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An endogenous 5-HT_7 receptor mediates pigment granule dispersion in *Xenopus laevis* melanophores

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1 Melatonin (5-methoxy *N*-acetyltryptamine) and serotonin (5-HT) exert rapid, but opposite effects on pigment granule distribution in *Xenopus laevis* melanophores. Low concentrations of melatonin (10^{-11}–10^{-9} M) cause a dramatic perinuclear aggregation of the melanin-containing granules, while 5-HT (10^{-8}–10^{-5} M) disperses pigment granules throughout the cell.

2 The present study found that pharmacological doses of melatonin ($\geqslant 10^{-6}$ M) induced a time- and concentration-dependent pigment granule dispersion, which was mediated by an endogenous melanophore 5-HT receptor.

3 5-HT produced a concentration-dependent elevation of melanophore cyclic AMP, and 5-HT-induced dispersion was blocked by H89 (10^{-4} M), an inhibitor of protein kinase A (PKA), but not by a PKC inhibitor (Ro 31-8220, 10^{-5} M), indicating a vital role for cyclic AMP in 5-HT-induced dispersion.

4 5-HT-mediated dispersion was not blocked by antagonists selective for G_s-coupled 5-HT_4 (GR113808) or 5-HT_6 (Ro 04-6790, Ro 63-0563, olanzepine) receptors, nor by 5-HT_{1-3} (pindolol, ketanserine, metoclopramide, MDL72222, tropisetron) receptor antagonists, but was inhibited by a selective 5-HT_7 receptor antagonist, DR4004, and other antagonists with a high affinity for 5-HT_7 receptors. The rank order of antagonist potency was: risperidone (mean pK_B 7.82) > methiothepin (7.43) > DR4004 (6.92) > mesulergine (6.83) > methysergide (6.60) > [±]-sulpiride (5.81) > spiperone (5.52). The agonist potency order [mean pEC_{50}, 5-CT (8.68) > 5-HT (7.13) > 5-MT (6.94) > 8-OH-DPAT (4.79) > sumatriptan (<4)] was also consistent with an action on 5-HT_7 receptors.

5 RT–PCR confirmed that melanophores express 5-HT_7 receptor mRNA. The pigment dispersing effect of high melatonin concentrations in melanophores is most likely mediated by activation of 5-HT_7 receptors. Conceivably some of the effects attributed to pharmacological doses of melatonin in mammals may be mediated by activation of 5-HT_7 receptors.

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Abbreviations: Cyclic AMP, cyclic adenosine monophosphate; PKA, protein kinase A; PKC, protein kinase C

Introduction

Melatonin (5-methoxy *N*-acetyltryptamine), the principal hormone of the pineal gland, is synthesized and released at night (Sugden, 1989) and acts on specific G-protein coupled receptors to regulate the timing of reproduction in seasonally-breeding, photoperiodic species and can entrain circadian rhythms in mammals (for a review see Arendt, 1995). These effects of melatonin are thought to be mediated through activation of specific, high affinity guanine nucleotide-binding protein (G-protein) coupled receptors on target cells in the basal hypothalamus and pituitary gland. Two mammalian melatonin receptors have been cloned, MT_1 and MT_2 (Reppert, 1997). Recently, a number of other functions have been proposed for melatonin. For example, melatonin can enhance electrically-evoked contraction of the rat caudal artery (Ting *et al*., 1999) and may play a role in circadian thermoregulation and sleep initiation (Krauchi *et al*., 2000). Melatonin also inhibits the proliferation of the oestrogen receptor-positive MCF-7 breast cancer cell line (Eck-Enriquez *et al*., 2000) and may play a role in regulating bone growth (Roth *et al*., 1999).

A well-established physiological action of melatonin is in the dermal layer of the skin of amphibians. Here, cells called melanophores containing the pigment, melanin, respond to a variety of hormonal 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 peri-nuclear position results in dramatic colour changes which are important for courtship and display, camouflage and self-defence, thermoregulation and photoprotection (reviewed in Rollag & Alderman, 1992; Fujii, 1993). In a clonal line of *Xenopus laevis* melanophores, pigment translocation can be triggered by activation of various cell surface receptors linked to a variety of intracellular signalling pathways (Graminski *et al*., 1993). Elevation of cyclic AMP or activation of PKC are known to cause dispersion of melanosomes throughout melanophores, while pigment aggregation is thought to be mediated by a reduction in intracellular cyclic AMP (reviewed in Lerner,

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1994). The pineal hormone, melatonin (5-methoxy *N*-acetyltryptamine) and 5-HT can both regulate pigment position. At physiological concentrations ($\leqslant 10^{-9}$ M), melatonin, causes a dramatic aggregation of pigment by activating a pertussis toxin sensitive melatonin receptor, and is responsible for the pallor typical of these animals at night. In contrast, 5-HT causes a concentration-dependent dispersion of pigment (Potenza & Lerner, 1994). A definitive pharmacological characterization of the endogenous melanophore 5-HT receptor(s) responsible has not been done and it is not known which 5-HT receptor subtype mediates melanosome dispersion.

The present study stemmed from a fortuitous observation that the pigment granule aggregation so readily apparent in melanophores at low concentrations of melatonin ($\leqslant 10^{-8}$ M) was not present if high concentrations ($\geqslant 10^{-6}$ M) of the pineal hormone were applied to cells. This reversal of aggregation was shown to be mediated by activation of an endogenous melanophore 5-HT receptor. The aim of the present study was to characterize and identify the 5-HT receptor subtype mediating pigment dispersion in *Xenopus* melanophores by defining the pharmacology of the response, examining the second messenger mechanism involved and identifying expression of the candidate 5-HT receptor subtype mRNA. We provide evidence that the endogenous melanophore 5-HT receptor mediating pigment granule dispersion is a 5-HT$_7$ subtype, and show that high concentrations of melatonin cause pigment dispersion by activating this receptor.

Methods

Quantification of melanosome translocation

A *Xenopus laevis* melanophore clonal cell line (Daniolos *et al*., 1990), provided by Dr M.R. Lerner (University of Texas, Dallas, U.S.A.), was grown as previously described in 96-well cell culture plates and was used at a density of 6000–8000 melanophores/well (Teh & Sugden, 1999). One hour prior to addition of drugs, the growth medium in each well was aspirated and replaced with 0.7 × Leibovitz L-15 medium (Sigma, Poole, U.K.) containing 1 mg ml^{-1} bovine serum albumin (BSA), 100 i.u. ml^{-1} penicillin, and 0.1 mg ml^{-1} streptomycin. Melanosomes remained evenly dispersed within melanophores in this medium. The change in distribution of pigment granules within melanophores was quantitated using a Bio-Tek microtitre plate reader (model EL311, Anachem, Luton, U.K.) by measuring the change in absorbance at 630 nm after drug treatment. The fractional change in absorbance, 1-(A_f/A_i) where A_i is the initial absorbance before drug treatment and A_f is the final absorbance at a given time after drug treatment, was calculated for each concentration of drug tested. All drugs were freshly prepared from 10^{-2} M stock solutions in methanol or DMSO kept at −20°C. The maximal concentration of solvent was 1% v v^{-1} which did not cause pigment redistribution in melanophores (data not shown). As light has been shown to cause pigment dispersion (Daniolos *et al*., 1990), melanophores were kept in the dark during drug treatment. As 5-HT could potentially be oxidized and/or its effective concentration diminished as a result of uptake and metabolism by cells, in a preliminary experiment an antioxidant, ascorbic acid (10^{-6} M), a 5-HT-uptake inhibitor, imipramine (10^{-5} M) and a monoamine oxidase inhibitor, pargyline (10^{-5} M) were added to the 0.7 × L-15 medium used for drug dilution. These drugs did not themselves alter pigment distribution, and the time-course and 5-HT concentration-response curves were unaffected by the presence of these drugs alone or in combination (data not shown).

To measure the pigment dispersing potency of 5-HT agonists (Potenza & Lerner, 1994) melanophores were pre-incubated with melatonin (10^{-9} M, 1 h) to stimulate complete aggregation prior to addition of 5-HT or 5-HT agonists. Antagonist potency (estimated pK_B) was determined by pre-incubating melanophores with melatonin (10^{-9} M, 1 h) in the presence of either vehicle or a single concentration of 5-HT antagonist prior to adding various concentrations of 5-HT (10^{-9}–10^{-4} M, 1 h). 5-HT potency in the presence and absence of the antagonist was determined and used to calculate the estimated pK_B value (see Data Analysis). None of the 5-HT antagonists alone (up to 10^{-4} M, 1 h) caused any pigment aggregation nor did they reverse aggregation in melatonin-pretreated cells, i.e. they had no activity as melatonin receptor agonists or antagonists (data not shown).

Determination of cyclic AMP

Melanophores grown in 24-well plates (~50,000 cells well^{-1}) were used. Prior to the experiment, growth medium was removed and cells were incubated in 0.7 × L-15 containing melatonin (10^{-9} M, 1 h) to reduce intracellular cyclic AMP before the addition of vehicle (H_2O) or 5-HT (10^{-8}–10^{-5}) for a further 1 h. At the end of drug incubation, the medium in each well was aspirated and 150 μl ice-cold acetic acid (50 mM) was added and cells were immediately scraped from the plate. A further 150 μl of acetic acid was added to collect the remaining cells in each well. Pooled samples (300 μl well^{-1}) were boiled for 5 min., then frozen at −20°C until used for cyclic AMP determination by RIA following acetylation as described previously (Harper & Brooker, 1975; Vanecek *et al*., 1985).

Reverse transcription-polymerase chain reaction (RT–PCR)

Melanophores (6–8 wells of a confluent 96-well plate) were disrupted in 400 μl ice-cold lysis buffer (100 mM Tris-HCl at pH 8.0, 500 mM lithium chloride, 10 mM EDTA, 1% lithium dodecylsulphate, 5 mM dithiothereitol) to inactivate cellular RNase activity. *Xenopus* brain was sonicated on ice in 400 μl ice-cold lysis buffer. Poly A$^+$ mRNA in each lysate was isolated using magnetic oligo $(dT)_{25}$ beads (Dynabeads; Dynal, Wirral, U.K.) and cDNA was synthesized from each mRNA sample immediately (Ting *et al*., 1999). Oligo $(dT)_{18}$ (1 μg; Promega, Southampton, U.K.) and random 10-mers (1 μg) were added to the mRNA sample and the mixture heated (70°C, 5 min) to remove secondary RNA structure, then cooled immediately on ice. Dithiothreitol (20 mM), dATP, dCTP, dTTP and dGTP (all 0.5 mM; Promega), recombinant ribonuclease inhibitor (80 u, RNasin; Promega), avian Moloney murine leukaemia virus-reverse transcriptase RNase H minus (200 u, MMLV-RT; Promega) and diethyl pyrocarbonate-treated distilled water were added to make the

final volume 20 μl. This mixture was incubated at 37°C for 1 h, then at 42°C for 15 min. MMLV-RT was inactivated by heating at 98°C for 3 min. All cDNA synthesized was diluted 1 : 10 in tRNA (10 μg/ml) and stored at −70°C until used.

The forward (5′-TTC TGC AAT GTG TTC ATT GCC ATG GA-3′) and reverse (5′-TCT TGA CTA ATC AGA CAC ACC-3′) primers used for PCR were designed from the published *Xenopus* 5-HT$_7$ sequence (Genbank accession no.: U10161; Nelson *et al*., 1995). The predicted size of the 5-HT$_7$ PCR product was 248 bp. Each reaction contained cDNA or mRNA, 0.5 μM of each primer, 100 μM of each deoxynucleoside 5′-triphosphate, $MgCl_2$ 1.5 mM, KCl 50 mM, Tris-HCl (pH 8.3) 10 mM, glycerol 0.5%, Triton X-100 0.1% and *Taq* DNA polymerase (Promega) 1 u. Thermal cycling conditions were: 1 min., 94°C; 1 min., 55°C; 2 min, 72°C for 40 cycles with a final extension of 10 min at 72°C. PCR products were resolved by agarose gel electrophoresis and stained with ethidium bromide (0.5 μg/ml).

The identity of the melanophore PCR product was confirmed using restriction digestion analysis (*Alu*I, Promega 10 u, 37°C overnight gave two bands as predicted at 132 + 116 bp) and direct sequencing on an ABI automated sequencer (Molecular Biology Unit, GKT School of Biomedical Science, King's College London, U.K.).

Data analysis

For 5-HT receptor agonists, mean $pEC_{50} \pm$ s.e.mean values for pigment dispersion were determined by curve-fitting individual data points using the four parameter logistic equation:

$$Y = \frac{A - D}{1 + (\frac{X}{C})^B} + D$$

where X is the concentration of agonist, Y is the fractional change in absorbance, A is the minimal absorbance (maximal pigment aggregation) in the absence of agonist, B is the slope factor, C is the concentration of the agonist producing half of the maximal response (EC_{50}) and D is the maximal absorbance (maximal dispersion). The potency of 5-HT in stimulating cyclic AMP accumulation was also determined using this equation.

Antagonist potency (estimated pK_B) was determined by constructing concentration-response curves to 5-HT in the absence and presence of a single concentration of each antagonist. Estimated pK_B values were calculated using the equation (Arunlakshana & Schild, 1959):

$$pK_B = Log\left[\left(\frac{^{Ant}EC_{50}}{^{Ctrl}EC_{50}}\right) - 1\right] - Log[Ant]$$

where $^{Ant}EC_{50}$ is the concentration of agonist producing 50% of the maximum biological response in the presence of the antagonist, $^{Ctrl}EC_{50}$ is the concentration of agonist producing 50% of the maximum biological response in the absence of the antagonist, and *Ant* is the concentration of the antagonist used.

Drugs

Melatonin, 5-HT HCl, 5-methoxytryptamine HCl (5-MT), risperidone, mesulergine, spiperone, S(−), R(+) and (±)-sulpiride, tropisetron (ICS 205-930), S(−), R(+) and (±)-pindolol, ketanserin, metoclopramide HCl, imipramine HCl, pargyline HCl, L-ascorbic acid and 4β-phorbol 12,13-dibutyrate (4β-PDBu) were purchased from Sigma. 5-carboxyamidotryptamine maleate (5-CT), (±)-8-hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT), methysergide and 1α-H,3α,5α-H-tropan-3-yl-3,5-dichlorobenzoate (MDL72222) were from Tocris Cookson (Bristol, U.K.). Methiothepin was from ICN Pharmaceuticals (Oxfordshire, U.K.). 2-methyl-4-(4-methyl-1-piperazinyl)-10H-thieno(2,3-b)-1,5) benzodiazepine (olanzepine or LY 170053) was from Eli Lilly (Indianapolis, U.S.A.). N-[4-methoxy-3-(4-methyl-L-piperazinyl)phenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadizol-3-yl) (GR127935), 1-[2-(methyl-sulphonylamino) ethyl]-4-piperidinyl-methyl 1-methyl-1H-indole-3-carboxylate maleate (GR113808), sumatriptan succinate were obtained from Glaxo (Stevenage, U.K.). 4-amino-N-(2,6-bis-methylamino-pyrimidin-4-yl)-benzene sulphonamide (Ro 04-6790) and 4-amino-N-(2,6-bis-methylamino-pyridin-4-yl)-benzene sulphonamide (Ro 63-0563) were from Roche Products Ltd. (Welwyn Garden City, U.K.). DR4004 (2a-[4-(4-phenyl-1, 2, 3, 6-tetrahydropyridyl) butyl] - 2a, 3, 4, 5- tetrahydro-benzo [cd]indol-2(1H)-one) was provided by Meiji Seika Kaisha Ltd. (Yokohama, Japan). Mesulergine was generous gift from Dr W.D. Hirst (SmithKline Beecham, Harlow, U.K.). H89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide 2HCl) and Ro 31-8220 (2-(1-[3-(amidinothio) propyl]-1*H*-indol-3-yl)-3-(1-methylindol-3yl)-maleimide methane sulphonate) were purchased from Alexis (Nottingham, U.K.).

Results

High concentrations of melatonin activate pigment dispersion

An extended melatonin concentration-response curve (10^{-12}–10^{-4} M) revealed a bi-phasic pigment translocation response of melanophores. Full pigment aggregation was apparent by 10^{-9} M, but higher concentrations ($\geqslant 10^{-6}$ M) produced a concentration-related pigment dispersion. As the chemical structures of melatonin and 5-HT are similar and 5-HT is known to cause pigment dispersion in these cells (Potenza & Lerner, 1994), activation of an endogenous melanophore 5-HT receptor by high concentrations of melatonin was suspected to be responsible. To test this, methiothepin, an antagonist with little 5-HT receptor subtype selectivity was incubated with melanophores before addition of melatonin. Methiothepin (10^{-6} M, 1 h) did not alter the potency of melatonin for inducing pigment granule aggregation (mean ± s.e.mean pEC_{50}, $n = 4$: methiothepin, 10.53 ± 0.03; + methiothepin, 10.65 ± 0.05) or alter the maximal response, but it did completely prevent the pigment dispersing action of high concentrations ($> 10^{-6}$ M) of melatonin (Figure 1). Risperidone (10^{-6} M, 1 h), another 5-HT receptor antagonist, had the same effect; mean ± s.e.mean melatonin pEC_{50} $n = 4$, + risperidone, 10.58 ± 0.12) These results indicate that the dispersing effect of melatonin is mediated by a 5-HT receptor.

Role of cyclic AMP in 5-HT induced pigment dispersion

5-HT stimulated a time- and concentration-dependent pigment dispersion in melanophores. The pigment dispersion

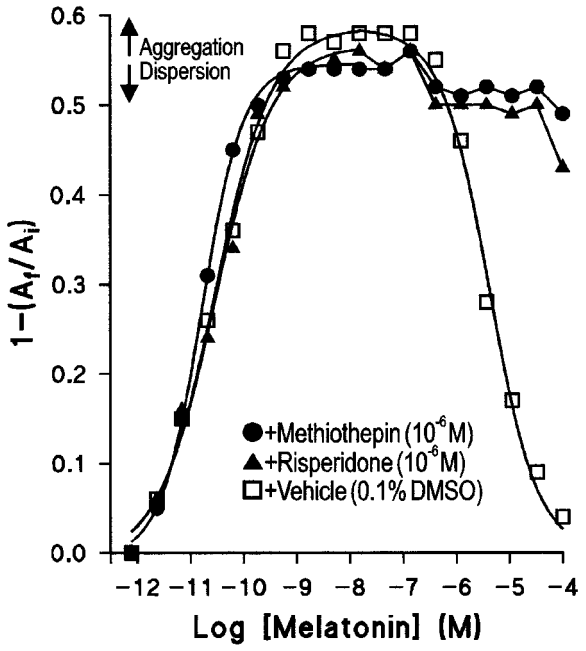

Figure 1 Effect of melatonin on melanosome translocation. Vehicle (0.01% DMSO), methiothepin (10^{-6} M) or risperidone (10^{-6} M) diluted in 0.7 × L-15 medium was pre-incubated with melanophores for 1 h before the addition of melatonin (10^{-12}–10^{-4} M). Absorbance was determined before (A_i) and 1 h after melatonin addition (A_f) and the relative change in absorbance calculated (1-(A_f/A_i). Pre-incubation of vehicle, methiothepin or risperidone did not affect the basal pigment position (i.e pigment remained fully dispersed). Each data point represent a mean ± s.e.mean, $n=4$. Error bars were omitted as s.e.mean were all <0.01 and fell within the area covered by the symbols.

response was nearly complete by 30 min. (Figure 2a). 5-HT potency was maximum after 30 min (Figure 2b) and remained stable for at least 120 min (data not shown). All subsequent determinations of the potency of 5-HT agonists used a 1 h incubation time. 5-HT also caused a concentration-dependent increase in melanophore cyclic AMP. The potency of 5-HT in elevating melanophore cyclic AMP (mean ± s.e.mean pEC$_{50}$ 6.61 ± 0.08, $n=3$; Figure 2c) was slightly lower than the potency of 5-HT for inducing pigment dispersion (mean ± s.e.mean pIC$_{50}$ 7.03 ± 0.01, $n=3$; Figure 2b).

The importance of these changes in melanophore cyclic AMP in triggering pigment dispersion was shown using a PKA inhibitor, H89 (Chijiwa *et al*., 1990). H89 pre-treatment (10^{-4} M, 1 h) significantly shifted the concentration-response curve for 5-HT-induced pigment dispersion to the right (4.4 fold, Figure 3a). As activation of PKC is also known to cause pigment dispersion (Sugden & Rowe, 1992; Graminski *et al*., 1993), Ro 31-8220, an inhibitor of PKC (Dieter & Fitzke, 1991), was used to investigate the possible involvement of PKC in 5-HT-stimulated pigment dispersion. In contrast to H89, Ro-31-8220 (10^{-5} M, 1 h) did not significantly shift the 5-HT concentration-response curve (Figure 3a).

To establish that these protein kinase inhibitors were acting selectively in melanophores at the concentrations used, cells were treated with Ro 31-8220 or H89 before using β-PDBu, an activator of PKC, to trigger pigment dispersion (Sugden & Rowe, 1992). Ro 31-8220, but not H89, significantly inhibited β-PDBu-induced pigment dispersion (Figure 3b) showing that Ro 31-8220 was acting specifically on PKC and that H89 was not able to inhibit PKC. Thus, H89 inhibition of pigment aggregation induced by 5-HT is most likely mediated by inhibition of PKA.

The pharmacology of the melanophore 5-HT receptor

The potency of several 5-HT receptor agonists, namely 5-CT, 5-HT, 5-MT, 8-OH-DPAT and sumatriptan, was determined. All of these agonists, except sumatriptan, caused a concentration-dependent pigment dispersion (Figure 4). The rank order of agonist potency on pigment dispersion was found to be: 5-CT > 5-HT ⩾ 5-MT > > 8-OH-DPAT > sumatriptan (Table 1).

The pharmacological characteristics of the melanophore 5-HT response were further investigated using numerous 5-HT receptor antagonists with varying selectivity for 5-HT receptor subtypes. Antagonist potency (pK_B) was determined (see Methods) for 10 5-HT antagonists (Table 2). The most potent antagonist was risperidone (pK_B 7.82) which is selective for the 5-HT$_6$ and $_7$ subtypes. DR4004, a selective 5-HT$_7$ antagonist (Kikuchi *et al*., 1999), was also effective (pK_B 6.92). Methiothepin (pK_B 7.43), mesulergine (pK_B 6.83) methysergide (pK_B 6.60), antagonists with affinity for several subtypes including 5-HT$_7$, also shifted the 5-HT concentration-response curve to the right (Figure 5). Sulpiride was found to be a weak antagonist of 5-HT-induced dispersion, with the R(+) enantiomer (pK_B 6.15) 30 fold more potent than the S(−) enantiomer (pK_B 4.67). The racemate having an intermediate potency (pK_B 5.81). This observation is interesting as it is the S(−) enantiomer which is the more potent enantiomer at D$_2$ and D$_3$ receptors (Seeman & van Tol, 1994).

Nine other 5-HT antagonists had no antagonist activity up to 10^{-4} M. These antagonists included compounds specific for the 5-HT$_3$ (metoclopramide, MDL72222) or 5-HT$_3$ and 5HT$_4$ subtype (tropisetron, Dumuis *et al*., 1988), the 5-HT$_6$ subtype (Ro 04-6790, Ro 63-0563, Sleight *et al*., 1998; olanzepine, Roth *et al*., 1994), the 5-HT$_4$ subtype (GR113808, Gale *et al*., 1994), the 5-HT$_{2A}$ (ketanserin) and 5-HT$_{1A/B}$ (pindolol) subtypes. This data suggested that 5-HT-induced pigment dispersion in melanophores is mediated by an endogenous 5-HT$_7$ receptor subtype.

Expression of 5-HT$_7$ mRNA in melanophores

To confirm that *Xenopus* melanophores express a 5-HT$_7$ receptor, the expression of mRNA for this receptor subtype was investigated using RT–PCR. A single PCR product of the appropriate size (248 bp) was amplified from cDNA prepared from *Xenopus* brain and melanophores (Figure 6). When melanophore poly A+ mRNA was used in PCR (i.e. it was not reverse transcribed to cDNA) no product was detected (blank, Figure 6). Incubation of the purified PCR product with the restriction enzyme *Alu*I (10 u overnight, 37°C) gave restriction products of the expected size (132 and 116 bp; data not shown), and the identity of the 248 bp band amplified from melanophore cDNA was confirmed by direct

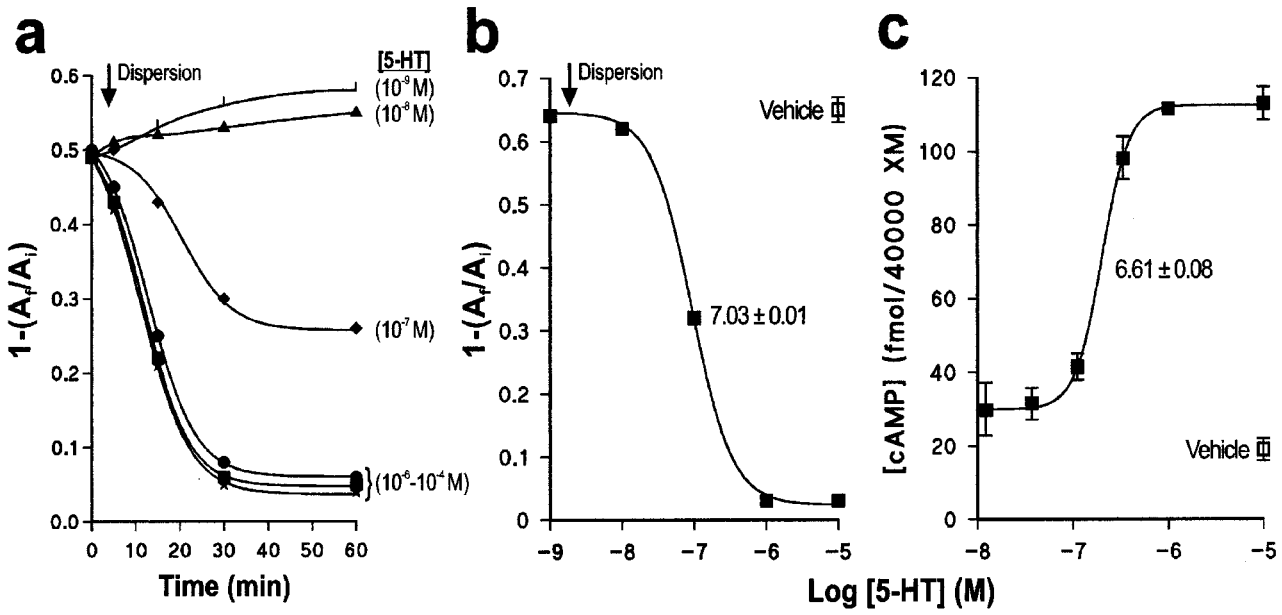

Figure 2 Effect of 5-HT on pigment distribution and melanophore cyclic AMP. (a) Time-course of pigment dispersion activated by 5-HT. Melanophores were pre-aggregated with melatonin (10^{-9} M, 1 h) before the addition of various concentrations of 5-HT. The response was determined at the indicated times. A similar time-course of pigment dispersion was also obtained for 5-CT and 5-MT (data not shown). Each point is the mean ± s.e.mean of triplicate measurements. Error bars are omitted as all s.e.mean were <0.02, and all are less than the area covered by the symbols. (b) Concentration-response curve for 5-HT. Each point is the mean ± s.e.mean of triplicate measurements. Vehicle (open symbol) had no effect on pigment position. (c) Concentration-response curve for 5-HT stimulation of melanophore cyclic AMP. Cyclic AMP was measured after 1 h of incubation with the 5-HT concentrations indicated. Each data point represents a mean ± s.e.mean of triplicate cell aliquots each measured in duplicate.

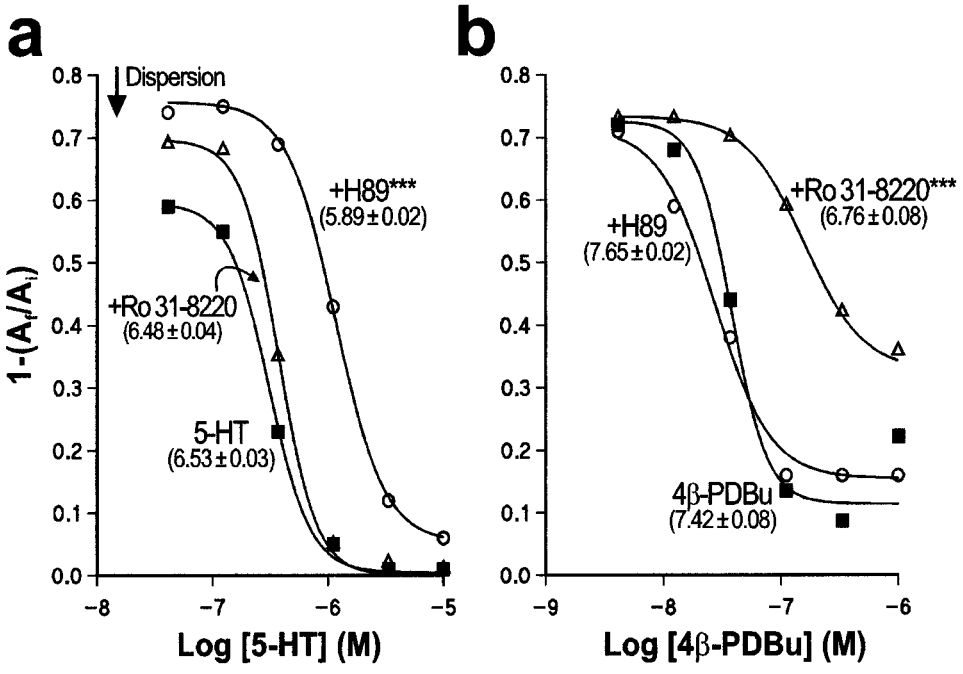

Figure 3 Effect of PKC and PKA inhibitors, Ro 31-8220 and H89, on 5-HT- and phorbol ester-stimulated pigment dispersion. Melanophores were pre-incubated with melatonin (10^{-9} M, 1 h) containing either Ro 31-8220 (10^{-5} M), H89 (10^{-4} M) or vehicle (1% DMSO) prior to the addition of various concentrations of 5-HT (a) or 4β-PDBu (b). The pigment dispersion response was measured after 1 h. Data points are mean ± s.e.mean of triplicates. Error bars are all within the area (<0.02) covered by the symbols. *** Indicates potency value was significantly ($P<0.001$) different from vehicle-treated controls.

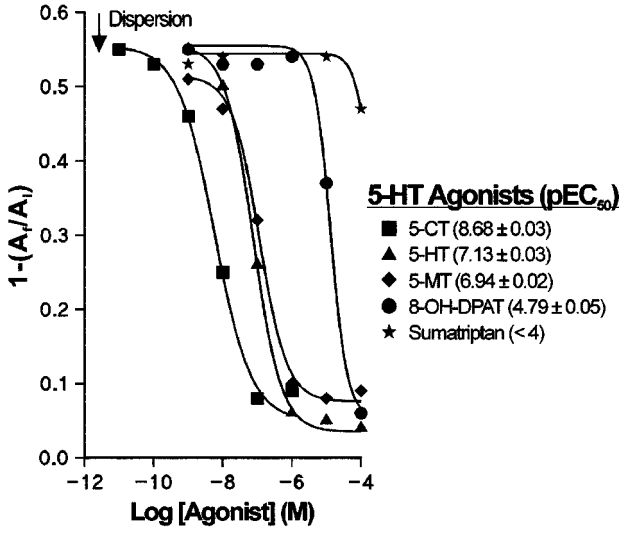

Figure 4 Examples of concentration-response curves for 5-HT agonists on pigment dispersion. Melanophores were pre-incubated with melatonin (10^{-9} M, 1 h) prior to the addition of various concentrations of the 5-HT agonists. Data points are mean ± s.e.mean of triplicates, and s.e.mean are within the area (<0.02) covered by the symbols.

Table 1 5-HT agonist potency (pEC_{50}) on melanosome dispersion

Agonists	*Subtype selectivity**	*pEC_{50}* (mean ± s.e.mean)	n
5-CT	1, 5, 6, 7	8.44 ± 0.28	11
5-HT	Non-selective	7.02 ± 0.27	36
5-MT	1B, 1D, 2C, 4, 7	6.76 ± 0.16	5
8-OH-DPAT	1A	4.88 ± 0.11	3
Sumatriptan	1B, 1D	<4	3

Melanophores were pre-incubated with melatonin (10^{-9} M, 1 h) prior to the determination of the concentration-response curve to the 5-HT agonists. Agonist potency (pEC_{50}) is the mean ± s.e.mean of the indicated number (*n*) of experiments each performed in triplicate. *From Hoyer *et al.* (1994), Eglen *et al.* (1997), Gerhardt & van Heerikhuizen (1997), Saxena *et al.* (1998), Terron & Falcon-Neri (1999) and Hemedah *et al.* (1999).

sequencing of the purified PCR product. These results confirm that a *Xenopus* 5-HT$_7$ subtype receptor mRNA is expressed in the clonal melanophore line.

Discussion

Our interest in 5-HT receptors in melanophores stemmed from the observation that a bi-phasic concentration-response curve for pigment translocation was generated if an extended range of melatonin concentrations (10^{-12}–10^{-4} M) was used (Figure 1). We (Sugden & Rowe, 1992) and others (Lerner, 1994) have shown previously that melatonin produces a concentration-related translocation of pigment towards the cell centre (i.e. aggregation) at concentrations between 10^{-12} and 10^{-7} M. We found that melatonin concentrations of 10^{-6} M and above produced an increasing pigment dispersion which overcame the aggregating action. As melatonin and 5-HT are similar in structure, and melanophores are known to express a 5-HT receptor which triggers pigment granule dispersion (Potenza & Lerner, 1994), we suspected that high concentrations of melatonin may activate pigment dispersion by activating an endogenous 5-HT receptor. The present study characterized the subtype of receptor mediating 5-HT-stimulated pigment dispersion in *Xenopus* melanophores using pharmacological, signal transduction and molecular criteria.

Pigment dispersion in *Xenopus* melanophores can be initiated by increasing intracellular cyclic AMP resulting in activation of PKA (Daniolos *et al.*, 1990), or by activation of PKC (Sugden & Rowe, 1992; Graminski *et al.*, 1993). G-protein coupled receptors linked to both cyclic AMP (e.g. MCl receptor, Potenza & Lerner, 1992) and PKC activation (e.g. endothelin$_C$ receptor, Karne *et al.*, 1993) are expressed in melanophores and their activation leads to pigment dispersion. Direct quantification of cyclic AMP in melanophores showed that 5-HT did cause a concentration-dependent increase (Figure 2c). The potency of 5-HT in elevating cyclic AMP (pEC_{50} = 6.61) was slightly lower than its potency in triggering pigment dispersion (pEC_{50} = 7.03) measured in the same experiment. Potenza & Lerner (1994) also observed a weaker potency of 5-HT on cyclic AMP synthesis than on pigment dispersion. The difference in potency may simply reflect the fact that both measurements were made after 1 h of treatment. At this time pigment movement was nearing completion, but the increase in cyclic AMP triggered by 5-HT may have reached a maximum well before 60 min. Alternatively, melanophores may have the capacity to synthesize more cyclic AMP in response to 5-HT than is required to initiate a full dispersion of pigment, leading to the slight difference in potency for the two responses. That the 5-HT-induced increase in melanophore cyclic AMP was important for inducing dispersion of pigment was shown by the finding that H89, a PKA inhibitor, significantly inhibited the 5-HT response (Figure 3a). A specific PKC antagonist (Ro 31-8220) was ineffective in blocking 5-HT-mediated dispersion, but markedly reduced dispersion induced by 4β-PDBu, a PKC activator (Figure 3b). H89, on the other hand, did not inhibit 4β-PDBu-induced dispersion showing clearly that in melanophores at the concentration used, it was acting specifically on PKA.

Together these results suggest that the 5-HT receptor subtype triggering pigment dispersion in melanophores is coupled to a stimulatory G_s-protein which activates the production of cyclic AMP. This argues against an involvement of 5-HT$_1$, 5-HT$_2$, 5-HT$_3$ and 5-ht$_5$ receptor subtypes which are not known to couple to an elevation of intracellular cyclic AMP (reviewed in Hoyer *et al.*, 1994). Indeed, 5-HT$_1$ and 5-ht$_5$ subtypes have been shown to inhibit the production of cyclic AMP, while the 5-HT$_2$ subtype is known to activate phosphatidylinositol hydrolysis and the 5-HT$_3$ subtype is an ion channel.

The pharmacological profile of 5-HT-stimulated pigment dispersion in melanophores was investigated using subtype-selective 5-HT receptor agonists and antagonists. The rank

Table 2 5-HT antagonists potency (estimated pK_B) on melanosome dispersion

Antagonists	*Conc.**	*Subtype selectivity†*	*pK_B* (mean ± s.e.mean)	n
Risperidone	[−6]	7,6	7.82 ± 0.32	9
Methiothepin	[−6]	7, 1, 2, 6, 5	7.43 ± 0.17	12
DR4004	[−6]	7	6.92 ± 0.12	5
Mesulergine	[−6]	7, 2, 6	6.83 ± 0.31	5
Methysergide	[−5]	2C, 7, 6	6.60 ± 0.19	5
Spiperone	[−5]	2, 6, 7	5.52 ± 0.20	6
R[+]-Sulpiride	[−6]	2A	6.15 ± 0.42	6
[±]-Sulpiride	[−5]	2A	5.81 ± 0.35	9
S[−]-Sulpiride	[−4]	2A	4.67 ± 0.43	9
GR127935	[−5]	1D	5.26 ± 0.29	3

Melanophores were pre-incubated with melatonin (10^{-9} M) in the absence or presence of a single concentration (*log [Antagonist] M) of the antagonists as indicated for 1 h prior to the determination of the concentration-response curve to 5-HT. Antagonist potency (pK_B) is the mean ± s.e.mean of the indicated number (n) of experiments each performed in triplicate. †From Hoyer *et al.* (1994), Gerhardt & van Heerikhuizen (1997), Saxena *et al.* (1998), Gale *et al.* (1994), Terron & Falcon-Neri (1999), Kikuchi *et al.* (1999) and Hemedah *et al.* (1999).

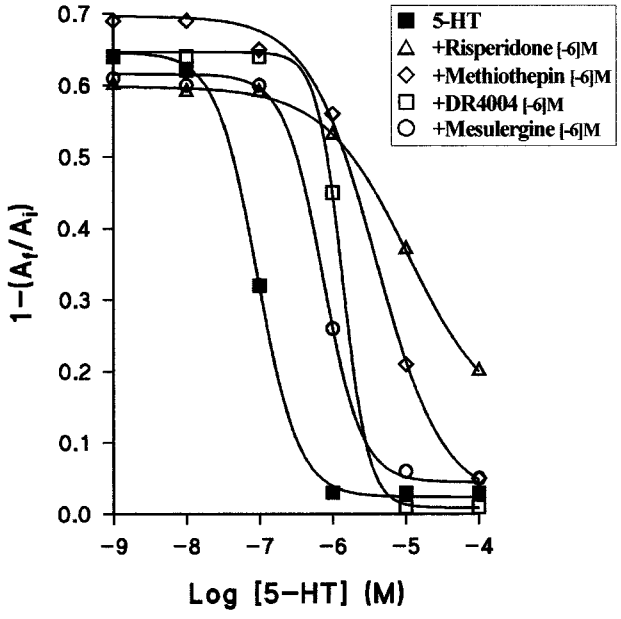

Figure 5 Examples of inhibition of 5-HT-induced pigment dispersion by 5-HT receptor antagonists. Melanophores were incubated for 1 h with melatonin (10^{-9} M) in the presence of risperidone, methiothepin, DR4004 and mesulergine (all 10^{-6} M) prior to the addition of various concentrations of 5-HT. The potency of 5-HT in the absence and presence of antagonist were determined after 1 h. Each data point represents the mean of triplicates with error bars omitted as s.e.mean were all <0.02.

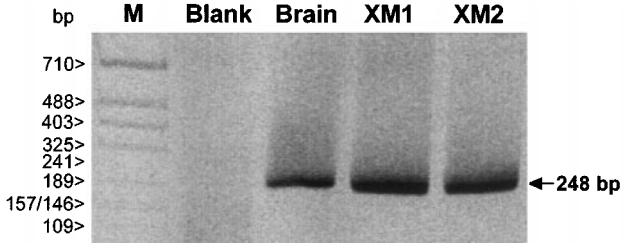

Figure 6 Detection of *Xenopus* melanophore 5-HT$_7$ receptor subtype mRNA expression in melanophores by RT–PCR. Ethidium bromide-stained agarose-gel image of the 248 bp PCR product amplified using *Xenopus* brain or melanophore (XM1, XM2) cDNA. Melanophore mRNA not subjected to reverse transcription (blank) did not amplify any products. M = molecular weight markers.

order of agonist potency (5-CT > 5-HT ⩾ 5-MT > > 8-OH-DPAT > sumatriptan) matches the pharmacology found for the 5-HT$_7$ receptor subtype in native tissues (Nelson *et al.*, 1995; Schoeffter *et al.*, 1996; Hirst *et al.*, 1997; Terron & Falcon-Neri, 1999; Hemedah *et al.*, 1999; Saxena *et al.*, 1998) and recombinant cell systems (Thomas *et al.*, 1998; Gerhardt & van Heerikhuizen, 1997). The very weak activity (pEC_{50} < 4) of sumatriptan argues against the involvement of 5-HT$_1$ receptor subtypes (Saxena *et al.*, 1998). 5-CT is either weakly active at 5-HT$_2$ and 5-HT$_4$ or inactive at 5-HT$_3$ receptors (Hoyer *et al.*, 1994) indicating these receptor subtypes are not responsible for mediating pigment dispersion in melanophores.

In order to unambiguously define the pharmacology of the 5-HT receptor subtype causing pigment dispersion, 16 5-HT receptor antagonists with varying degrees of subtype selectivity were tested. The rank order of antagonist potency (risperidone > methiothepin > DR4004 > mesulergine ⩾ methysergide ⩾ R(+)-sulpiride > (±)-sulpiride ⩾ spiperone > GR127935 > S(−)-sulpiride) is consistent with a 5-HT$_7$ subtype, as the most potent compounds share high affinity at this subtype. Risperidone (pK_i 8.9, Roth *et al.*, 1994) and mesulergine (pK_D 8.15, Hoyer *et al.*, 1994) have a high affinity for the 5-HT$_7$ subtype with about 300 fold lower affinity at 5-HT$_6$ receptors. Mesulergine does not interact with receptors in the 5-HT$_1$ family. DR4004, a recently described antagonist (Kikuchi *et al.*, 1999), has been shown to have considerable selectivity (50–500 fold) for the 5-HT$_7$ subtype over 5-HT$_{1A}$, 5-HT$_2$, 5-HT$_4$ and 5-HT$_6$ receptors. The inability of various 5-HT receptor subtype specific antagonists to block 5-HT-induced pigment granule disper-

sion was particularly informative. Thus, three selective 5-HT$_6$ receptor antagonists (olanzepine, Ro 04-6790, Ro 63-0563) were completely ineffective even at 10^{-4} M, as were GR113808 (5-HT$_4$ selective, Gale *et al*., 1994) and metoclopramide, MDL72222 (5-HT$_3$ selective) and tropisetron (5-HT$_3$ and $_4$ selective). A 5-HT$_2$ (ketanserin) and 5-HT$_{1A/B}$ antagonist (pindolol) were also without effect. The low potency of GR127935 is not consistent with an action at 5-HT$_1$ receptors.

The dopamine D$_2$ receptor antagonist, sulpiride was a weak antagonist of 5-HT mediated dispersion. Interestingly the R(+)-enantiomer was 30 fold more potent than the S(−)-enantiomer. This is opposite to dopamine D$_2$ and D$_3$ receptors where the S(−)-enantiomer has a higher affinity (Seeman & van Tol, 1994). Indeed, in *Xenopus* melanophores which have been made to express human D$_2$ or D$_3$ receptors by transient transfection with a plasmid encoding the appropriate receptor subtype (Potenza *et al*., 1994), the racemate was 50 fold more potent than the (+)-enantiomer of sulpiride in triggering pigment aggregation. The higher potency of R(+)-sulpiride on the *Xenopus* 5-HT response may be a property of the interaction of this ligand with 5-HT receptors in general, or may be a unique pharmacological feature of the 5-HT$_7$ receptor subtype. If so, it may provide a useful means of distinguishing 5-HT$_7$-mediated responses.

RT–PCR using primers based on the published *Xenopus laevis* 5-HT$_7$ gene sequence (Nelson *et al*., 1995) confirmed that the 5-HT$_7$ receptor mRNA is expressed in melanophores. The predicted amino acid sequence of the *Xenopus* 5-HT$_7$ receptor has a high homology ($\sim$64% identity and $\sim$77% similarity) with mammalian 5-HT$_7$ receptors.

The present study found that concentrations of melatonin considerably greater than physiological circulating levels (maximum of $\sim 0.5-1\times10^{-9}$ M at night) were able to activate the endogenous 5-HT$_7$ receptor on *Xenopus* melanophores to trigger pigment granule aggregation. Given the similarity of chemical structure between melatonin and 5-HT, perhaps it is not surprising that high concentrations of melatonin may also activate a 5-HT receptor subtype. Melatonin administration has been reported to counteract the effects of both the 5-HT$_2$ receptor agonist, DOM (1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane) and the antagonist, ritanserin on sleep (Dugovic *et al*., 1989), and to attenuate head-shake behaviour triggered by another 5-HT$_{2A}$ agonist, DOI (±-1-(2,5-dimethoxy-4-iodophenyl)2-aminopropane, Eison *et al*., 1995). However, in radioligand binding assays melatonin has little affinity for 5-HT$_{1A}$, 5-HT$_{1B}$ or 5-HT$_2$ subtypes (Guardiola-Lemaitre, 1991), and cannot activate the 5-HT$_4$ subtype in guinea-pig isolated proximal colon (Lucchelli *et al*., 1997). Melatonin and 5-HT (potentially acting through a 5-HT$_7$ receptor) can both phase-advance circadian rhythms by acting on neurons in the suprachiasmatic nucleus (McArthur *et al*., 1997; Lovenberg *et al*., 1993). The high potency of melatonin (pEC$_{50}\sim10^{-11}$ M) and the different phase-dependency of the sensitivity of SCN neurons to the effects of melatonin and 5-HT indicate that these effects on circadian physiology are mediated by the cognate receptors for these ligands.

A number of psychopharmacological actions have been reported after administration of melatonin, with perhaps the most convincing evidence showing that melatonin has hypnotic properties in both man (Waldhauser *et al*., 1990) and animals (Holmes & Sugden, 1982). Analgesic, anxiolytic and anti-convulsant activity has also been reported after administration of high doses (Guardiola-Lemaitre *et al*., 1992; Sugden, 1983). It has been suggested that these effects may be mediated by an interaction with the benzodiazepine receptor as melatonin and some of its metabolites have micromolar affinity for this site (Marangos *et al*., 1981), although the effects are not prevented by flumazenil, a benzodiazepine receptor antagonist (Green *et al*., 1982; Sugden, 1995). It is possible that activation of 5-HT$_7$ receptors may account for some of the pharmacological effects reported after melatonin administration, although its affinity at mammalian 5-HT$_7$ receptors has not yet been determined.

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