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Review

VERTEBRATE MELANOPHORES AS POTENTIAL MODEL FOR DRUG DISCOVERY AND DEVELOPMENT: A REVIEW

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Abstract: Drug discovery in skin pharmacotherapy is an enormous, continually expanding field. Researchers are developing novel and sensitive pharmaceutical products and drugs that target specific receptors to elicit concerted and appropriate responses. The pigment-bearing cells called melanophores have a significant contribution to make in this field. Melanophores, which contain the dark brown or black pigment melanin, constitute an important class of chromatophores. They are highly specialized in the bidirectional and coordinated translocation of pigment granules when given an appropriate stimulus. The pigment granules can be stimulated to undergo rapid dispersion throughout the melanophores, making the cell appear dark, or to aggregate at the center, making the cell appear light. The major signals involved in pigment transport within the melanophores are dependent on a special class of cell surface receptors called G-protein-coupled receptors (GPCRs). Many of these receptors of adrenaline, acetylcholine, histamine, serotonin, endothelin and melatonin have been found on melanophores. They are believed to have clinical relevance to skin-related ailments and therefore have become targets for high throughput screening projects. The selective screening of these receptors requires the recognition of particular ligands, agonists and antagonists and the characterization of their effects on pigment motility within the cells. The mechanism of skin pigmentation is incredibly intricate, but it would be a considerable step forward to unravel its underlying physiological mechanism. This would provide an experimental basis for new pharmacotherapies for dermatological anomalies. The discernible stimuli that can trigger a variety of intracellular signals affecting

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Abbreviations used: ACh – Acetylcholine; AChE – acetylcholinesterase; GABA – gamma aminobutyric acid; 5HT – 5 hydroxy tryptamine; mAchR – muscarinic acetylcholine receptor; MCR – melanocortin receptor; NE – nor-epinephrine; POMC – proopiomelanocortin

pigment granule movement primarily include neurotransmitters and hormones. This review focuses on the role of the hormone and neurotransmitter signals involved in pigment movement in terms of the pharmacology of the specific receptors.

Key words: G-protein-coupled receptors, Melanocytes, Skin pigmentation, Neurotransmitter, Pigment cells, Melanocyte-stimulating hormone, MSH, Drug discovery

INTRODUCTION

Research on the pigment-bearing cells called melanophores has played a significant role in the advancement of modern skin pharmacotherapy. Melanophores belong to the most diverse class of cells, the chromatophores, which originate in the neural crest and migrate to the basal layer of the epidermis and dermis during embryogenesis [1]. These cells synthesize and store the dark brown and black biopolymer pigment melanin, and package it into membrane-bound intracytoplasmic vesicles called melanosomes [2]. The broad range of colors seen in many fish, amphibians, reptiles and invertebrates together with the remarkable ability of sudden chromatic variation (physiological color change) seen in many such species stand in contrast to the more subtle variations in skin tone seen in mammals including humans (morphological color change). However, the two phenomena are directed by related cell types, the melanophores of the lower vertebrates and the melanocytes of humans and other mammals.

The melanophores are by far the most widely studied chromatophores. They have an instinctive ability to quickly reposition pigment granules within the cells when they are given the appropriate stimulus. This property differentiates them from their non-motile mammalian kin, the melanocytes. The responses of melanophores to external stimuli are highly coordinated: external signals are received and integrated, eliciting a concerted and appropriate response. This cellular communication depends largely on the transmission of signal couriers (i.e. “ligands/agonists”), which are received via the cell surface and intracellular recognition molecules (i.e. “receptors”) on the recipient cells. This results in remarkably coordinated bidirectional movement of pigment granules within the cells. It is this property of the melanophores combined with the receptor sites for a large number of ligands that makes them highly robust and sensitive potential targets for drugs and pharmaceutical compounds.

This paper is an overview of the detailed investigations done on melanophores together with an attempt at extrapolating the mammalian pigmentary system for use in drug cell responses at the neuro-melanocyte junction. Keeping in mind that melanin-based pigmentation biology is conserved across vertebrates [3], a deeper understanding of the physiology of the lower vertebrates can easily be translated into testable hypotheses for studying the cellular basis of pigmentation variation in the natural vertebrate cell system, including that of humans. Both

melanogenesis and hair follicle pigmentation are highly complex phenomena governed by an array of enzymes, hormones and neurotransmitters, which are in turn regulated by the signal transduction pathways intrinsic to the skin and hair follicles. These pathways are receptor dependent, and independently act through auto-, para-, or intracrine mechanisms [4, 5], which certainly offers a mosaic picture of the complex cascading interactions that researchers are slowly but surely revealing.

BACKGROUND

Physiological color change has been a subject of investigation for a number of researchers. Investigators like Frisch [6] and Parker [7] have proferred that individual chromatophores can "aggregate or disperse" when given an appropriate external stimulus, but the underlying mechanism that results in such a highly coordinated and integrated response was unexplained until the middle of the century. The involvement of the nervous system and direct innervations of chromatophores was the view that came to the fore, and it has since been widely accepted by a number of researchers. A significant number of reports have been published by pioneers like Parker [7], Pye [8], Fujii [9], and David *et al.* [10]. Pouchet [11] reported that in teleost fish, the sectioning of peripheral nerves or the electrical stimulation of spinal nerves respectively led to darkening or paling in definite areas. Earlier, Brücke [12] had suggested that the color control in chameleons is purely neuronal, but this is not a general phenomenon in lizards [13]. This finding broadened the perception of the mechanism of chromatophore control, and the indirect control of chromatophores by neurotransmitters and hormones came to light [7, 9, 13]. The release of the endocrinal catecholamines epinephrine and nor-epinephrine from the adrenal medulla directly affects melanophores, bringing about a marked contraction of the pigment granules within the cells. This hormonal nexus was seen as a significant cue for the phenomena of pallor or paling due to excitement seen in many fish, amphibians and reptiles. It has also very interestingly been reported that autocrine catecholamine biosynthesis and the beta-adrenoceptor signal promote pigmentation in human epidermal melanocytes [14]. However, the involvement of catecholamines in the case of lower vertebrates is secondary to that of pituitary hormone alpha-MSH (alpha-melanocyte-stimulating hormone), which stimulates the production and release of melanin in the melanophores [15]. In the case of amphibians, alpha-MSH plays a pivotal role in the process of skin color adaptation. In turn, the skin of amphibians contains a number of regulatory peptides, some of which have been found to regulate the activity of pituitary melanotrope cells. Also, the skin of certain species of amphibians harbors considerable amounts of thyrotropin-releasing hormone (TRH), a highly potent stimulator of alpha-MSH release [16]. Interestingly, the TRH gene is also expressed in human skin [17]. Expression of functional thyroid-stimulating

hormone receptor in the skin may have significant physiological and pathological consequences.

This concept is further sustained by the significant experimental evidence showing that the melanocytes of mammals produce classical stress neurotransmitters, neuropeptides and hormones, and that this production is stimulated by ultra-violet radiation, biological factors and other agents that act within the skin neuroendocrine system [18]. On the other hand, melanin-concentrating hormone (MCH) is synthesized in the brain and secreted from the pars nervosa of teleost fish. This hormone stimulates melanosome aggregation within the integumental melanophores of fish, but stimulates melanosome dispersion within tetrapod (frog and lizard) melanophores [19]. Also, the color change in amphibians is under hormonal control, unlike that in fish and reptiles [20]. Interestingly, mammalian skin is a well-characterized target organ for neuropeptides like proopiomelanocortin (POMC), alpha-MSH, beta-endorphin and ACTH, and it is also a source of these peptides. The receptors for POMC peptides expressed in the skin are functional, recognizing MSH and ACTH. The powerful effects of POMC peptides on the skin pigmentary, immune and adrenal systems are consistent with the stress-neutralizing activity addressed at maintaining skin integrity to restrict disruptions of internal homeostasis [21]. Therefore, bearing in mind the expanding list of neuroendocrine elements expressed in the skin, there is evidential support for a strong role for this system in cutaneous biology.

Besides catecholamines, the role of other neurotransmitters like acetylcholine, 5-hydroxytryptamine and histamine has also been quite significant in pigment translocation. It is quite apparent that the color-changing phenomenon is under neuronal or hormonal control or the cumulative action of both [13, 22-25].

In view of this, the responses of melanophores and melanocytes to various neurotransmitters and biogenic amines and compounds have been analyzed, and it has been confirmed that the effector cells contain several types of receptor for different neurotransmitters and hormones. Much of the drug development today is focused on finding the chemicals that demonstrate the ability to bind with specific receptors, thereby either inhibiting or accelerating the cellular processes. These receptors have been linked to the regulation of a variety of metabolic processes, and are crucial to many biological functions, including skin pigmentation. Considering this, the current state of knowledge on the subject is incomplete, especially in terms of the molecular mechanism of receptor stimulation by various drugs and hormones. It is well known that there is a great deal of inconstancy in the response of lower vertebrate melanophores to different stimuli. This diversity is not only observed within species, but also within individuals [13, 26]. The regulatory pathway controlling pigment transport has been partially elucidated using live melanophores. By analyzing the effect of various agonists and antagonists on cell surface receptors, and the effect of inhibitors or activators of signal transduction pathways, researchers have begun to identify components of the signaling pathways that ultimately control the

motility of pigment granules. While there are several stimuli (internal and external) that can trigger a variety of intracellular signals affecting pigment granule motility, this review focuses on the pharmacological effects of various biogenic agents and pharmaceutical compounds that have been explored so far, and their actions on melanophores with respect to the phenomenon of color change in fish, amphibians and reptiles.

Interestingly, it has been reported that the melanophores and their mammalian counterpart melanocytes act as sensors for computing environmental signals, and that normal epidermal MC are sensory and regulatory cells operating in the context of a regulatory network for the maintenance of human epidermal homeostasis. On the other hand, altered regulatory MC functions may play a role in marked skin ailments and affect other cutaneous functions [27]. Furthermore, this presumption that melanocytes produce classical stress neurotransmitters, neuropeptides and hormones owing to their response to intrinsic or extrinsic signals represents a clear-cut neuro-endocrinal involvement of melanocytes in coordinating cutaneous homeostasis [28]. This interesting connection has definitely paved the way towards a better comprehension of mammalian pigment biology and its related abnormalities, and thus given ideas for corrective therapies.

Since the skin is situated at the interface between the external and internal environments, it has evolved to detect, integrate and respond to a variety of ligands in a discernible fashion through consequential phenotypic effects, including skin pigmentation. The role of skin as an important neuro-endocrinal modulator actively networked with the central nervous system to maintain body homeostasis has been strongly established [29].

The gamut of cell surface receptors for various neuropeptides, hormones and neurotransmitters are the key players in the detailed and intricate network of signaling pathways involved in the process of skin pigmentation. Research focused on devising the putative mechanism underlying the process of skin pigmentation has yielded intriguing insights into the cell surface metabotropic receptors. A number of investigators have contributed to the understanding of the pharmacology of these receptors, and the ramifications are manifold. The in-depth characterization and knowledge of these receptors on the pigment cells would create novel platforms for deeper investigations and open up endless possibilities for composite clinical trials directed towards various specific ligand-receptor targets, which could be further used for therapeutic strategies.

THE PHARMACOLOGY OF ADRENOCEPTORS

The powerful action of adrenaline in bringing about the aggregation of pigment granules has been well documented. Spaeth and Barbour [30] observed that adrenaline at a threshold dose of the order of 1:50,000,000 could aggregate the melanophores of *Fundulus*. Fujii and Miyashita [31] found that adrenaline causes a considerable response in *Lebistes melanophores*. By contrast, Breder and Rasquin [32] reported that some teleost fish such as *Chaetodipterus faber*

showed dark markings indicating pigment dispersion after the injection of adrenaline. Enami [33] also reported that the catfish *Parasilurus* darkens after injections of adrenaline. Rasquin [34] surveyed its effects in a variety of fish, and noted that the melanophores of some fish are unresponsive to adrenaline. Watanabe *et al.* [35] described that *Oryzias* melanophores sometimes respond to adrenaline by dispersion. There have been reports that indicate that nor-adrenaline is also effective in arousing melanophore aggregation. These are by Umrath [36] on *Rhodeus*, Fujii [37] on *Chasmichthys*, by Fange [38] on *Gadus* and *Lebistes*, by Scheline [39] on *Labrus*, Ali [40] on *Channa punctatus*, Martensson [41] on cichlid fish, and Aspengren [42] on Atlantic cod.

The contradictory results in the literature regarding this ambiguity in the action of adrenaline on melanophores suggest that species variation is a major factor in the adrenaline response. The skin of teleost fish is innervated by a dense plexus of autonomic nerve fibers [43-45]. Sympathetic fibers identified by electron microscopy and catecholamine fluorescence are plentiful around the melanophores. Ahlquist [46] classified the adrenergic receptors present on the smooth muscle as alpha and beta types, with the further sub-classification of alpha as alpha-1 and alpha-2 [47]. Later, Lands [48] classified the beta adrenoceptors as beta-1 and beta-2. It has been found that the teleost melanophores have either alpha or beta receptors, or may have both types, each of which controls the responses that are opposite in nature to each other [13, 26, 49]. It is also possible that individual melanophores are innervated by more than one sympathetic neuron [50]. In most species studied so far, the effects of sympathetic stimulation of the melanophores, erythrophores and iridiophores have been found to be mediated by alpha adrenoceptors, generally of the alpha-2 subtype [51, 52]. Melanophore aggregation results from the inhibition of adenylate cyclase activity [53] resulting in pigment aggregation. It was originally suggested [54] that the sympathetic nerve to melanophore transmission in teleosts was mediated by alpha adrenoceptors, since dibenamine effectively antagonized the melanin-aggregating effect of adrenaline and nor-epinephrine. Fujii and Miyashita [31] later demonstrated that adrenergic monoamines caused aggregation in guppy melanophores, suggesting the role of post-synaptic alpha adrenoceptors. The effects of 37 agents that are known to act upon the autonomic nervous system in mammals were thoroughly investigated in *Fundulus heteroclitus* by Abbott [55]. It was found that catecholamines and related substances caused aggregation *in vivo* and *in vitro*, confirming the presence of an aggregating mechanism. Various reports also suggest the co-existence of both alpha-1 and alpha-2 adrenoreceptors and the predominance of one over the other. For instance, *Oreochromis mossambicus* has been demonstrated to possess predominant alpha-2 receptors causing aggregation [56], and *Siganus canaliculatus* has post-synaptic alpha-2 receptors [57]. Also interestingly, one of the reports on cryptic patterning in *Pleuronectes americanus* [58] suggested that the *in vitro* responsiveness to the alpha adrenoceptor agonists phenylephrine and clonidine and to the antagonists yohimbine and prazosin demonstrated that melanosome aggregation in this species is mediated through

both alpha-1 and alpha-2 adrenoceptors, the alpha-2 subtype being predominant in each pattern component. Therefore, it cannot be generalized that teleost melanophore alpha adrenoceptors are universally of one subtype, or that there is intraspecific variation in subtypes of these receptors associated with the cryptic patterning mechanism.

On the other hand, the activation of beta adrenoceptors can cause melanophore dispersion as a result of the stimulation of adenylase cyclase. Furthermore, beta adrenoceptors mediating melanophore dispersion have been reported in the guppy and *Parasilurus asotus* [59, 60]. Also, autoradiographic demonstrations of beta adrenergic receptors were achieved by light microscopy in the melanophores of isolated scales of a teleost fish, *Oryzias latipes* [61]. The co-existence of beta adrenergic receptor subtypes 1 and 2 in the melanophores of the marine gobies *Tridentiger* and *Chasmichthys* has been reported to cause pigment dispersion [62]. Earlier subtypes of beta adrenergic receptors mediating pigment dispersion in the chromatophores of *Oryzias latipes* have also been reported [63]. Strangely, the co-existence of muscarinic cholinoceptors and alpha adrenoceptors, which both mediate pigment aggregation, has also been reported from *Corydoras* melanophores [64].

In the case of amphibians, the functional location of adrenergic binding sites was studied in frog skin melanophores by injecting nor-epinephrine (NE) outside and inside a melanophore. It has been shown that both alpha and beta receptors may be present on amphibian chromatophores. While both epinephrine and nor-epinephrine lighten the skins of *R. pipiens* by overriding the MSH effects [65], catecholamines darken the skin of both *Xenopus* [66] and *Scaphiopus* [67]. Apparently, alpha receptors are present in *R. pipiens* [68, 69], accounting for the paling reaction, whereas in *Xenopus* [70, 71] and *Scaphiopus* [67], beta receptors predominate, allowing darkening to occur in the presence of catecholamines. It has also been reported that adrenoceptors may be totally absent, as in *Bufo ictericus* melanophores [72]. Although the advances in the pharmacology of alpha and beta receptors have elaborated subclasses of these receptors, little has been done with respect to their definition in amphibians and reptiles. By contrast, the adrenoreceptors of fish have been better characterized in this regard [9].

Among reptiles, the diverse and wide-spread New World genus *Anolis* has been studied the most. It has become a useful model for physiological studies of color-changing phenomena. In particular, the green anole, *Anolis carolinensis*, has been well investigated and documented by Greenberg *et al.* [73]. In this species, dermal chromatophores are known to be free of sympathetic innervation [74], leaving body color subject only to the influence of circulating chromoactive hormones: epinephrine, nor-epinephrine and MSH. For instance, a color change from green to brown, or darkening involving speckling and the appearance of a small “eyespot” just behind the eye, indicate specific patterns of activation of alpha-2 and beta-2 adrenoceptors and MSH. Despite early beliefs about the background adaptation in *A. carolinensis* [75], more recent studies in the field indicate that body color is most typically affected by social activities [76]. The

aggregation of melanin granules within *Anolis* melanophores in response to sympathomimetic stimulation is regulated by alpha adrenergic receptors, whereas their dispersion is controlled through the beta adrenergic receptors of the melanophores. Most *Anolis* melanophores possess both alpha and beta adrenergic receptors, but some melanophores possess only beta adrenergic receptors [67]. In the normal physiology of the lizard, under conditions of stress, the stimulation of alpha adrenergic receptors by catecholamines leads to an "excitement-pallor" followed by an "excitement-darkening" resulting from the stimulation of beta adrenergic receptors which causes dispersion of melanin granules within localized populations of melanophores. Thus, in *Anolis*, the dispersion of melanin granules within the melanophores is regulated by both MSH and by catecholamines. Evidence is presented that the intracellular level of cyclic 3',5'-AMP within the melanophores may be responsible for the regulation of movement of melanin granules [77]. Reports on wall lizard *Hemidactylus flaviviridis* have been published [78] stating that adrenaline and isoprenaline (beta agonist) caused dispersion of the melanophores, whereas phenylephrine (alpha-1 agonist) caused aggregation, suggesting the presence of both alpha and beta receptors.

Interestingly, in humans it has been reported that both the alpha-1 adrenoceptor and the beta-2 adrenoceptor can exercise a major influence on melanogenesis in melanocytes. The nor-epinephrine/alpha-1 adrenoceptor signal initiates the IP3/diacylglycerol/PKC cascade, leading to increased melanocyte dendricity and melanin biosynthesis [79, 80]. Furthermore, the release of calcium from intracellular stores by IP3 causes an increase in the Ca^{2+} content in the cytosol, which could foster cellular L-phenylalanine uptake followed by its intracellular turnover to L-tyrosine, finally providing sufficient levels of this substrate for melanogenesis [81]. Interestingly, in another report, it was shown that a symbiotic mechanism exists where the secretion of catecholamines by keratinocytes brings about the stimulation of melanocytes resulting in a time-dependent induction of alpha-1 adrenoceptors in melanocytes [82]. Furthermore, it is worth mentioning that the human keratinocytes have a full capacity for the biosynthesis and degradation of catecholamines [83]. Enzymes involved in the synthesis of an essential co-factor (6R)L-erythro-5,6,7,8-tetrahydrobiopterin (6-BH4) are expressed in keratinocytes producing epinephrine and nor-epinephrine, which in turn control beta-2 adrenoceptor density and the expression of alpha-1 adrenoceptors on melanocytes. The receptor density correlates with the signal transduction system in the regulation of calcium homeostasis in the epidermis. This system has been linked to a pigmentation disorder called vitiligo, where the overproduction of 6-BH4 leads to a dysfunction of catecholamine biosynthesis with a high number of beta-2 adrenoceptors in differentiating keratinocytes, and defective calcium uptake in both keratinocytes and melanocytes [83]. This important finding has led to major hypotheses regarding the physiology and therapy of vitiligo, and can be further explored for a deeper understanding of the disparate facets of this disease. A detailed understanding of the physiology and

pathophysiology of the adrenergic network in the skin could therefore lead to the discovery of specific drugs for novel treatment modalities.

THE PHARMACOLOGY OF CHOLINOCEPTORS

The great importance of acetylcholine derives from its role as a neurotransmitter for cholinergic neurons, which innervate many tissues, including the smooth skeletal muscles. The action of acetylcholine in the periphery is the result of the activation of either the ionotropic nicotinic receptor (nAchR) or the metabotropic muscarinic receptor (mAchR). In the mammalian central nervous system (CNS), both nicotinic and muscarinic receptor subtypes are present on neurons, although there is as yet very limited evidence for a physiological role for nicotinic receptors in synaptic function in the mammalian brain [84].

A number of researchers have investigated the action of acetylcholine on pigment cells, but the results were inconsistent and conflicting. Parker [7] reviewed the action of acetylcholine in a number of teleost fish and presented the evidence for dispersing fibers as cholinergic. There have been other reports on the effectiveness of acetylcholine at inducing melanin dispersion [85-88]. Umrath [36] reported that acetylcholine acts to disperse erythrophore pigment in *Rhodeus*. On the other hand, a very high concentration of acetylcholine has been reported to produce a detectable antagonizing effect of epinephrine action on *Oryzias* melanophores [35]. There have been studies where acetylcholine was found to be ineffective on fish melanophores [30, 89]. The melanophores of *Fundulus*, *Scopthalamus* and *Tilapia melanopleura* were all found to be unaffected by acetylcholine [90-92]. Acetylcholine and/or eserine, an anticholenesterase agent, was not found to induce darkening in the *Phoxinus*, unlike carbachol, a parasympathomimetic drug, which did [93]. Among the lower vertebrates, the sympathetic innervation to the melanophores of the siluroid fish *Parasilurus* is the sole instance of such innervation in which the peripheral transmission to the effector cells is peculiarly cholinergic [94]. By contrast, in the glass catfish *Kryptopterus*, catecholamines were found to be ineffective, whereas acetylcholine and its analogues were found to be potently active in aggregating pigment [95]. Atropine or scopolamine interfered with the action of both nervous stimulation and acetylcholine. One the other hand, physostigmine (parasympathomimetic drug) augmented the cholinergic effects, indicating the presence of cholinoceptors of muscarinic type. Like the melanophores of many teleosts, those of the dark chub, *Zacco temmincki*, and the common minnow, *Z. platypus* (Cyprinidae, Cypriniformes), responded to nor-epinephrine (NE) with an aggregation of pigment. It was further found that some melanophores were responsive to acetylcholine (ACh) in the same way. The response to NE was blocked by an alpha-adrenergic blocker, phentolamine, whereas the response to ACh was not. By contrast, two muscarinic cholinoceptor antagonists, namely, atropine and scopolamine, were found to be effective in blocking the action of ACh. This report was the first to describe the presence of

cholinoceptors on the chromatophores in species of fish other than that of *Siluriformes* [96]. Another report by Ovais *et al.* [97] suggested that acetylcholine, carbachol, pilocarpine and nicotine induced a dispersal effect on the melanophores of *Cirrhinus mrigala*. Physostigmine was found to disperse the melanophores while atropine and scopolamine failed to block the dispersal effects of cholinergic agonists. However, nicotine was found to block the dispersal effect of carbachol. Hexamethonium and atropine aggregated the *C. mrigala* melanophores. These findings clearly indicate the involvement of cholinergic nicotinic receptors in the dispersal responses of melanophores in this fish species. Later Ovais [98] found that cholinomemetic drugs induce a significant dispersal in the melanophores of the catfish, *Clarius batrachus*.

In the case of amphibians and reptiles, acetylcholine has been reported to have varying effects [13]. Moller *et al.* [99] reported that the dermal and epidermal melanophores of *Rana pipiens* possess cholinergic receptors where ACh caused a significant aggregation *in vitro* to melanophores previously darkened by MSH. Further reports by Goldman and Hadley [67] on *Anolis* suggested that a very high concentration of acetylcholine is needed to bring about a detectable response in the melanophores. Later it was reported that acetylcholine produced melanin aggregation and blanching of skin color in *Rana tigerina* (now referred as *Hoplobatrachus tigerinus*), the common Indian frog, and the effects were more prolonged in frogs pretreated with an anticholinesterase agent [100]. In another study, the responses of isolated skin melanophores of *Rana tigerina* and *Bufo melanostictus* to cholinergic drugs were investigated [101]. It was found that acetylcholine caused dispersion of the skin melanophores of *R. tigerina* and *B. melanostictus* and that the effects were blocked by atropine and hyoscine.

Eserine was found to augment the melanophore dispersal effects of ACh. This potentiated dispersal effect of ACh by eserine was also antagonised by hyoscine. Carbachol, another specific cholinergic agonist, caused significant dispersion of the melanophores of both of the amphibian species. The effects were also blocked by atropine and hyoscine. These results indicated that cholinergic receptors of muscarinic type are present on the melanophores of *R. tigerina* and *B. melanostictus*, and that they mediate the dispersion of integumental melanophores leading to darkening of the skin. Further studies revealed that the secretion of alpha-MSH from the intermediate lobe of the frog pituitary is regulated by multiple factors, including classical neurotransmitters and neuropeptides. In particular, acetylcholine (ACh), acting via muscarinic receptors, stimulates alpha-MSH release from frog neurointermediate lobes (NILs) *in vitro* [102].

It has been shown that the intra- and intercellular cholinergic signal transduction network in the human epidermis contributes significantly to homeostatic and compensatory responses regulating vital functions in keratinocytes as well as melanocytes [103]. The mammalian cholinergic system is highly complex, consisting of both muscarinic and nicotinic receptors with multiple subtypes, coupled to classical intracellular second messenger pathways, including c-AMP,

c-GMP and calcium-mediated downstream processes. Interestingly, normal human skin melanocytes express the m1, m2, m3, m4 and m5 subtypes of classic muscarinic acetylcholine receptors on their cell membrane, and these receptors regulate the concentration of intracellular free Ca^{2+}, which may play an important physiological role in melanocyte behavior and skin pigmentation [104]. Muscarinic acetylcholine receptors (mAChRs) regulate the activity of numerous fundamental central and peripheral functions, and have been linked to various skin-related ailments [105]. For instance, in vitiligo the response of melanocytes to ACh depends on the activity/amount of the ACh-degrading enzyme acetylcholinesterase (AChE). The AChE activity is lowered in vitiliginous skin during depigmentation, but returns to normal on repigmentation [106].

Furthermore, acetylcholine has been shown to elicit pigmentation acting via its nicotinic receptors. In one study, melanin pigmentation was found in oral lesions at the site of application for 3 to 6 months of a sublingual 2 mg nicotine tablet [107]. The lack of small-molecule ligands that can block or activate specific mAChR and nAChR subtypes with high selectivity has remained a major obstacle in defining the roles of the individual receptor subtypes and in the development of novel muscarinic and nicotinic drugs.

Therefore, high throughput characterization of these receptors is necessary in order to get a deeper insight into the physiology of their responses to various drugs and pharmaceutical agents. The knowledge of animal melanophore responses to ACh together with the involvement of a specific receptor type can be further extrapolated to devise chemicals and drugs that may rectify and cure skin-related issues in human systems.

THE PHARMACOLOGY OF MELATONIN RECEPTORS

Melatonin (5-methoxy N-acetyltryptamine) is a hormone synthesized and released from the pineal gland at night. It acts on specific high affinity G-protein-coupled receptors to regulate various aspects of physiology and behavior, including circadian and seasonal responses. Poikilothermic vertebrates exhibit a circadian rhythmic color change, with nocturnal blanching, usually related to melatonin secretion. In amphibians, melatonin exhibits a potent skin-lightening activity. However, in fish and reptiles, the melatonin effects vary with the species, the developmental stage, and the pigment cell location [108, 109]. Melatonin also exerts inhibitory or excitatory activity on the amphibian reproductive system, regulates circadian locomotory activity in reptiles, and modulates amphibian metamorphosis. Binding sites of melatonin have been detected in the central nervous system and peripheral tissues of various vertebrates. The relative potencies of melatonin analogues demonstrated two subtypes of melatonin receptors (ML-1 and ML-2). A transmembrane melatonin receptor has been cloned from *Xenopus laevis* melanophores. It belongs to the family of the G-protein-coupled receptors and exhibits 85% homology with the mammalian nervous system receptor. Melatonin binding sites in the nucleus of

many cell types and its potent intracellular anti-oxidant action suggest mechanisms of action other than through the G-protein-coupled receptor [109]. It was first recorded that melatonin effectively aggregated melanophores inclusions in the goby, *Chasmichthys golosus* [37]. This action of melatonin was also found in other species like *Scardinus erythrophthalmus* [110], *Carassius auratus* [111], *Phoxinus Phoxinus* [93] and *Salmo gairdneri* [112, 113]. However, in some species like *Fundulus heteroclitus* [55], *Lepidosiren paradoxa* and *Potamotrygon reticulates* [114], there was a weak or negative response.

In amphibians such as *Xenopus laevis*, a very low concentration of melatonin activates the Mel (1c) receptor subtype triggering granule aggregation and lightening of skin color. It was also reported that melatonin exerts rapid effects on pigment granule distribution in *Xenopus laevis* melanophores [115]. Low concentrations of melatonin (10^{-11} to 10^{-9} M) cause a dramatic perinuclear aggregation of the melanin-containing granules [115]. Additionally, Mel (1c) receptor activation reduces intracellular cAMP via a pertussis toxin-sensitive inhibitory G-protein (Gi), but how this and other intracellular signals regulate pigment movement is not yet fully understood [116]. In another study, melatonin was found to bind to a melatonin receptor, which causes melanosome aggregation in Atlantic cod [42]. It has also been reported that the melatonin-mediated melanosome aggregation in *Xenopus* is coupled with tyrosine phosphorylation, and is of the utmost importance for melanosome aggregation mediated by both NE and melatonin in cod melanophores. Together with cAMP fluctuations, tyrosine phosphorylation functions as a switch for melanosome aggregation and dispersion in these cells.

It is known that melatonin is also produced and metabolized in mammalian skin, where it exerts its effect through cell surface and putative nuclear receptors expressed in the skin [117]. It was demonstrated that hamster skin can acetylate serotonin to NAS (N-acetylserotonin), which is then further metabolized by the skin to form melatonin, which subsequently transforms into 5-MT (5-methoxy tryptamine) [117]. Later, it was confirmed that fully developed, local serotonin and melatonin biosynthetic pathways do exist in human skin, and human skin and cultured skin-derived cells express the intrinsic capability to transform L-tryptophan to serotonin and to metabolize serotonin to NAS and melatonin [118]. The conversion of L-tryptophan to serotonin and melatonin in human melanoma cells was also presented [119]. The widespread expression and pleiotropic activity together with the intra-, auto-, and paracrine mechanism of the cutaneous melatonergic system brings forth a high level of functional and cell-specific selectivity [120]. Another interesting study [121] on the role of melatonin in skin pigmentation and physiology found that melatonin is able to suppress UV-induced skin damage and could counteract or buffer external or internal stresses to preserve the biological integrity of the skin. Also, the existence of a biosynthetic pathway involved in the sequential transformation of L-tryptophan to serotonin and melatonin has been subsequently confirmed [121].

The melanophore pigment aggregation response has also played a vital role in the ongoing effort to devise specific melatonin receptor antagonists. Much of what has been learnt about the parts of the melatonin molecule required for receptor binding and activation has come from detailed structure-activity data using novel melatonin ligands. Work aiming to devise ligands specific for the distinct melatonin receptor subtypes should soon deliver selective agonists and antagonists which will be valuable tools in understanding the role of this enigmatic hormone in skin-related ailments.

THE PHARMACOLOGY OF SEROTONIN RECEPTORS

Serotonin or 5-hydroxytryptamine, one of the primary monoamine neurotransmitters in the central nervous system, was first reported to cause dispersion in the melanophores of the guppy, *Lebistes reticulates* [122]. Later Fujii, *et al.* [54] reported contradictory results showing aggregation on the melanophores of the same species. Also Scheline [39] and Scott [91] had observed the aggregation of melanophores of *Labrus* and *Scopthalamus*. Later Healy and Ross [93] and Ruffin *et al.* [123] did not find any effect of 5 HT on melanophores of *Phoxinus* and *Nannostomus*, respectively.

5 HT has been reported to cause dispersion in the melanophores of the frog, *Rana pipiens*, after it had undergone a hypophysectomy [124]. This was later confirmed by Veerdonk *et al.* [125] on *Xenopus laevis*. By contrast, Lerner [126] demonstrated a concentrating effect on the melanophores of *Rana pipiens*. It has been emphatically reported that amphibian skin is rich in bioactive peptides and amines including 5 HT [127, 128]. Further reports indicating the role of serotoninergic activity in the background adaptation response in red-spotted newts (*Notophthalmus viridescens*) have been published [129]. Systemic injection of 5-hydroxytryptophan (5-HTP), the precursor to serotonin, stimulated melanin dispersion within dermal melanophores, whereas the injection of para-chlorophenylalanine (pCPA, an inhibitor of serotonin synthesis) blocked melanin dispersion. The results indicated a role for serotoninergic activity in the background adaptation response in this amphibian. Later, the response of a cell line of *Xenopus laevis* melanophores to serotonin was reported [130]. Serotonin increased the intracellular levels of cAMP and induced pigment dispersion in the cells. Furthermore, Teh and Sudgen [115] reported the involvement of an endogenous 5-HT7 receptor in pigment granule dispersion in *Xenopus laevis* melanophores. 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. 5-HT-mediated dispersion was not blocked by antagonists selective for G(s)-coupled 5-HT(4) or 5-HT(6) receptors, nor by 5-HT(1-3) receptor antagonists, but was inhibited by a selective 5-HT(7)

receptor antagonist, and other antagonists with a high affinity for 5-HT(7) receptors.

There have been reports that melanocyte-stimulating hormone (MSH) stimulates the production and release of melanin, which itself has been stimulated by injections of serotonin into eels and some amphibian species, producing a strong darkening in the skin [131]. Interestingly, para-chlorophenylalanine (pCPA), an inhibitor of tryptophan hydroxylase which depletes brain serotonin in higher vertebrates, was injected into freshwater eels. After 4 or 6 injections (200 mg/kg/day) or 10 injections (100 and 140 mg/kg/day), the animals were paler, with a low melanophore index [131]. It has also been reported that the injection of 5-hydroxytryptophan (5-HTP), a precursor of serotonin, induces the dispersion of melanin in the amphibians *Pleurodeles waltlii* (Urodela) and *Xenopus laevis* (Anura), in the goldfish *Carassius auratus*, and in the carp *Cyprinus carpio*. This is accompanied by a dispersion of erythrophore pigments [132].

In fact, both early and late serotonin embyogenic functions and morphogenic activities are evolutionarily conserved from lower vertebrates to mammals [133]. Interestingly, in the case of humans, the enzyme tryptophan hydroxylase, TPH (TPH1 gene), which catalyzes the rate-limiting step of serotonin synthesis, is expressed in whole human skin and in a wide array of normal and transformed human skin cells [17]. Also, whole skin and skin cells express membrane-bound receptors that mediate serotonin action [134]. This is supported by detailed molecular analyses that identified the expression of m-RNA encoding receptors 5-HT1A, 1B, 2A, 2C, and 7 in human skin [135].

The first experimental evidence that serotonin is synthesized in the skin cells including melanocytes was reported for hamster skin [136]. It was was reported that human melanoma cells can synthesize and metabolize serotonin [119]. Later, a characterization of the serotonergic system in the C57BL/6 mouse skin was presented emphasizing that mouse skin has the molecular and biochemical apparatus necessary to produce and metabolize serotonin and *N*-acetylserotonin [137]. Furthermore, the immunofluorescence of skin biopsies tended to localize TPH protein and serotonin immunoreactivity to normal and malignant melanocytes *in vivo*, and the cutaneous pathway for local serotonin synthesis in melanocytes was also reported [138, 142], implicating the skin as a target of bioregulation by serotonin.

Serotonin may be involved in the regulation of apoptosis and proliferation of melanocytes through receptors expressed by normal and malignant melanocytes [135]. Although serotonin receptors were detected to be present in the skin cells of different mammalian species, including the melanocytes of humans [135], the role of serotonin in the regulation of mammalian pigmentation has yet to be clarified [3].

Interestingly, it has been reported by Iyenger [139] that after the incubation of vitiligo tissue cultures with tryptamine (1 mg/ml) and serotonin (0.5 mg/ml), serotonin uptake was immunohistochemically revealed in the marginal dendritic melanocytes of vitiligo lesional skin. In addition, highly dendritic melanocytes

in pigmented basal cell carcinoma and the epidermis overlying melanomas were also positive for serotonin [139]. Since melanophores originate from the neural crest [1], they are associated with the nervous system. In the brain, nerve terminals containing serotonin are located at synaptic and non-synaptic sites [140], and serotonin is considered a neurotransmitter and neuromodulator. The release of neurotransmitters in the central and peripheral nervous system can be modulated by serotonin receptors, which are located on various nerve terminals of the central and peripheral nervous system [141]. Serotonin was proposed to be manufactured in the cutaneous melanocytes [118, 135, 142] and this has been verified [118, 135, 143]. Another interesting report showed that the increase in UV exposure results in an elevation of the serotonin concentration in skin cells [144]. Serotonin may also be involved in the pathogenesis in manifestations of the skin dermatoses [145, 146] including cholestatic and uremic pruritis [147, 148], urticaria, and itch reaction [149]. Ideally anti-histamines are taken as the primary treatment, but serotonin antagonists may offer an attractive alternative [147, 150].
Developmentally, if serotonin affects melanophores and melanoblasts in the neural crest, and their maturation and migration to the skin, a serotonin receptor regulation on the surface of these cells may be involved. However, until the localization and type of such a receptor is determined, this can only be considered speculation. Therefore, further research should be done with respect to serotonin receptors and their putative involvement in demystifying the intricate and complicated phenomenon of skin pigmentation.

THE PHARMACOLOGY OF HISTAMINE RECEPTORS

Histamine is a biogenic amine involved in local immune responses and in the regulation of physiological function in the gut, and as a neurotransmitter [151]. Histamine is known to stimulate smooth muscles in a variety of tissues. The action of histamine is mediated through its specific receptors which have been identified and classified as H1, H2, H3 and H4 [152]. Earlier reports on histamine have been documented in the literature, where it was indicated to show a slight aggregating effect, while anti-histamine drugs produced marked dispersion in rainbow trout melanophores [153] and [85]. When injected into minnow, histamine had no definite action on the melanophores [93]. Recently Acharya *et al.* [154] reported on both dispersion and aggregation from the melanophores of fish Tor Khudree (Sykes). It was found that histamine induced a mild dispersion in a low dose-range, while at higher doses, it showed consistent aggregation in the fish melanophores, suggesting that both H1 and H2 receptors may be present. Bhattacharya *et al.* [155] reported that histamine causes blanching in the frog, *R. tigerina* (syn. *Hoplobatrachus tigerinus)*, with no substantial approach towards the role of histamine receptors. Fernando *et al.* [156] studied the effect of histamine on the melanophores of *Pleuronectes* and *Lebistes*, but did not confer any definite role of histamine and its receptors in the physiology of pigment cells. Histamine receptors have been well studied and

documented by Ali *et al.* and have contributed quite a number of reports on the significance of histamine receptor involvement in skin pigmentation. For the first time [40], it was reported that the H1 and H2 receptors of histamine are present on the melanophores of the teleost fish *Channa punctatus*. Later, the effects of histamine and specific H1 and H2 receptor agonists were investigated on isolated web skin melanophores of the frog, *R. tigerina*. Histamine, 2-methyl-histamine and 4-methyl-histamine all induced dose-dependent dispersion in the frog melanophores *in vitro*, which were mediated partially through specific H1, H2 and beta adrenergic receptors [158]. Furthermore, histamine and 2-methyl histamine were found to cause dose-dependent aggregation of the integumental melanophores of *Rana tigerina* both *in vitro* and *in vivo*, whereas the specific H2 receptor agonist 4-methyl histamine was reported to cause dispersion [159]. The tail melanophores of *Bufo melanostictus* have been reported to have a dominant population of the H2 type of histamine receptors along with a sparse population of the H1 receptor [157]. These findings were supported by the fact that metiamide, a specific H2 receptor antagonist, completely blocked the aggregation of pigment cells, while mepyramine, a H1 receptor blocker, partially blocked the aggregating response. The strong melanin-aggregating effect of 4-methyl histamine, a specific H2 receptor agonist, and its complete blockade by metiamide further supported this finding. Later, integumental melanophores of *Bufo melanostictus* were investigated [160] and the involvement of H1 receptors in bringing about melanophore aggregation was suggested.

Very interestingly, Tomita *et al.* [161] reported that normal human epidermal melanocytes became swollen and more dendritic and the immunoreactive tyrosinase increased markedly when they were cultured for 2 days with 5 μM of histamine. These results suggest that high dermal concentrations of histamine may be responsible for the induction of the skin pigmentary changes associated with local proliferation of mast cells such as in urticaria pigmentosa and systemic mastocytosis. Another interesting report with the possible involvement of histamine in skin pigmentation was published by Niekerk and Prinsloo [162], where the effects of injecting histamine phosphate intradermally into three groups of nonatopic healthy volunteers with varying degrees of skin pigmentation were studied. It was found that the wheal sizes in the Negroid subjects with darkly pigmented skins were consistently greater than those in both the Caucasian subjects with light skin pigmentation and the mixed caucasian/negroid subjects with light brown skins. From this study, it appears that skin pigmentation has a profound effect on the wheal response to intradermally injected histamine. It is speculated that this difference in response may be related to the melanin pigment in the skin. Very recently it was reported that histamine induces melanogenesis of human cultured melanocytes by protein kinase A activation via H2 receptors [163]. The effects of histamine (0.1 to 10 μM) on cultured human melanocytes were examined, and it was found that histamine evoked morphological changes and an increase in tyrosinase activity, thereby bringing about a marked increase in the melanin content of the cells. In another

study by Lassalle *et al.* [164], the effect of nitric oxide (NO) and histamine on the production of black colored eumelanin and the reddish-yellow pigment pheomelanin in cultured human melanocytes was investigated. It was found that NO and histamine are melanogenesis-inducing factors, and that the amount of eumelanin production significantly increased with independent stimulation by histamine. These findings suggest that NO and histamine, as in the case of alpha-MSH may contribute to UV-induced hyper-pigmentation by enhancing eumelanogenesis.
Based on observations that changes in skin coloration are frequently accompanied by inflammatory reactions, a number of inflammatory factors have been reported as melanogens. However, the mechanism of hyper-pigmentation accompanied by inflammation remains obscure. In human skin, histamine is mostly produced and released by dermal mast cells, and this histamine release from mast cells leads to the development of inflammatory reactions. Thus, it could be speculated that histamine may often participate in the hyper-pigmentation of human skin. For example, hyper-pigmentation after UV irradiation is a physiological reaction combining acute inflammation and pigmentation. An elevation of histamine in UV-irradiated skin was reported by Gilchrest *et al.* [165]. In addition, hyper-pigmentation is a frequent outcome of several diseases with proliferation and infiltration of the mast cells in the skin, e.g. urticaria pigmentosa and atopic dermatitis. Thus, it is easy to infer that histamine levels are high at the infiltrating site of mast cells. Also, in sun-burnt skin, the increased proinflammatory mediators like arachidonic acid and histamine are thought to stimulate melanocytes in the process of hyper-pigmentation. Thus, tanning after sun exposure may be induced not only by the effect of Vitamin D3 and direct UV irradiation, but also by the effect of inducing factors like histamine. Mast cells massively proliferate in the skin lesions of urticaria pigmentosa, so hyper-pigmentation in the skin lesions is quite likely to be induced by chemical mediators including histamine and leucotrienes [166]. This finding has indeed opened up new avenues to deduce the physiology and pathophysiology of histaminergic receptors in relation to skin pigmentation. Also recently, Chou *et al.* [167] investigated the variances in the histamine control skin-testing response between Asian/Pacific islanders and other racial groups. It was found that Asian-Americans did not have a statistically significant difference in the histamine skin test when compared to Hispanic-Americans and caucasians. By contrast, African-Americans had a higher reaction than the other groups. These different responses may be related to skin pigmentation and may bring forth a possible mechanism underlying the physiology of the histamines in the cutaneous biology of pigmentation. The core analysis of histaminergic receptors with the newly discovered subtypes may be investigated for future studies. These findings may bring to light a new pharmacological property of histamine antagonists that might be a remedy for those diseases related to hyper-pigmentation of skin.

THE RECEPTOR PHARMACOLOGY OF MELANOCORTIN SYSTEMS

The melanocortin system primarily consists of melanocortin peptides derived from the proopiomelanocortin gene (POMC), in particular MSH melanocyte-stimulating hormones and adrenocorticotropic hormone (ACTH), and five melanocortin receptor subtypes (MC1R through MC5R). There is limited knowledge regarding the melanocortin system in fish, but the body of information on the receptor part of the system is very rapidly growing [168]. The melanocortin receptors (MCRs) have recently been cloned from several species of fish, and the amino acid sequences appear remarkably well conserved. Pharmacological characterization studies of the first identified piscine MCRs indicate that ACTH may be the original ligand for the MCRs. In mammals, MC4R is exclusively expressed in the central nervous system, while in the fish species examined so far, it is expressed peripherally [168]. In cold-blooded animals, MC1R is reported to affect the distribution of the pigment within the cell rather than the synthesis of different pigment types [25]. Zebrafish also have a family of three MC-receptors, homologous to the human MC1R and MC2R, with a duplicated MC1R. The molecular and evolutionary analysis of MC1R was investigated using three major fish models, the zebrafish *Danio rerio*, the medaka *Oryzias latipes* and the platyfish *Xiphophorus maculates* [169]. It was found that in contrast to some other melanocortin receptor genes, mc1r (the melanocortin receptor-1 gene) was conserved as a single copy gene in divergent fish species. A protein sequence comparison between fish and mammalian Mc1r also revealed a remarkable concordance between evolutionary and functional analyses for the identification of residues and regions critical for receptor function. Richardson *et al.* [170] also reported that the loss of the mc1r gene in zebrafish embryos lead to concentrated melanosomes that do not disperse in dark conditions. These findings were the first to indicate a crucial role of MC1R the regulation of melanosome dispersal in fish and lay an important foundation for further characterization of melanocortin receptors and their physiology in skin pigmentation in different animal models and humans. In another report, the cloning of two river lamprey MC receptors, designated MCa and MCb, respectively showed orthology to the MC1 and MC4 receptor subtypes. Expression and pharmacological characterization showed that the lamprey MCa receptor was able to bind and be activated by both lamprey and human MSH peptides [171]. This study shows the presence of MC receptors in agnathans, indicating early signs of specific functions of melanocortin receptor subtypes.

Recently, cloning, pharmacological characterization, tissue distribution and detailed brain mapping of melanocortin 5 receptor in goldfish (gMC5R) were presented by Cerda-Reverter *et al.* [172]. The goldfish orthologue protein is 69% identical to human MC5R and is conserved in important functional domains. The gMC5R showed similar potency to alpha-, beta- and gamma-MSH peptides in radioligand binding as the mammalian orthologues. The gMC5R-mRNA was found in the peripheral tissues including the skin. The cloning and

characterization of this receptor provides an important tool to elucidate its participation in the neuroendocrinology of cutaneous functions including skin pigmentation.

Interestingly, there is a lack of evidence that POMC peptides are implicated in the regulation of skin color in humans [173]. Moreover, it has been reported that the skin and hair follicles are the local targets for POMC-derived peptides including ACTH, alpha- and beta-MSH, and beta- endorphin [174-179]. Furthermore, POMC, which is a precursor of ACTH, is secreted by human epidermal keratinocytes and melanocytes and stimulates melanogenesis [180].

Alpha-MELANOCYTE-STIMULATING HORMONE (alpha-MSH)

Alpha-melanocyte stimulating hormone (alpha-MSH) is classically known for its ability to induce pigment dispersion within pigment cells [13]. It is also involved in the regulation of melanin synthesis in mammals and fish [181, 182]. Along with these pigment regulatory functions, in fish alpha-MSH serves as a satiation signal in feeding behavior [183] and acts as corticotrope in the chronic phase of the stress response [184]: plasma alpha-MSH levels increase after temperature shock [185], during confinement combined with air exposure [186] and during chronic exposure to acidified water [187]. It has been reported that MSH produces melanin dispersion in melanophores by increasing the cyclic AMP content of the cell. MSH is capable of stimulating the adenylcyclase of melanoma and adrenocortical tissue [188]. The involvement of alpha-MSH in pigmentation in fish has been reported for several species, such as catfish, eel, trout [189], and cyprinid *Zacco temmincki* [190], while in *Fundulus*, arctic charr, flounder, gilthead sea bream and red porgy, such an involvement could not be demonstrated [189, 191-194]. The role of alpha-MSH has been demonstrated in *Oreochromis mossambicus*, where it binds to the melanocortin 1 receptor and can change the hue of its body in response to a change in background [195].

Interestingly, the involvement of ACTH and alpha- and beta-MSH in human skin pigmentation was first demonstrated upon systemic application on human volunteers, where these peptides were found to induce a marked skin darkening [196-198]. Furthermore, both alpha-MSH and ACTH are proposed to be the key players in human skin pigmentation via the melanocortin 1 receptor (MC1-R)/c-AMP second messenger pathway [175-177]. It was reported that ACTH, alpha-MSH and beta-endorphin could stimulate melanogenesis and proliferation of epidermal and hair follicle melanocyte and modulate cell dendricity [173, 178, 199]. Pathologically increased plasma ACTH levels (Addison's disease or Nelson's syndrome) induce hyper-pigmentation and skin atropy [21]. On the other hand, patients with a mutation in the POMC gene have a defective production of POMC pro-hormone in all tissues with a consequent red hair phenotype [200, 201]. Recessive mutations in the MC1R produce unresponsiveness of epidermal melanocytes to alpha-MSH, also resulting in the red hair phenotype in humans [202] or yellow fur in mice, whereas the dominant allele produces a constitutively

active MC1R resulting in uniform jet black fur in somber mice [203]. Futhermore, another report showed that POMC knockout in mice with a C57/BL6 genetic background yielded abundant eumelanin hair pigmentation despite the congenital absence of melanocortin ligands, suggesting that either the mouse MC1R has sufficient basal activity to trigger and sustain eumelanogenesis *in vivo* or that some compensatory non-melanocortin pathway(s) might play a part [204].

MELANOPHORE-CONCENTRATING HORMONE (MCH)

Enami [33] observed pigment aggregation in catfish (*Parasilurus asotus*) melanophores after injecting crude extracts of the pituitary of the same species. He termed the effective principle melanophore-concentrating hormone of "MCH" then indicated that the substance was neither a catecholamine nor an acetylcholine. Using the rainbow trout, Baker *et al.* [205] reported that the melanin-concentrating hormone is a peptide with a molecular weight of less than 2000, and that most of its bioactivity occurs in the hypothalamus, favoring the hypothalamic origin of the hormone theory, which had originally been summarized by Enami [33]. Using immunohistochemical electron microscopy, the results of Naito *et al.* [206] supported these findings. Meanwhile, Kawauchi *et al.* [207] determined the primary structure of MCH peptide purified from chum salmon (*Oncorhynchus*) pituitary. Unrelated to any known hormonal peptide, it was found to be a novel hormone, consisting of 17 amino acids and a sulphydryl link. Subsequently, the same molecular species of MCH was synthesized and its action to aggregate melanosomes was confirmed in a few teleost species by Wilkes *et al.* [208]. Its specific receptor remained an enigma until very recently when it was identified as the orphan G-protein-coupled receptor SLC-1 [209]. MCH binds to and activates two G-protein-coupled receptors, MCH1R and MCH2R [210]. Nagai *et al.* [211] studied the action of the melanin-concentrating hormone (MCH) on melanophores in 27 teleost species and a very detailed comparison of the action of MCH on these fish was presented. It was found that MCH caused melanosome aggregation in all of the teleosts studied, including two siluroid catfish in which melanin-aggregating nerves are known to be cholinergic. In most fish, the minimal effective concentration of MCH was estimated to be 10^{-6} M, while in the three swellfish examined it was higher than 10^{-8} M. The mode of action of the peptide was identical in either adrenergically or cholinergically innervated melanophores, and suggested that it may act through specific receptors on the melanophore membrane. These results suggest that MCH is a biologically active hormone common to teleosts. Another report by Takahashi *et al.* [212] suggested the involvement of the MCH-2 receptor in the background color adaptation of barfin flounder.

The mechanism of MCH action has also been studied in detail in blue damselflsh (*Chrysiptera cyanea*) melanophores [213]. The effect of the hormone on

melanophores of several teleost species was compared in an attempt to determine whether MCH is a general color change hormone in the Teleostei. By contrast, MCH stimulates melanosome aggregation within teleost melanophores but also exhibits MSH-like (melanosome dispersing) activity on melanocytes from the tetrapod frog, *R. pipiens* and the lizard, *Anolis* [208, 214]. Also, it was reported that a reduction in the c-AMP level results in the aggregation of pigment mediated by MCH suggesting the role of c-AMP as a second messenger in the physiological action of MCH [215].

Interestingly, MCHR1 has also been identified as a novel autoantigen in patients affected with vitiligo [216]. Furthermore, very recently Kemp *et al.* [217] stated that there is a lack of evidence to support functions for melanin-concentrating hormone (MCH) and melanin-concentrating hormone receptors (MCH-R) in mammalian skin physiology including pigmentation, inflammation and skin cell proliferation. Thus, it is quite apparent that the MCH/MCHR1 system is functional in human skin and may regulate skin pigmentation through modifications of melanocortin signaling. Intensive research is therefore still needed to define the roles of the hormone and its receptors in mammalian skin. This will be a crucial step to identify pathogenic mechanisms that may involve the MCH/MCH-R system in the context of inflammatory and other skin pigmentation-related disorders.

THE PHARMACOLOGY OF ENDOTHELIN RECEPTORS

The endothelins (ETs) are potent vasoactive peptides which are involved in diverse biological processes, such as contraction, neuromodulation and neurotransmission. The diversity of action of ETs may be attributed to (i) the existence of a number of receptor subtypes, and (ii) the G-protein-mediated activation of different signal transduction pathways. Interestingly, endothelin peptides have been demonstrated to induce pigment dispersion in melanophores [218]. Moreover, the ability of ET-3 specifically to cause pigment dispersion shows that these cells express the ET cell receptor subtype [218]. In many species of teleost and in zebrafish (*Brachydanio rerio*) in particular, endothelin-1 (ET-1) has been reported to cause an aggregation of pigment within the melanophores in a dose-dependent manner [219]. ET-1 appeared to act directly on the melanophores, since denervated melanophores responded to the peptide quite similarly to the normally innervated cells. The alpha adrenergic blockers, namely, phentolamine and tolazoline, and a muscarinic cholinergic blocker, scopolamine, did not interfere with the action of ET-1 on the melanophores. By contrast, BQ-123, an inhibitor of mammalian ET-1 receptors (ETA receptors), did not block the action of ET-1 on melanophores. Presumably, teleostean ET receptors have very different pharmacological characteristics from those of mammalian species, suggesting that ETs may be involved in the subtle and delicate modification of the hues and patterns of the integument that are associated with their elaborate and effective chromatic strategies for survival [219].

Furthermore, mammalian endothelins (ET-1, -2 and -3) proved effective in dispersing the light-scattering organelles (leucosomes) in the leucophores of the medaka *Oryzias latipes*, in a dose-dependent manner [220]. They were almost equally effective, and their minimal effective concentrations were less than 100 pM. Endothelins are reported to act directly on the leucophores, since denervated cells responded to the peptides quite similarly. Also, phentolamine, an alpha-adrenergic blocker, propranolol, a beta-adrenergic blocker, and BQ-123, an inhibitor of the mammalian ET_A receptor, did not interfere with the action of ETs. By contrast, BQ-788, an inhibitor of the mammalian ET_B receptor, potently blocked the action of ETs. Sarafotoxin S6c and IRL 1620, both mammalian ET_B receptor-selective agonists, were also found to disperse leucosomes effectively, mimicking the effect of ETs. In another report, Murata *et al.* [221] suggested that mammalian endothelins-1, -2 and -3 effectively aggregated pigmentary organelles in the xanthophores of the medaka, *Oryzias latipes*. IRL 1620 and sarafotoxin S6c, both selective agonists of the mammalian ET_B receptor, were also found to aggregate xanthosomes in those cells. Therefore, it was suggested that ETs may act through the mediation of ET receptors on the medaka xanthophores, which do not resemble mammalian ET receptors pharmacologically. Thus, along with their effects on other kinds of chromatophores, ETs may take part in the delicate control of integumentary hues and patterns. Many reports clearly indicate that endothelins exert several functions in human melanocytes, including proliferation, dendrite formation, melanin synthesis and the regulation of skin pigmentation [222]. Among the ET receptors, the non-selective endothelin-B (ETB) receptor is the major receptor in melanocytes and malignant melanoma (MM) cells [223]. It is also reported that endothelin receptors are expressed in the pigment cell lesions of the skin [223]. In spite of the important role of ETs and their receptors in the growth and differentiation of melanocytes, the distribution and expression levels of ET receptors in the tissue sections of pigment cell lesions is still unknown. Therefore, through proper screening and characterization of these receptors, many unsolved mysteries related to skin abnormalities could be solved.

OTHER IMPORTANT RECEPTORS

It has been reported that dopamine is a potent inhibitor of alpha-MSH release from the neurointermediate lobe (NIL) in mammals, amphibians and fish. However, dopamine can also be a stimulatory secretagogue for goldfish growth hormone cells. Indeed, two distinct classes of receptor subtypes for dopamine with opposite effects are known. The first subtype consists of the dopamine D1-like receptors D1a and D1b and the recently described D1c receptor in lower vertebrates. These are stimulatory receptors coupled to adenylyl cyclase (AC). The dopamine D2-like subtypes (D2, D3 and D4) are inhibitory receptors, coupled to AC or other secondary messengers [224]. Kemenade *et al.* [225] examined the characteristics of the receptors involved in the dopamine-induced

inhibition of MSH secretion. It was suggested that the receptor system involved has characteristics of both classical D-2 receptors and alpha adrenergic receptors and indicated that in living animals, there must also be a non-catecholaminergic system involved in the inhibition of MSH release from the pars intermedia. Another report by Ovais *et al.* [226] suggested the presence of gamma aminobutyric acid (GABA) receptors on the melanophores of teleost *Cirrhinus mrigala*. It was reported that the GABA-ergic agonists induced significant dispersion in the melanophores, while the antagonists blocked the effect, suggesting the involvement of both GABA A and GABA B receptors. Furthermore, the presence of a receptor for vasoactive intestinal polypeptide (VIP) has been functionally characterized in melanophores of *Xenopus laevis* [227]. It has been reported that the activation of these receptors stimulates intracellular c-AMP accumulation and pigment dispersion, suggesting a Gs protein-mediated response. In addition, evidence for the presence of receptors responding to oxytocin and beta-CGRP (calcitonin gene-related peptide) has also been reported [228]. Further characterization of these receptors has yet to be accomplished.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The discovery of signaling systems in pigment-bearing cells has radically changed our concept of the intracellular communication that occurs within these cells. The classical ligand-receptor modules which are rooted on the principle of specificity together with the dynamics involved in the intercellular communication pathways of the melanophores and melanocytes are remarkable. The phenomenon of skin pigmentation is highly coordinated and works as a well-established companion to the nervous system. Understanding how the cell interprets the signals and the crosstalk between the second messenger pathways that determine the outcome or "translate" downstream responses is just a prelude to the richness of the knowledge in this field. The intracellular messenger pathways are linked to each other forming an intricate network that is richly expressed on the cell surface of pigment cells leading to a highly coordinated cellular response. Our understanding of the complex physiology and pharmacology that underlies these cell surface metabotropic systems for signal transduction has grown dramatically over the past few years, and the consistent new discoveries surrounding these receptor systems continue to fascinate and challenge researchers to go further. Since the science of drug discovery is a field of constant evolution, the specific techniques employed to link chemicals and neurotransmitters and the receptors with which they interact will certainly undergo change or can quickly become outdated. From these studies, it is apparent that one cannot freely apply the results of experiments to human pigmentation. Nevertheless, equipped with a knowledge of experiments using animal melanophores, together with an awareness of clinical abnormalities of pigmentation in human beings, it may be possible to unravel the mechanism of skin pigmentation in humans and to devise possible and better ways to curb

dermatological abnormalities. The amount of research and studies done so far together with the research still in the pipeline indicates that the agonists and antagonists directed towards specific adrenergic, histaminergic, cholinergic and serotonergic receptors could definitely be useful in connection with the treatment of skin-related diseases. It is known that the epidermis *in situ* is exposed to intrinsic and extrinsic challenges from ultra-violet rays, light, temperature, hormones, growth factors, and fluctuating second messengers on a regular basis. Therefore, before broad clinical interventions with agonists and antagonists can be undertaken, we need more information on the responsiveness of the epidermis *in vivo*. Based on our increasing knowledge concerning these receptors and their plasticity, future research would focus on the development of drugs that exert metabotropic effects on the cells of the skin without affecting the central nervous system. Future investigations should be focused to fully elucidate how the adrenergic, cholinergic, serotonergic and histaminergic systems are integrated with their ligands and receptors, and more work should be undertaken to obtain information on the responsiveness of the epidermis *in vivo*. It is quite plausible that in-depth characterization of these receptors would create novel platforms for deeper investigations and open substantial possibilities for composite clinical trials for specific ligand-receptor targets subjected for diagnostics and therapeutics in clinical dermatology.

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