$ receptor agonists, sumatriptan and myristicin. also induced dose-dependent dispersion. Yohimbine and metergoline synergistically antagonized sumatriptan-mediated dispersion, whereas trazodone partially blocked myristicin-induced dispersion. Conversely, 5-HT$_3$ and 5-HT$_4$ receptor agonists, 1 (3 chlorophenyl) biguanide (1,3 CPB) and 5-methoxytryptamine (5-MT), caused a dose-dependent pigment aggregation. The aggregatory effect of 1,3 CPB was completely blocked by ondansetron, whereas L-lysine partially blocked the effect of 5-MT.

CONCLUSIONS AND IMPLICATIONS

The results suggest that 5-HT-induced physiological effects are mediated via distinct classes of receptors, which possibly participate in the modulation of pigmentary responses in amphibian.

Abbreviations

1,3 CPB, 1 (3 chlorophenyl) biguanide; 5-MT, 5-methoxy tryptamine; ARS, amphibian ringer saline; MMSI, Mean Melanophores Size Index; NAS, N-acetyl-5-HT

Introduction

5-HT (serotonin) is one of the primary monoamine neurotransmitters in the CNS. The release of 5-HT is modulated by 5-HT receptors, which are located on various nerve terminals of the central and peripheral nervous systems (Julius, 1991; Göthert *et al.*, 1995). The fundamental advancement towards understanding the physiology of neurotransmitters acting on transmembrane receptors in numerous effector cells has radically reconditioned our concept of down-stream signalling pathways that occur within the cells. Interestingly, it has been reported that frog skin contains various biologically active compounds such as biogenic amines, neurotransmitters and neuropeptides (Erspamer, 1992). Also, neuropeptides detected in frog skin have led investigators to elucidate their mammalian counterparts (Tatemoto *et al.*, 1982). 5-HT is one of the compounds detected in frog skin (Bennett *et al.*, 1982; Roseghini *et al.*, 1988; 1989). It stimulates smooth muscle contraction (Ross *et al.*, 1995) and also functions as an important modulator and transmitter in vertebrate nervous system (Dicke *et al.*, 1997). It is also suggested that 5-HT plays a role in self-defence as well as in colour change and water exchange in frog skin (Kramer, 1970; Lillywhite, 1971; Bennett *et al.*, 1981).

A well-established physiological action of 5-HT takes place in the dermal layer of the skin. Here, cells called melanophores, which contain the pigment melanin respond to neuronal stimuli by initiating a rapid, reversible translocation of pigment granules (melanosomes) within the cell cytoplasm. Dispersion of melanosomes throughout the cell or their aggregation in a perinuclear position results in dramatic colour changes which are important for courtship, camouflage, self-defence, thermoregulation and photo protection (Rollag and Adelman, 1992; Fujii, 1993). This bidirectional translocation of pigment to the centre (aggregation) or towards the periphery (dispersion) gives the corresponding lighter or darker hue respectively. It is known that melanophores of lower vertebrates especially amphibians and fishes are highly sensitive in their responses to external cues, which makes them an excellent model tissue for studying the effect of compounds and their implications (Gele and Lambert, 2011).

The role of 5-HT in bringing about a discernible pigmentary response has been revealed by the presence of 5-HT receptors on pigment cells, melanophores (Potenza and Lerner, 1994). Despite their detection on melanophores, there are very few, yet conflicting reports regarding the putative role of 5-HT receptors in controlling pigmentary responses within these cells. As early as 1957, Kahr and Fischer had found that 5-HT was incapable of causing any response in black background-adapted *Rana temporaria*, but it did induce pigment dispersion in normal white- background adapted frogs (Kahr and Fischer, 1957). 5-HT has been reported to cause dispersion in the melanophores of the hypophysectomized frog, *Rana pipiens* (Davey, 1959). Later, this was confirmed by Vande Veerdonk (1960) on *Xenopus laevis*. In contrast, Lerner and Case (1960) demonstrated a concentrating effect of 5-HT on the melanophores of *R. pipiens* and it has recently been reported that 5-HT causes a concentration-dependent dispersion as well as aggregation in *X. laevis* (Teh and Sugden, 2001).

5-HT exerts its pleiotropic function by interacting with specific G-protein cell surface membrane-bound receptor families (Peroutka, 1990; Hoyer *et al.*, 2002; Bockaert *et al.*, 2006). The physiological activities of these receptors on the effector/target cells differ, depending upon the signalling pathway downstream to them. For instance, 5-HT_4 and 5-HT_7 stimulate the activity of adenylyl cyclase, thereby increasing the levels of cAMP (Branchek, 1995). Conversely, 5-HT_1 attenuates the activity of adenylyl cyclase, resulting in a decrease in cAMP. On the other hand, 5-HT_2 receptors enhance the activity of phosphoinositol hydrolases and 5-HT_3 functions as a ligand-gated ion channel (Maricq *et al.*, 1991; Bohlen and Dermietzel, 2002).

The relationship between pigmentary function and 5-HT receptor type is complex. Detection of 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2B} and 5-HT_7 receptors on mammalian melanocytes and dermal fibroblasts is consistent with a putative function for 5-HT as a growth factor (Seuwen and Pouyssegur, 1990). 5-HT stimulates proliferation of melanocytes in a medium deprived of growth factors, while it inhibits cell growth in the presence of growth factors (Slominski *et al.*, 2003c). However, the direct involvement of 5-HT receptors in pigment generation in melanocytes is still obscure and needs to be investigated.

As melanin-based pigmentation biology as well as the 5-HT embryogenic and morphogenic functions are conserved across lower to higher vertebrates (Goodrich *et al.*, 1980; Slominski *et al.*, 2004), a deeper understanding of the physiology of lower vertebrates translates easily and directly into testable hypotheses for studying the cellular basis of pigmentation variation in natural vertebrate cell systems including humans. It is now known that the 5-hydroxytryptaminergic system is fully expressed in various vertebral systems including human skin (Johansson *et al.*, 1998; Slominski *et al.*, 2002b, 2003b, 2005), and the first experimental evidence that 5-HT is synthesized in skin cells was obtained in hamster skin (Slominski *et al.*, 2002a). Interestingly, the tryptophan hydroxylase gene (TRH 1) that catalyzes the synthesis of 5-HT is expressed in whole human skin cells (Slominski *et al.*, 2003a). Furthermore, there is a dearth of evidence that the skin has molecular and biochemical apparatus to produce and metabolize 5-HT and N-acetyl-5-HT (Slominski *et al.*, 2002c, 2003a). Although 5-HT receptors are reported to be present in an array of skin cells across animal species, their explicit role in the process of skin pigmentation is still unclear (Slominski *et al.*, 2004). Also, looking at the variable and fragmentary reports in literature, our present study was undertaken to characterize the presence of recently classified novel 5-HT receptors and their putative role in the pigmentary responses within the melanophores of commonly found amphibian, *Hoplobatrachus tigerinus* (synonym, *Rana tigerina*), which offers an excellent system for *in vitro* pharmacological studies. This investigation would definitely be a considerable step towards bridging the gaps in our understanding of the properties of the 5-hydroxytryptaminergic system and its role in pigmentation biology together with its evolutionary significance.

Methods

Studies were performed on the isolated dorsal skin melanophores of *H. tigerinus* (*syn. R. tigerina*) weighing 100–125 g.

Adult frogs were acquired legally from an authorized dealer following the legislation of the country where the study was conducted, and no damage was caused to the environment or wild animal populations. The animals were kept in a laboratory froggery for 3–4 days with a 12 h light-dark phase and temperature maintained between 20 and 25°C. A mud substratum was built at the base of the tank, which was kept wet. The tank was covered with a wire mesh that had provision for withdrawing and introducing frogs. Food, temperature, humidity and sanitation were taken care of properly. Overfeeding was avoided and any animals with signs of disease or lethargy were left out. Suitable care was taken to minimize any causes of stress to the animals.

The ethical committee for Animal Examination and Research, Saifia College of Science, Bhopal, India, certified the use of animal experimentation and handling. The research was done under strict compliance with the guidelines of Indian Council of Medical Research, Guidelines for Use of Laboratory Animals in Medical Colleges (2001) as per Breeding and Experiments of Animal Amendments Rules (2001) and Prevention of Cruelty to Animal Act (1966).

Preparation of dorsal skin pieces for in vitro *studies*

For experimentation, the animals were carefully decapitated and the dorsal skin pieces measuring approximately 2–3 mm were removed and immediately immersed in amphibian ringer saline (ARS); 111 mM of sodium chloride, potassium chloride 2 mM, calcium chloride 1 mM and sodium hydroxide 2 mM; in 100 mL of double distilled water with pH 7.4. The skin pieces were incubated in ARS for 15–20 min for equilibration with frequent shakings. The temperature of the experimental setup remained between 20 and 25°C. After 20 min of equilibration in the ARS, the skin pieces containing approximately 50–100 melanophores were incubated with known concentrations of drugs for 7–10 min. All drugs were dissolved freshly in doubled distilled water and their solutions were added to the Petri dishes containing the ARS, the total volume of which was kept constant (10 mL).

The responses of control as well as of those melanophores that were incubated in 10 mL ARS containing various concentrations of receptor-specific agonists were measured according to the method used by Bhattacharya *et al*. (1976) based on the Hogben and Slome (1931) Melanophore Index. In this method, the individual melanophores were measured with the help of Leitz ocular micrometer calibrated by a stage micrometer, by marking the maximum vertical and horizontal diameters. Ten such randomly selected melanophores from each skin piece were measured and the Mean Melanophore Size Index (MMSI) was calculated. When the melanophores disperse, that is, the melanin pigment granules within the melanophores move to the periphery, the diameter of the cells increases and *vice versa*. The calculated value is the MMSI, expressed in terms of μm.

Drugs

5-HT hydrochloride (≥99% HPLC solid) and 1-(3- chlorophenyl) biguanide hydrochloride (≥97% solid) were purchased from AlphaAesar (Karlsruhe, Germany); sumatriptan succinate (≥98% HPLC solid) was purchased from Sun Pharmaceuticals India Ltd. (Mumbai, India); yohimbine hydrochloride (≥98% TLC) was purchased from HiMedia Laboratories, (Mumbai, India); myristicin (>99.5% clear liquid) from parsley oil, was obtained from MP Biomedicals LLC (USA); metergoline (≥98% HPLC solid), mirtazapine (≥98% HPLC solid), trazodone hydrochloride (≥99% HPLC powder), ondansetron hydrochloride dihydrate (≥98% HPLC solid), 5-methoxy tryptamine (5-MT; ≥97%) and L-lysine (≥98% TLC) were purchased from Sigma-Aldrich (USA); L-adrenaline bitartrate (≥98% solid) was purchased from C.H. Boehringer Sohn AG & Co. KG (Ingelheim, Germany).

Control

The control position was established by pretreating the melanophores with a known concentration of adrenaline (2×10^{-8} g·mL^{-1}). This value was determined by treating the melanophores with varying concentrations of adrenaline. Adrenaline has been reported to cause the melanophores to aggregate (Borisky and Rodionov, 1999). Therefore, a dose was selected where the melanophores were brought to an intermediate stage of neither aggregation nor dispersion as per the modified method of Ali *et al*. (1998). This facilitated the accurate measurement of the dispersal responses to 5-HT as well as the other agonists.

Employment of 5-HT receptor agonists

To investigate the pharmacological characteristics of 5-HT receptors on melanophores, various specific agonists were tested for their ability to initiate pigment mobility (Table 1). The analysis of data reveals several points of interest. The agonists selected were highly specific for their respective active site whereas they also possess varying abilities to activate a wide range of subtypes of the receptor.

Employment of 5-HT receptor antagonists

To characterize further the pharmacology of the 5-HT receptor system in the skin melanophores, and to confirm the presence of 5-HT receptors, various antagonists were tested for their ability to inhibit the agonist-induced effect (Table 1). Antagonists acting at 5-HT$_{1-4}$ receptors with individual as well as synergistic antagonistic effects were investigated. Individual effects of the drugs were tested on the pigment motility of *H. tigerinus* (summarized in Table 1).

The antagonist was applied to the incubating media before addition of the agonist. The final concentration of the antagonist was selected from a series of dose-dependent pre-incubated pilot experiments, with the dose ranging from 1×10^{-7} g·mL^{-1} to 6.4×10^{-5} g·mL^{-1}. The concentration of antagonist that was found to have no effect on the melanophore responses on its own was finally selected as the dose to use in the antagonist experiments.

Statistical data analysis

Statistical data analyses are presented as mean ± SEM (represented by vertical bars) and n represents the number of dose concentrations (treated) used for a particular experiment. Comparisons were made between treated and control groups by use of Student's t-test. All data were analysed using GraphPad Prism software (UK). $P < 0.05$ indicates statistically significant difference.

Table 1

Summary of the pharmacology and effects of drugs used in this study on melanin dispersion within the melanophores of *Hoplobatrachus tigerinus*

Drug	Pharmacology	Receptor efficacy	Effect on melanin dispersion
5-HT hydrochloride	Stimulation of 5-HT receptor	Non-selective endogenous agonist at 5-HT receptors	+
Sumatriptan succinate	Stimulation of 5-HT_1 receptor	Highly potent/selective 5-HT_1 agonist	+
Myristicin or methoxysafrole	Stimulation of 5-HT_2 receptor	Selective 5-HT_2 agonist	+
1, 3 CPB	Stimulation of 5-HT_3 receptor	Highly potent/selective 5-HT_3 agonist	–
5-MT	Stimulation of 5-HT_4 receptor	Full 5-HT_4 agonist	–
Yohimbine	Blockage of 5-HT_1 and 5-HT_2 receptors	Potent 5-HT_1 antagonist	–
Metergoline	Blockage of 5-HT_1 receptor	5-HT_1 and 5-HT_2 antagonist	+
Trazodone	Blockage of 5-HT_2 receptor	Potent 5-HT_2 antagonist	–
Mirtazapine	Blockage of 5-HT_2 receptor	Potent 5-HT_2 antagonist	+
Ondansetron	Blockage of 5-HT_3 receptor	Highly potent/selective 5-HT_3 antagonist	None
L-lysine	Blockage of 5-HT_4 receptor	Partial antagonist at 5-HT_4	None

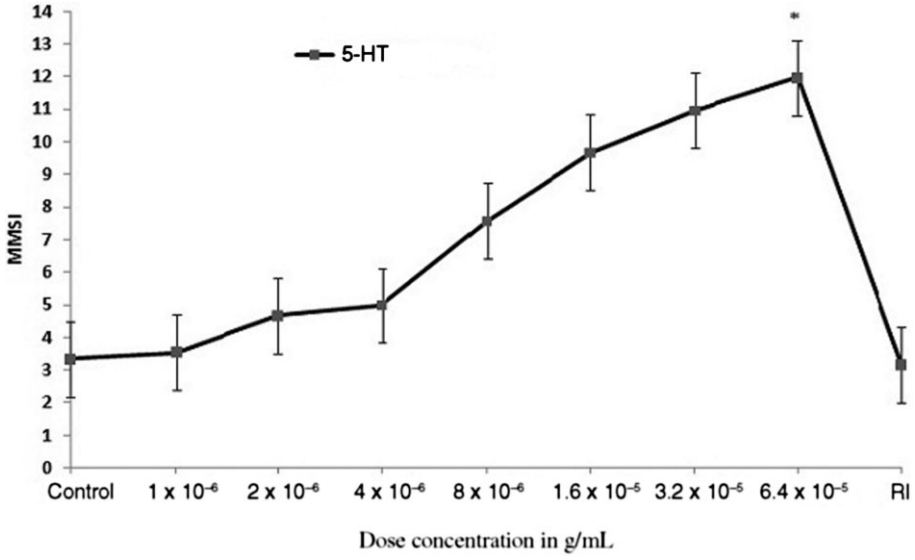

Figure 1

Dose-response curve for 5-HT on the dorsal skin melanophores of *Hoplobatrachus tigerinus* shown by (■) closed squares. Vertical bars indicate SEM. $P < 0.0001$ signifies level of significance.

Results

5-HT induces pigment dispersion in melanophores of H.tigerinus

It was found that 5-HT induced pigment dispersion in a dose-dependent manner. The minimum threshold concentration of 5-HT that could bring about a discernible response in melanophores was 1×10^{-6} g·mL^{-1}, where the MMSI increased to 3.55 ± 0.04 from the control value of 3.04 ± 0.49. The effect followed a dose-dependent curve with the maximum degree of dispersion recorded at 6.4×10^{-5} g·mL^{-1}, with the MMSI at 11.96 ± 0.20 (Figures 1 and 2). The dispersing effect of 5-HT was challenged with a combination of antagonists. The 5-HT_1 antagonist yohimbine (2×10^{-7} g·mL^{-1}) could not completely block the effect of 5-HT with the highest dispersion peak at 6.4×10^{-5} g·mL^{-1}, with MMSI 7.55 ± 0.34. Thereafter, a combination of 5-HT_1 antagonists' yohimbine (2×10^{-7} g·mL^{-1}) and metergoline (4×10^{-6} g·mL^{-1}) was employed. The synergistic blocking effect of the two drugs could completely attenuate the dispersion caused by 5-HT where the MMSI recorded at highest dose concentration of 5-HT was found to be 5.87 ± 0.12 (Figure 3).

In another set of experiments, 5-HT was challenged with the 5-HT_2 antagonist trazodone. It was found that trazodone did bring about a slight attenuation in the 5-HT response, with the MMSI at highest concentration to be 8.98 ± 0.32. Thereafter, a combination of trazodone (2×10^{-7} g·mL^{-1}) and mirtazapine (4×10^{-6} g·mL^{-1}) was employed to challenge the dispersion caused by 5-HT. It was found that the synergistic blockage of the two antagonists potentiated the 5-HT response and the MMSI increased to 9.59 ± 0.29. This finding indicated the presence of both 5-HT_1 and 5-HT_2 receptors with 5-HT_1 being the predominant receptor type. Also, the affinity of 5-HT seems to be higher for 5-HT_1 receptors (Figure 3).

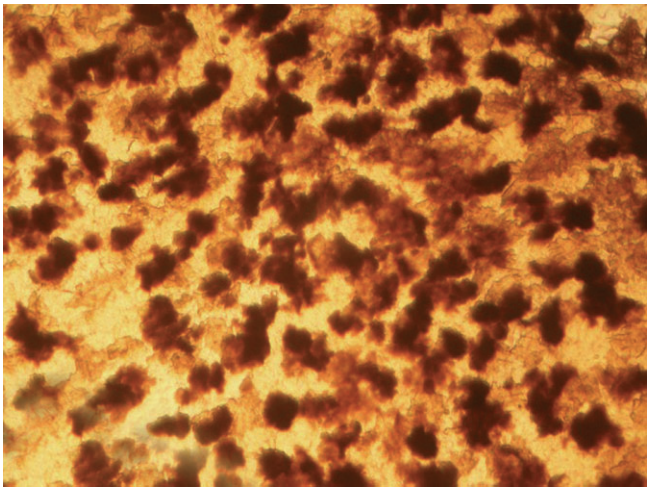

Figure 2
Dispersion caused by 5-HT 6.4×10^{-5} g·mL^{-1}.

5-HT_1 receptors

The potent and selective 5-HT_1 receptor agonist sumatriptan succinate caused dispersion in the melanophores in a dose-dependent manner. Sumatriptan at a concentration as low as 1×10^{-7} g·mL^{-1} produced a measurable response that was evident from the increase in MMSI 5.20 ± 0.13 in comparison with the control MMSI 3.53 ± 0.18. The maximum effect was observed at 6.4×10^{-6} g·mL^{-1} where the MMSI increased to 13.48 ± 0.21. A comparative analysis of the effects of non-specific 5-HT *per se* and sumatriptan on melanophores revealed that sumatriptan caused a higher degree of dispersion than 5-HT (Figure 4). This finding indicated the presence of dominant 5-HT_1 receptors in the melanophores of *H. tigerinus*. To confirm this finding, the drug yohimbine was utilized, which is an antagonist at the 5-HT_1 receptor. Melanophores pre-treated with yohimbine 2×10^{-7} g·mL^{-1} and subsequently subjected to sumatriptan, reduced the MMSI response to 6.78 ± 0.16, at highest dose of sumatriptan (6.4×10^{-6} g·mL^{-1}). Thereafter, the effects of metergoline (4×10^{-7} g·mL^{-1}) were tested; metergoline attenuated the dispersion induced by the highest concentration of sumatriptan, with the MMSI reduced to 5.99 ± 0.13. Furthermore, the combination of yohimbine (2×10^{-7} g·mL^{-1}) and metergoline (4×10^{-7} g·mL^{-1}) effectively and completely block the dispersion caused by

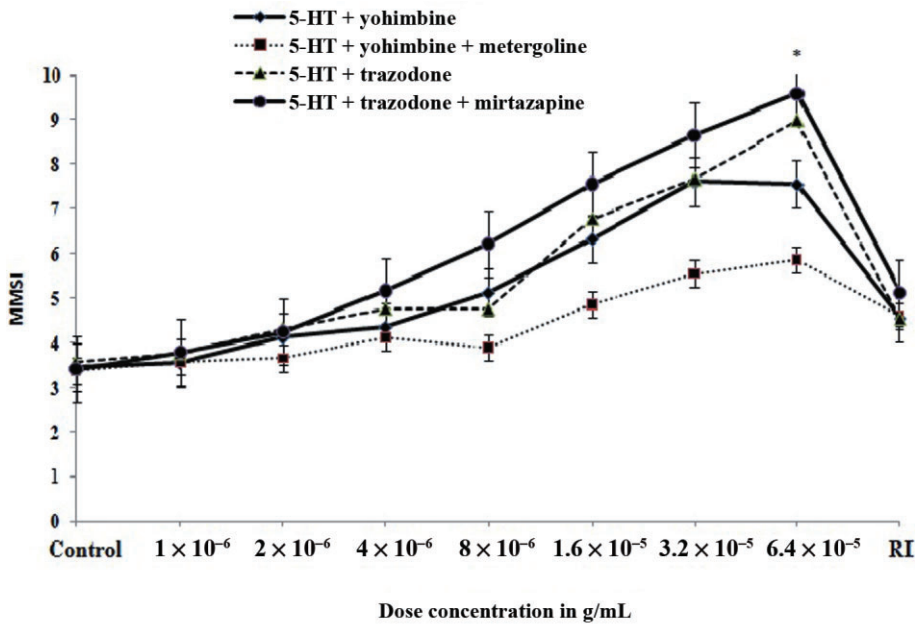

Figure 3
Dose-response curves depicting the dispersal effect caused by 5-HT being challenged by a combination of antagonists on the dorsal skin melanophores of *Hoplobatrachus tigerinus*. The blocking effect of yohimbine (2×10^{-7} g·mL^{-1}) is shown plus the synergistic blockage by the combination of yohimbine (2×10^{-7} g·mL^{-1}) and metergoline (4×10^{-6} g·mL^{-1}). The inhibition of 5-HT-induced dispersion by trazodone (2×10^{-7} g·mL^{-1}) is shown plus the synergistic blockage effect of the combination of trazodone (2×10^{-7} g·mL^{-1}) and mirtazapine (4×10^{-6} g·mL^{-1}). Vertical bars represent SEM. *$P < 0.0002$ signifies level of significance.

sumatriptan, with MMSI (4.68 ± 0.14) confirming the presence of 5-HT_1 receptors (Figure 5).

5-HT_2 receptors

The 5-HT_2 receptor agonist myristicin was found to cause a weak dispersion in the melanophores in a dose-dependent manner. The concentrations in low range in the dose-response curve had little effect, with an MMSI of 5.59 ± 0.24 at 4×10^{-7} g·mL^{-1}, with a slight increment from the control value 3.98 ± 0.17 (Figure 6). However, at a higher dose range, that is, above 1×10^{-6} g·mL^{-1} to 6.4×10^{-5} g·mL^{-1}, there was a marked increase in dispersion with the MMSI 9.79 ± 0.15 with $P < 0.0001$ (Figures 7 and 8). The presence of the 5-HT_2 receptor was confirmed by antagonizing the dispersion caused by myristicin with trazodone (2×10^{-7} g·mL^{-1}), which is a potent 5-HT_2 receptor antagonist; trazodone did not completely block the effect of myristicin. In another series of

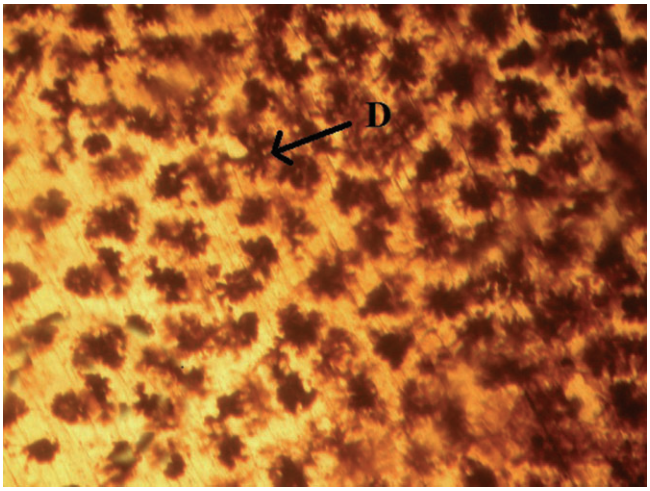

Figure 4

Dispersion caused by sumatriptan 6.4×10^{-6} g·mL^{-1}. D, dispersion.

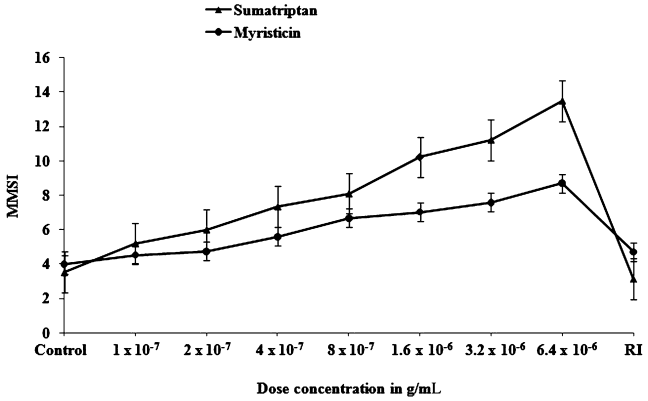

Figure 6

Dose-response curves for the effects of the 5-HT_1 selective agonist sumatriptan and the 5-HT_2 agonist myristicin on the dorsal skin melanophores of *Hoplobatrachus tigerinus*. Vertical bars represent SEM. Note that within the dose range 1×10^{-7} g·mL^{-1} to 6.4×10^{-6} g·mL^{-1} myristicin caused little dispersion, whereas the same dose range resulted in a substantial response to the specific agonist sumatriptan. $P < 0.0001$ signifies level of significance.

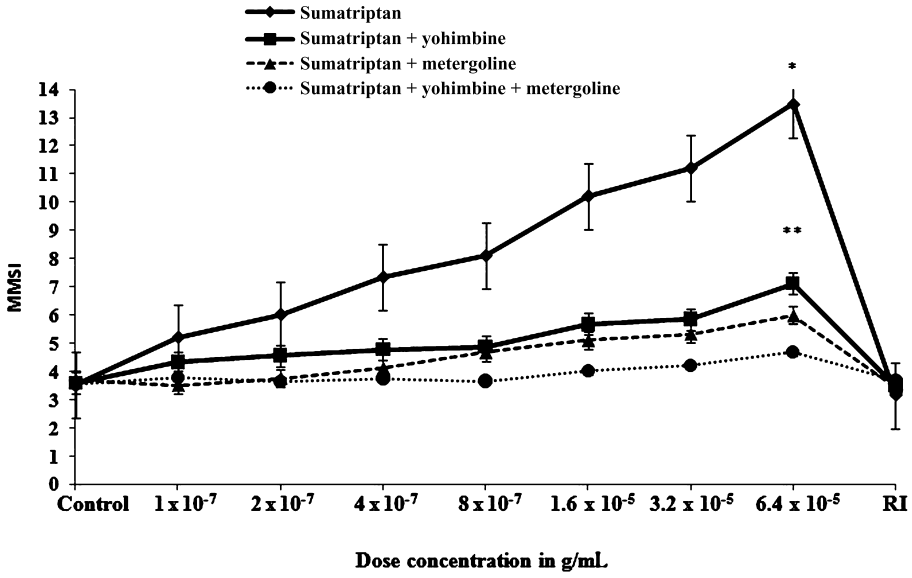

Figure 5

Dose-response curve for the effect of sumatriptan on the dorsal skin melanophore of *Hoplobatrachus tigerinus*. The inhibitory effect of yohimbine (2×10^{-7} g·mL^{-1}) and metergoline (4×10^{-7} g·mL^{-1}) alone are shown plus the combined effect of yohimbine (2×10^{-7} g·mL^{-1}) and metergoline (4×10^{-7} g·mL^{-1}), which induced complete synergistic blockage of the dispersion induced by sumatriptan. Vertical bars represent SEM. *$P < 0.0001$ and **$P < 0.0002$ signify level of significance.

experiments, the melanophore preparation was pretreated with a combination of trazodone and yohimbine, 2×10^{-7} g·mL^{-1} and 4×10^{-7} g·mL^{-1}, respectively; the combination of these two antagonists produced a synergistic blocking effect of myristicin response with MMSI 4.75 ± 0.12 (Figure 8). The dose-response curve for myristicin *per se* revealed that the degree of dispersion was less than that induced with sumatriptan, which indicates the dominance of 5-HT$_1$ receptors (Figure 6).

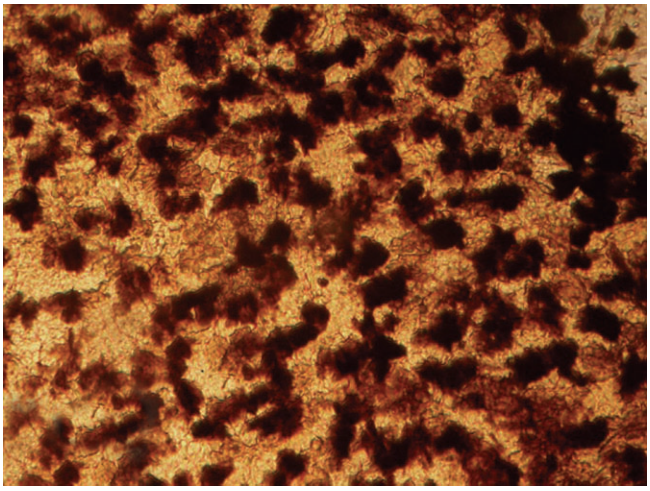

Figure 7
Dispersion caused by myristicin 6.4×10^{-5} g·mL^{-1}.

5-HT$_3$ receptors

The potent and selective 5-HT$_3$ receptor agonist 1-(3-chlorophenyl) biguanide was employed to confirm the presence of 5-HT$_3$ receptors in the melanophores. The response was weak and barely detectable at a dose of 1×10^{-8} g·mL^{-1}, with an of MMSI 4.11 ± 0.07. Further increasing the doses on a logarithmic scale produced a dose-dependent curve. The maximum concentration (6.4×10^{-5} g·mL^{-1}) of 1 (3 chlorophenyl) biguanide (1,3 CPB) produced an intense aggregation of all the melanophores ($P < 0.001$) (Figures 9 and 10), with the MMSI reduced to 1.15 ± 0.40. Further increasing the dose 1,3 CPB did not produce any measurable change in the response.

To further investigate the possible involvement of 5-HT$_3$ receptors in this aggregating response, the specific antagonist ondansetron was used. Ondansetron 4×10^{-7} g·mL^{-1} completely blocked the aggregation effect caused by 1,3 CPB. This blockage persisted on increasing the concentration of 1,3 CPB, and even at the highest dose of 6.4×10^{-5} g·mL^{-1}, 1,3 CPB produced no observable aggregation; the MMSI recorded was 3.93 ± 0.21. This finding further confirmed the presence of 5-HT$_3$ receptors in the melanophores of *H. tigerinus* (Figure 12).

5-HT$_4$ receptors

5-Methoxytryptamine, which is a potent agonist at 5-HT$_4$ receptors, was tested and it was found to cause pigment aggregation in a dose-dependent manner. 5-MT was employed in the dose range of 1×10^{-8} g·mL^{-1} to 6.4×10^{-5} g·mL^{-1}. It was observed that 5-MT has a very potent ability to cause pigment aggregation. Also, the lowest dose previously mentioned caused a marked aggregating response,

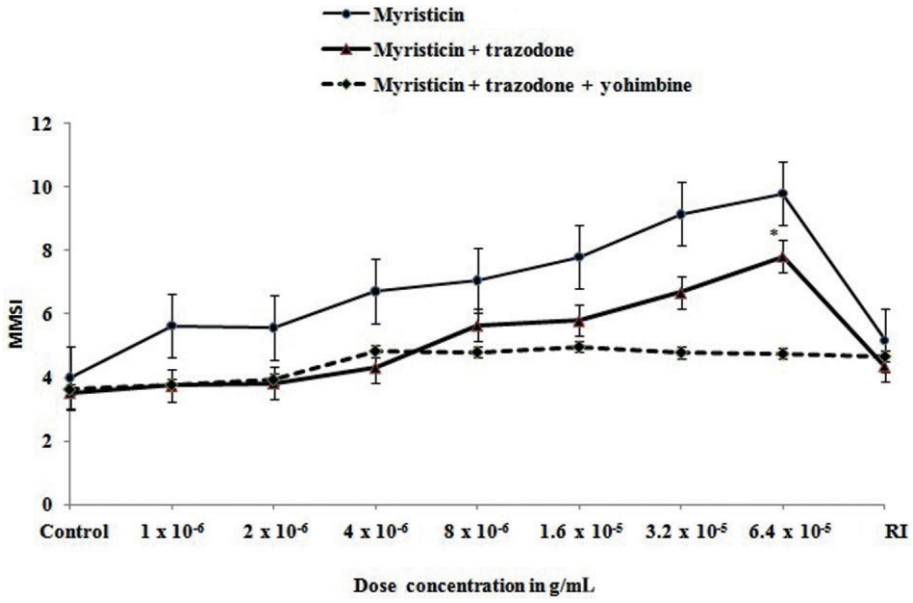

Figure 8
The dose-response curve for the effect of myristicin on the dorsal skin melanophores of *Hoplobatrachus tigerinus* alone and in the presence of trazodone (2×10^{-7} g·mL^{-1}). The synergistic blockage effect of trazodone (2×10^{-7} g·mL^{-1}) and yohimbine (4×10^{-7} g·mL^{-1}) is also shown. Vertical bars represent SEM. Note the blocking effects of yohimbine alone with the synergistic blockage shown by yohimbine and trazodone together. *$P < 0.0004$ signifies the level of significance.

in which the melanophores showed an instantaneous decrease in MMSI from 4.33 ± 0.47 to 3.23 ± 0.14 at 4×10^{-5} g·mL^{-1}. The maximum aggregation was recorded at concentration 6.4×10^{-5} g·mL^{-1}, with the MMSI reduced to 0.66 ± 0.14 (Figures 10 and 11).

However, although the antagonist L-lysine, employed at a dose of 2×10^{-7} g·mL^{-1}, only partially blocked the aggregating action of 5-MT, the strong aggregation caused by 5-MT on melanophores indicates that 5-HT$_4$ receptors are probably involved in substantial blanching of the skin (Figure 12).

Discussion

The presence of distinctive classes of 5-HT receptors in the amphibian system has paved a way to decipher their significant involvement in the modulation of skin pigmentary responses. Also, 5-HT is an important mediator of bidirectional interactions between the neuroendocrine system and the skin (Nordlind *et al.*, 2008). Moreover, 5-HT may be involved in responses to tissue damage as a consequence of stress (Majno and Palade, 1961; Majno *et al.*, 1961) and injury (Coderre, 1993), which may induce 5-HT release (Kawahara *et al.*, 1993). The pigmentary responses of frog, caused by environmental stress or intrinsic factors, with the induction of

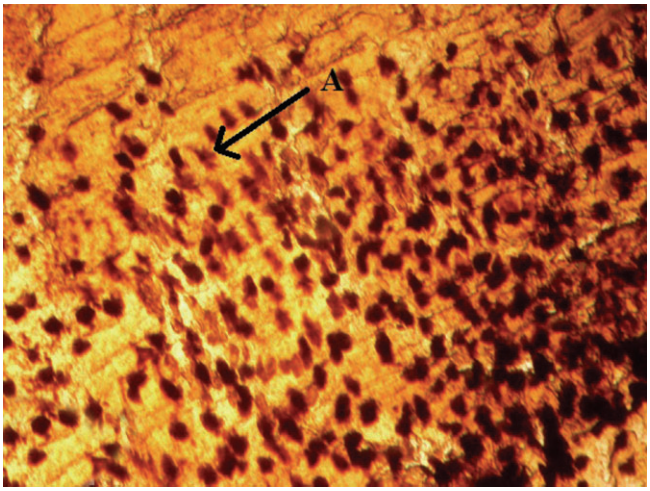

Figure 9
Aggregation caused by 1, 3 chlorophenyl biguanide 6.4×10^{-5} g·mL^{-1}. A, aggregation.

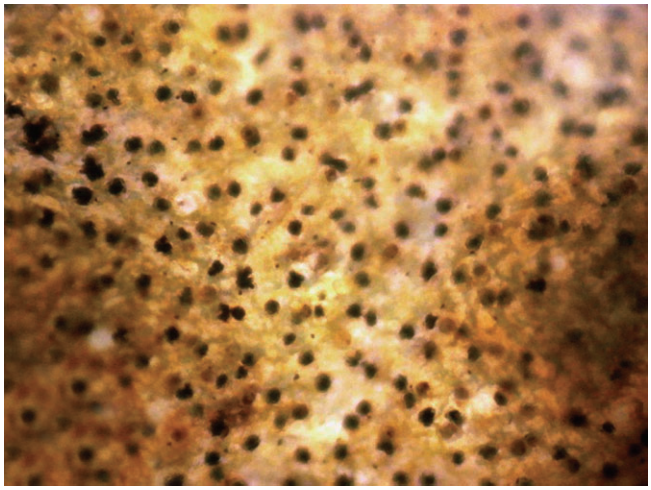

Figure 11
Aggregation caused by 5-MT 6.4×10^{-5} g·mL^{-1}.

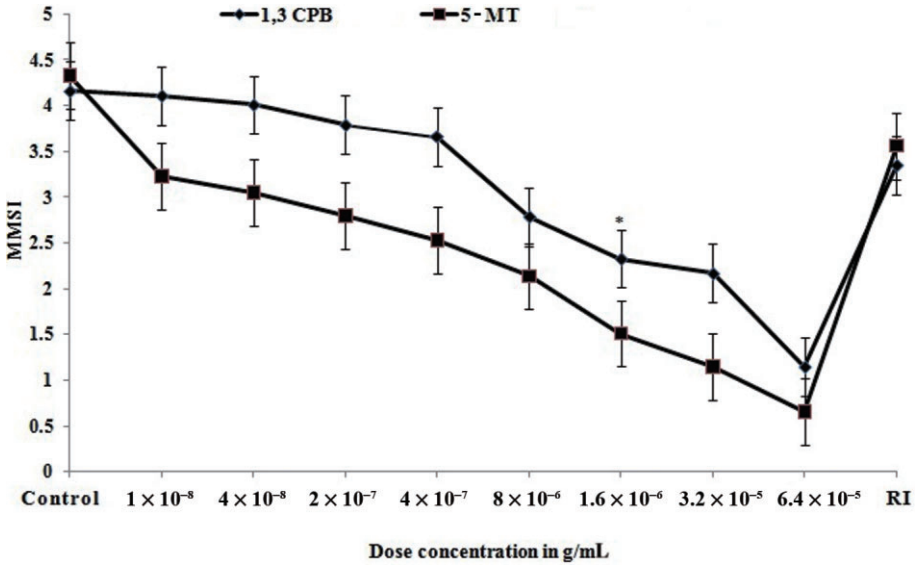

Figure 10
Dose-response curve for the aggregation induced by 1, 3 CPB and 5-MT of the dorsal skin melanophores of *Hoplobatrachus tigerinus*. Vertical bars represent SEM. *$P < 0.0001$ signifies the level of significance.

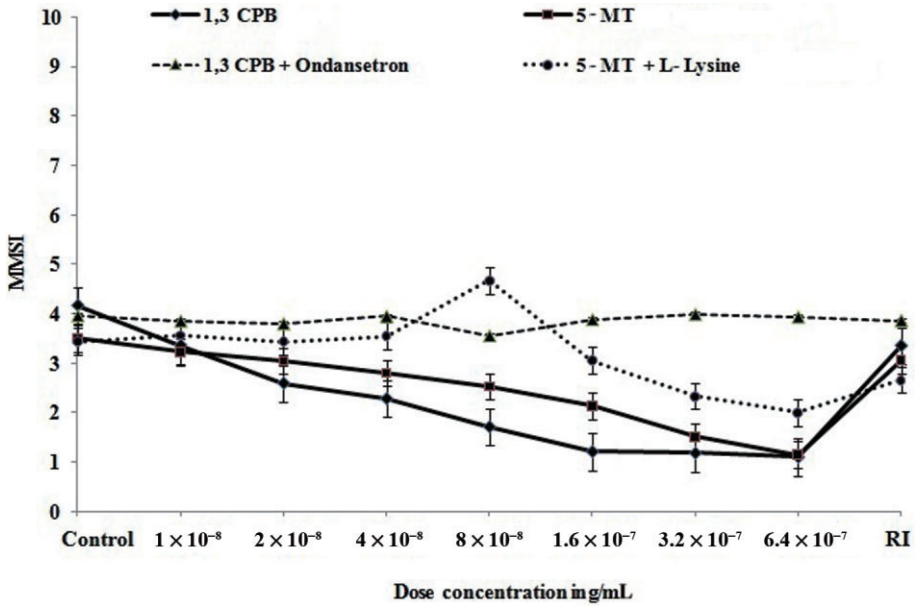

Figure 12

Dose-response curve for the aggregation induced by 1, 3 CPB and 5-MT of the dorsal skin melanophores of *Hoplobatrachus tigerinus* in the absence and presence of ondansetron (4×10^{-7} g·mL^{-1}) and L-lysine (2×10^{-7} g·mL^{-1}), respectively.

5-HT release and subsequent darkening or lightening of skin would allow the animal better camouflage and defence. This finding holds significance from the evolutionary standpoint as well; skin cells, such as melanocytes, have been demonstrated to produce 5-HT (Slominski, 2009). Skin cells also express functionally active, membrane-bound receptors for 5-HT, as well as proteins that transport 5-HT. The interactions of 5-HT with various proteins determines the nature, magnitude and duration of the 5-HT response. Moreover, there is evidence that the 5-hydroxytrptaminergic system is fully expressed in mammalian skin (Slominski *et al.*, 2002a). Also, human skin and cultured skin-derived cells have the ability to transform L-tryptophan to 5-HT and to metabolize 5-HT to N acetyl-5-HT and melatonin (Slominski *et al.*, 2002c).

In our present study, the presence of 5-HT receptors in a commonly found amphibian has been demonstrated. The major unanswered questions about the evolution and characteristics of the 5-hydroxytrptaminergic system in lower vertebrates might explain their role in the complex systems of mammals and humans. As the discovery and putative involvement of numerous receptor subtypes have baffled the physiology of skin pigmentation, the knowledge of lower vertebrates would certainly add to the fragmentary picture of skin pigmentation with respect to 5-HT involvement in higher vertebrates.

The precise level of 5-HT in the amphibian system is unknown; however, there are enough data to support the fact that the levels of 5-HT fluctuate rhythmically depending upon the environmental conditions as well as internal factors (Firth and Heatwole, 1976; Firth *et al.*, 1979; Martin and Burggren, 1992). The results with 5-HT *per se* indicate that the affinity of 5-HT$_1$ receptor for 5-HT is sufficiently high for it to be stimulated by normal circulating levels of this ligand. Also, the interaction of sumatriptan with the 5-HT$_1$ receptor seems to be of greater potency, with greater ligand efficacy as compared with the 5-HT$_2$ agonist myristicin, with MMSIs of 13.48 ± 0.21 and 9.79 ± 0.22, respectively. In contrast, the 5-HT$_2$ receptor is a low-affinity receptor activated only by high levels of 5-HT [as compared by the dose-response curves (Figure 6)]. This indicates the predominant presence of 5-HT$_1$ rather than 5-HT$_2$ receptors.

On the other hand, the presence of 5-HT$_3$ receptors has been indicated as well. Neurophysiological experiments have demonstrated the existence of 5-HT$_3$ receptors on c-fibres being activated by 5-HT (Christian *et al.*, 1989; Liang-Wu Fu and Longhurst, 1998). Also, it has been reported that c-fibres are present in frog skin, which get activated by numerous neuropeptides (Ciancio and Chang, 1992) and also by neurotransmitters like 5-HT (Fjällbrant and Iggo, 1961). In our study, we employed a potent and selective 5-HT$_3$ agonist, 1, 3 CPB, which elicited a response in the form of pigment aggregation and the complete blockage of this response by ondansetron strongly suggests the presence of 5-HT$_3$ receptors in the skin melanophores.

Interestingly, previously it was reported that 5-HT is present in frog skin and is involved in physiological functions in connection with defence and camouflage (Welsh and Zipf, 2005). Unfortunately, there are no data to reveal the exact location of 5-HT in the skin. 5-HT has been shown to cause pigment dispersion in *Xenopus* melanophores, initiated by an increase in intracellular cAMP resulting in the activation of PKA (Daniolos *et al.*, 1990), or PKC (Sugden and Rowe, 1992; Graminski *et al.*, 1993). On the other hand, a decrease in cAMP levels resulted into pigment aggregation. GPCRs have been linked to both cAMP (e.g. MC-l receptor, Potenza and Lerner, 1992) and PKC activation (e.g. endothelin-c receptor, Karne *et al.*, 1993) and are expressed in melanophores and stimulation of these GPCRs leads to pigment translocation. Our

present results are in agreement with the previously mentioned findings demonstrating the role of 5-HT receptors and their site of action on the melanophores together with their involvement in optimizing skin pigmentary responses.

Conclusion and future perspective

It is concluded that 5-HT, induces powerful, dose-dependent, physiologically significant, melanin dispersal effects in the isolated skin melanophores of *H. tigerinus*. This is the first report to demonstrate the presence of newly classified 5-HT receptors. The use of receptor-specific agonists and antagonists revealed that 5-HT receptors are involved in the skin pigmentary responses mediated by 5-HT. It is suggested that the stimulation of 5-HT_1 and 5-HT_2 receptors brings about pigment darkening, whereas 5-HT_3 receptor stimulation results in blanching of the skin. 5-MT caused rapid pigment aggregation, but the presence of 5-HT_4 receptors could not be confirmed as L-lysine failed to completely block this aggregating action of 5-MT. It is speculated that post-synaptic adrenoceptors are also involved in this aggregation response to 5-MT, but this needs clarification. Given the intriguing possibility that the 5-HT_4 receptor might represent the hitherto elusive reactivity that could directly contribute to melanin translocation within the cells could open novel perspectives for the physiology of skin pigmentation. Nonetheless, the pharmacological characterization of 5-HT receptors from our present study adds to the credence that 5-HT receptors are responsible for optimizing the pigmentary responses of animals with subsequent darkening and lightening of skin. The results we obtained strongly suggest that skin cells such as melanophores contain 5-HT receptors and add to our understanding of the evolutionary significance of this novel class of metabotropic receptors in the amphibian system.

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Conflict of interest

The authors state no conflict of interest.

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