PHD

Factors affecting the release of melanin-concentrating hormone (MCH) and melatonin in rainbow trout

Lyon, Vivienne Elizabeth

Award date:
1991

Awarding institution:
University of Bath

Factors affecting the release of melanin-concentrating hormone (MCH) and melatonin in rainbow trout.

Submitted by Vivienne Elizabeth Lyon for the degree of PhD of the

University of Bath 1991.

Vivienne Lyon

Vivienne Lyon.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ACKNOWLEDGEMENTS

This thesis is dedicated to my mother who has often said I would write a book - so here it is! Thank you very much for all your support. I would also like to thank my supervisor, Dr Bridget Baker, for all her help, encouragement and advice during the course of this work, and especially for her boundless enthusiasm. Thanks are also extended to Jim Knight, Dave Molesworth, Molly Powell and Pete Clark for their technical help.

INDEX

ABSTRACT

This thesis discusses the control of melanin-concentrating hormone (MCH) release in rainbow trout. The effects on MCH release of darkness and black or white illuminated backgrounds were investigated. On a white background, fish released more MCH (214.2 ± 34 pg/ml, n=8) than on a black background (55.4 ± 5.8 pg/ml, n=8), while those kept in the dark released least of all (27.9 ± 3.4 pg/ml, n=8). MCH release in the light depended upon background colour rather than intensity of illumination. Specific illumination of different retinal areas, suggested that a low ratio of incident to reflected light entering the eye may be important for initiating the paling response in trout. Illumination of the dorso-lateral retina was associated with high plasma MCH concentrations (94.2 ± 20 pg/ml, n=6); it was not possible to illuminate the dorsal retina alone as the fish oriented themselves nose-downwards, facing the light source.

Trout plasma MCH concentrations varied with a distinct diurnal rhythm. Values were low during the dark (<70pg/ml) but rose with light onset to reach a peak (200-300pg/ml) at about midday, before declining prior to dark onset. This rhythm could be manipulated by changing the photoperiod regime, but did not persist under conditions of continuous darkness.

The work investigated the potential influence of MCH, acting alone or in conjunction with catecholamines, on the secretion of melatonin by the isolated pineal gland. Melatonin secretion by illuminated pineals was low (< 1ng/ml/pineal/h) but increased rapidly in darkness (>10ng/ml/pineal/h). Melatonin production appears to be mediated by cAMP since it could be enhanced by forskolin. It is stimulated by 10^{-5}M dopamine, while the dopamine receptor blocker, haloperidol (10^{-4}M), blocked the effects of exogenous dopamine and partially blocked the response to darkness. Norepinephrine (10^{-4}M), but not specific adrenergic agonists, will also enhance melatonin production, apparently acting on dopaminergic receptors, since this effect could be blocked by haloperidol. α-MSH, VIP, ACh and GABA did not appear to affect pineal melatonin release; neither did MCH (alone or in conjunction with norepinephrine) reliably influence melatonin secretion.

Autoradiographic studies of the trout brain showed di-iodo-melatonin binding sites in the area thalamica lateralis and the optic tectum. Binding was observed also in the retina and on melanophores located in the meninges and on trout scales. No iodo-melatonin binding was observed in the pineal itself. The overall implications of the results are discussed.

ABBREVIATIONS

AChE - Acetylcholinesterase
ACTH - Adreno-corticotrophic hormone
Athl - Anterior thalamus lateralis
B - label bound to antibody in presence of unlabelled peptide
B_o - label bound to antibody in absence of unlabelled peptide
BSA - Bovine serum albumen
BST - British summer time
$CaCl_2$ - Calcium chloride
cAMP - Cyclic adenylate monophosphate
cm - centimetre
CNS - Central nervous system
cpm - counts per minute
CRF - Corticotrophin releasing factor
°C - degrees centigrade
Ci - Curie
µCi - micro Curie
DA - dopamine
DD - constant darkness
dpm - disintegrations per second
EDTA - Ethylene-diamine-tetra-acetic acid
eg. - for example
et al. - and others
Fig. - Figure
g - grammes
mg - milligrammes
µg - microgrammes
ng - nanogrammes
pg - picogrammes
x g - force due to gravity
GABA - Gamma-amino butyric acid
cGMP - cyclic guanine monophosphate
h - hour
^3H - Tritium
HCl - hydrochloric acid
HIOMT - Hydroxyindole-O-methyltransferase
HPLC - High perfomance liquid chromatography
HPA - Hypothalamo-Pituitary-Adrenal
^{125}-I - radioactive iodine
KIU - Killicrein inhibitory units
l - litre
ml - millilitre
µl - microlitre
LD - light/dark cycle
LL - constant light
M - Molar
mM - millimolar
µM - micromolar
nM - nanomolar
pM - picomolar
MCH - Melanin-concentrating hormone

rMCH - Rat melanin-concentrating hormone
sMCH - Salmon melanin-concentrating hormone
ME - Median eminence
MEM - Minimal essential medium
min - minute
α-MSH - Alpha-melanocyte-stimulating hormone
MT - Melatonin
n - number of individuals in experimental group
nm - nanaometer
NAT - N-acetyltransferase
NE - Norepinephrine
NIL - Neurointermediate lobe of pituitary
NLT - Nucleus lateralis tuberis
NSB - Non-specific binding
PBS - Phosphate-buffered saline
PBSG - Phosphate-buffered saline with gelatine
PD - Pars distalis
% - percent
RIA - radioimmunoassay
s - seconds
SCG - superior cervical ganglion
SCN - suprachiasmatic nucleus
vSCN - visual suprachiasmatic nucleus
TFA - Trifluoroacetic acid
TLC - Thin layer chromatography
VIP - vasointestinal polypeptide
v:v - volume : volume
w:v - weight : volume

CHAPTER ONE

GENERAL INTRODUCTION

This thesis concerns the control of melanin-concentrating hormone (MCH) release in rainbow trout, Onchorhynchus mykiss (formerly, Salmo gairdneri, R.). MCH is a novel vertebrate neuropeptide of hypothalamic origin which was first discovered through its effects on integumentary colour change responses in teleosts in which it is a neurohypophyseal hormone; but is now supposed also to have central effects in the brains of other vertebrates, in which MCH has a wider distribution throughout the brain. Various comparative studies suggest that the role of MCH as a colour change hormone is an evolutionary novelty in fish.

In trout, MCH is released in response to the perception of a pale background. It is likely, therefore, that in this group of vertebrates, MCH release is either directly, or indirectly controlled by the retina and this thesis therefore briefly examines the effect of illuminating different regions of the retina. Although background colour influences the release of MCH, another section of this work examines the level of release at different times of day, and presents evidence for a strong diurnal and seasonal effect on circulating levels of MCH. Recent observations (Naito *et al.*, 1985) raise the possibility that the pineal gland might also be involved in the control of MCH secretion. Alternatively, it is possible that MCH might act as a neuromodulator to alter pineal activity,

Investigations of the possible influence of circulating MCH on the release of melatonin necessitated a study of pineal control which directed research away from MCH itself, and more towards the pineal.

To appreciate some of the problems relating to MCH that have been explored in this thesis, a brief outline of what is known about MCH and the gaps in our knowledge will be described.

1.1 The discovery of MCH

The control of integumentary colour change responses in lower vertebrates has been investigated for many years. Animals are able to adapt the background colour of their environment through the movement of melanin granules (melanosomes) within melanophores (pigment cells). On a white background, melanosomes aggregate towards the centre of the melanophore, so that the animals appear pale in colour, while on a dark background, they disperse throughout the cell giving a dark appearance. In teleosts, such integumentary colour change is generally achieved by both nerves and hormones, their relative importance being species dependent (Pickford and Atz, 1957). Melanocyte-stimulating hormone (α-MSH) is the main hormone responsible for melanophore dispersal and integumentary darkening. It is of hypophyseal origin and is a 13-residue peptide, derived from pro-opiomelanocortin (POMC), together with ACTH, β-endorphin, β-lipotrophin, and CLIP. For a long time it was believed that background adaptation could be explained exclusively in terms of the levels of α-MSH secretion from the pars intermedia of the pituitary, since in amphibia and elasmobranchs, hypophysectomy led to

melanosome aggregation, while injection of pituitary extracts caused melanosome dispersion.

The idea that body coloration was under dual hormonal control, was first proposed by Hogben and Slome (1931). Lesions in the pars tuberalis of the amphibian pituitary resulted in intense body darkening which suggested the existence of a paling hormone in this region. It was later shown, however, that this body darkening was due to the nervous dis-inhibition of α-MSH release (Etkin, 1962) and as no further evidence for a pituitary paling hormone was forthcoming in this group of vertebrates, the concept of an amphibian paling agent was subsequently abandoned.

The situation in teleosts was somewhat different. The first good experimental evidence for a pituitary paling agent was provided by Healey (1948) in the minnow, Phoxinus laevis who showed that after removing the sympathetic nervous control to melanophores, fish could still pale if part of the pituitary remained. Later, Enami (1955) showed that the pituitary of the silurid catfish, Parasilurus asotus also contained a paling agent which was not a catecholamine, and since hypothalamic extracts also contained a paling agent, he suggested that it might originate from the hypothalamus. Over the following years, interest in the existence of a paling hormone waned because of the lack of a sensitive MCH bioassay, variable interspecific responses of teleost melanophores to pituitary extracts, and the lack of any detectable MCH-bioactivity in the pituitaries of other vertebrate species. Yet, 20 years later, Baker

and Ball (1975) finally proved the presence of a teleostean hormonal paling agent (MCH) in the pituitary of the molly, Poecilia latipinna. After severing local nervous connections to the melanophores, fish became pale when placed on a white background, unless the pituitary was removed. Subsequent evidence (Rance and Baker, 1979), suggested that the paling agent was a neurohypophyseal hormone, synthesised by neurones in the hypothalamus and stored in the pars nervosa (PN) of the pituitary before being released into the circulation. This has now been confirmed for a number of teleost species.

In fish, the pituitary concentrations of MCH change with adaptation to different coloured backgrounds: transfer of long-term white-adapted fish to a black background results in a significant increase of immunoreactive MCH (irMCH) content (Barber *et al.*, 1987; Powell and Baker, 1988). Conversely, pituitary MCH content may decrease significantly after transfer from a black to a white background. These changes have been interpreted as the release of MCH in response to retinal perception of a pale background colour; on a black background secretion is inhibited but synthesis continues, resulting in an accumulation of MCH stores within the pituitary.

The purification and chemical characterization of salmonid MCH (Kawauchi *et al.*, 1983) permitted the manufacture of synthetic salmonid MCH. Intra-peritoneal implantation of slow-release capsules containing synthetic MCH (Baker *et al*, 1986), and intra-peritoneal injection (Kawauchi *et al*, 1986) of MCH in black-adapted trout, confirmed the role of MCH as an antagonistic hormone to α-MSH *in*

vivo. Recently a solid-phase radioimmunoassay has been developed to measure MCH in trout plasma (Eberle *et al*, 1989; Kishida *et al*, 1989). Plasma MCH concentrations are higher in long-term white- than black-adapted trout and titres increase after transferring fish from black to white backgrounds.

1.2 The localization of MCH

Teleosts

In order to investigate how MCH secretion is controlled, it is useful to know where the MCH cells are localized within the teleostean brain, and thus which other neurone types might be involved. Baker and Rance (1983) provided the first information about the distribution of MCH neurones in trout by mapping the distribution of MCH bioactivity in frozen brain sections. They found that although the majority of bioactive MCH in the brain was found in the ventral third of the hypothalamus, up to 30% was found in the dorsal hypothalamus. Following the characterization of MCH (Kawauchi *et al.*, 1983), specific antisera were raised to map the distribution of immunoreactive MCH neurones within the brain and pituitary of a variety of vertebrates. In salmonids, (Naito *et al.*, 1985; Bird *et al.*, 1989), carp (Bird *et al*, 1989) and molly (Batten and Baker, 1988) MCH nerve cell bodies are located in the nucleus lateralis tuberis (NLT) near the ventral floor of the basal hypothalamus, though a small number of cell bodies are also found scattered dorsolaterally in the caudal part of the NLT. The majority of MCH-positive axons arising from these cell bodies project ventrally into

the pituitary. There, they can be traced next to blood vessels and secretory cells of the pars nervosa (PN) and pars intermedia (PI). A few fibres are also found near the corticotrophs in the pars distalis (PD) though no MCH-immunoreactivity is found within any pituitary cells. Such observations suggest that MCH might also act in a paracrine manner to influence the secretory activity of both melanotrophs and corticotrophs. In trout, and to a lesser extent in carp, some prominent but less numerous MCH-positive fibres also pass caudo-dorsally through the hypothalamus to the pretectal area where they are found lateral to the posterior commissure (Naito *et al.*, 1985; Bird *et al.*, 1989). A few MCH-like fibres have also been traced to the preoptic area of the anterior hypothalamus in trout (Naito *et al*, 1985), while MCH perikarya have also been found in the preoptic area in the molly, Poecilia, (Batten and Baker, 1988).

It is not yet known whether these dorsally-projecting MCH fibres arise from the same perikarya as those whose fibres extend into the pituitary. Nor is it clear whether MCH release occurs simultaneously at both sites. Preliminary work (Green *et al.*, 1991) suggests that both ascending and descending axons may arise from the same cell bodies, since MCH tissue concentrations in both ventral and dorsal hypothalamus were reduced when fish were transferred from black to white backgrounds or during stress. However, this does not necessarily mean that the two populations of fibres are functionally integrated. Naito and co-workers (1985) recently postulated that the MCH axons which extend to the pretectal region might be involved with the regulation of MCH release. In trout, this area of the brain also

receives axonal projections from part of the optic nerve (Pinganaud and Clairambault, 1979) and from the pineal (Hafeez and Zerihun, 1974). Naito's group therefore suggested that these fibres might communicate with each other such that MCH release might be controlled (directly or indirectly) by either or both of these photosensory organs. This raised the question of how light mediates the release of MCH in rainbow trout.

Ultrastructural studies have shown that in teleosts, MCH is contained within oval neurosecretory granules (Powell and Baker, 1987; Batten and Baker, 1988). Unlike the trout (Salmo) or the eel (Anguilla), approximately 40% of the MCH-positive neurones in the neurointermediate lobe of the Chinese grass carp (Ctenopharyngdon) also appear to show α-MSH-like immunoreactivity (Powell and Baker, 1987). These double-staining neurones apparently project into the pituitary rather than projecting dorsally into the brain. Furthermore, they do not show any cross-reactivity with antisera for other POMC-derived peptides (ACTH or salmonid N-terminal POMC peptide: NPP). This suggested that the α-MSH-like immunoreactivity does not represent the presence of true α-MSH but may result from antibody cross-reactivity with an epitope of the MCH precursor and this idea has been confirmed by subsequent analysis of the MCH precursor for rats (Nahon *et al.*, 1989a). This precursor may be cleaved differently in the ventrally- and dorsally-projecting neurones to reveal the α-MSH-like epitope or not (Powell and Baker, 1987).

Localization of MCH in other vertebrates

Neurones immunostaining with salmonid MCH (sMCH) antisera have also been demonstrated within the brains of all other classes of vertebrates so far examined: elasmobranchs (Vallarino *et al.*, 1989), amphibia (Andersen *et al*, 1986, 1987), rats (Skofitsch *et al*, 1985; Naito *et al*, 1986; Zamir *et al*, 1986), pigs, guineapigs and humans (Sekiya *et al*, 1988; Bresson *et al*, 1989), where it is thought to act as a neuromodulator, neurotransmitter or hypophyseal regulatory peptide. All vertebrate hypothalami seem to contain MCH perikarya. Unlike the situation in teleosts, however, where the MCH-like cell bodies are restricted to the ventral floor of the hypothalamus, they are generally more widely dispersed in other vertebrates. Moreover, the MCH-like perikarya are often clustered in several groups, as in the dogfish, Scyliorhinus canicula, (Vallarino *et al.*, 1989) and the amphibian, R. ridibunda (Andersen *et al.*, 1987), although this is not necessarily true for other amphibians such as Necturus, R. temporaria and Xenopus (review: Baker, 1988b). MCH-like perikarya have also been observed in the dogfish pars distalis. As in the carp, some of the MCH-like neurones in the hypothalami of dogfish (Vallarino *et al.*, 1989), frogs (Andersen *et al.*, 1987), and rats (Naito *et al.*, 1986; Pelletier *et al.*, 1987) also immunostain with α-MSH antisera, but not with those raised against other POMC-derived peptides. It therefore seems likely that this α-MSH-like immunoreactivity represents cross-reactivity with an α-MSH-like epitope of the MCH precursor, rather than co-localization with true α-MSH. In most vertebrates, immunoreactive MCH fibres do not predominantly course towards the pituitary (as in fish) but extend from the hypothalamic cell bodies

to many other regions of the brain. In rats, fibres extend to the fore- mid- and hindbrain, and to the dorsal horn of the spinal chord (Skofitsch *et al.*, 1985; Naito *et al.*, 1986). Some MCH-like fibres can, however, be traced to the median eminence of the frog, R. ridibunda (Andersen *et al.*, 1987). The widespread distribution of immunoreactive MCH neurones suggests that MCH may be involved in several different functions. These central effects of MCH may be more important than its pigmentary role, since the latter is confined to teleosts, and therefore probably represents an evolutionary specialization of this vertebrate group.

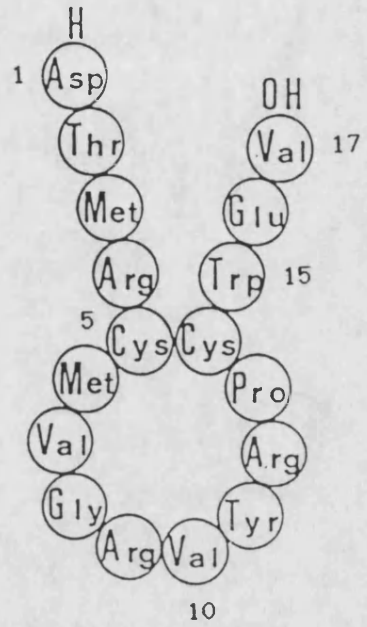
1.3 The structure of MCH and its precursor

Melanin-concentrating hormone was first characterized from chum salmon, Onchorhynchus keta, pituitaries (Kawauchi *et al*, 1983) and found to be a cyclic heptadecapeptide, with a disulphide bridge between two cysteine residues at positions 5 and 14 (Fig. 1.1). The structure was later confirmed by peptide synthesis (Okamoto *et al.*, 1984; Wilkes *et al*, 1984b; Eberle *et al.*, 1986). MCH does not resemble any other known molecule, except for a slight resemblance to the cyclic carboxy-terminal disulphide loop of salmonid prolactin (Kawauchi *et al*, 1983). The MCH-like bioactivity of this isolated C-terminal region is, however, only about 0.04% of sMCH, while that of intact prolactin is undetectable.

The molecular structures of MCH has since been elucidated for other teleost species. The structure of MCH in the marine fish, bonito (Katsuwonus pelamis) is identical to that of chum salmon, while the

FIGURE 1.1

THE STRUCTURE OF SALMONID MCH



PRIMARY STRUCTURE OF MCH IN DIFFERENT SPECIES

Salmonids and Bonito

H-Asp. Thr. Met. Arg. Cys. Met. Val. Gly. Arg. Val. Tyr. Arg. Pro. Cys. Trp. Glu. Val-OH

Eel

H-Asn. Thr. Met. Arg. Cys. Met. Val. Gly. Arg. Val. Tyr. Arg. Pro. Cys. Trp. Glu. Val-OH

Rat and Human

H-Asp. Phe. Asp. Met. Leu. Arg. Cys. Met. Leu. Gly. Arg. Val. Tyr. Arg. Pro. Cys. Trp. Gln. Val-OH

eel, Anguilla japonica, has one substitution of an asparagine for the aspartic acid residue at the N-terminal (Kawauchi, 1989). Such work suggests that the structure of MCH has been highly conserved through teleostean evolution. Rat MCH has also been isolated and characterized (Vaughan *et al*, 1989) and only differs from salmonid MCH by an N-terminal extension of two amino acids and four additional substitions. The amino acid sequence between the two cysteine residues appears to have been highly conserved since there is only one conservative amino acid substitution in this part of the molecule. Human MCH is identical to rat MCH (Presse *et al.*, 1990).

In 1987, Kawazoe and co-workers (1987b) reported that both MCH bioactivity and immunoreactivity increased if large molecular weight molecules from hypothalamic extracts were digested with lysylendopeptidase. This suggested that, like many other peptides, MCH was cleaved from a large molecular weight precursor. Working independently, two groups (Ono *et al*, 1988; Minth *et al*, 1989) isolated MCH cDNA clones from two species of salmon: Onchorhynchus keta and O. tshawytscha, respectively. They each found two different MCH cDNA sequences: cDNA MCH-1 and MCH-2, thus supporting the suggestion that salmonids are tetraploid (Ohno *et al.*, 1968). Each cDNA sequence coded for a 132 amino acid precursor sequence in which MCH was found at the C-terminus. Both MCH genes were later isolated from chum salmon genomic DNA and shown to be intronless sequences (Takayama *et al*, 1990). The cDNA sequences from the two salmonid species are virtually identical (coding for only five amino acid substitutions) suggesting that species separation may be a relatively

recent event. Tetraploidy may have occurred much earlier, since there is significantly more variations between MCH-1 and MCH-2. In chum salmon, the nucleotide sequence homology between cDNA MCH-1 and MCH-2 was found to be 86%, with an 80% homology between the two amino acid precursor sequences (Ono *et al.*, 1988), while chinook salmon cDNA MCH-1 and MCH-2 sequences showed an 80% homology and an 81% homology at the amino acid level (equivalent to 107/132 identities) (Minth *et al.*, 1989). The last 17 amino acid residues at the C-terminus of both MCH precursors code for identical MCHs, while the neighbouring sequence (residues 101-113), which is separated from MCH and the N-terminus by classical dibasic amino acid cleavage sites, shows only two amino acid substitutions. This second sequence has been termed MCH-gene-related peptide (Mgrp) (Fig. 1.2). Most of the differences between the two precursors reflect single base changes in the DNA sequences and occur towards the N-terminus. Several potential cleavage sites have been located along the precursor but which of these are actually used has not yet been established.

Bird and co-workers (1990) isolated MCH and its precursor fragments by SDS polyacrylamide gel electrophoresis and Sephadex G-75 chromatography, following biosynthetic isotope labelling of the proMCH molecule with either ^{3}H-leucine or ^{35}S-methionine. They separated five labelled MCH-molecules. This work suggested that the MCH precursor is cleaved to MCH via several intermediates as it is transported to the pituitary and stored in the NIL. Bird and co-workers (1990) suggested that the MCH precursor (proMCH) might be initially cleaved at the first Arg Arg cleavage site, prior to the

FIGURE 1.2

Comparison of salmon, rat and human preproMCH sequences

: amino acid identities
. amino acid substitutions
▼ potential cleavage sites

Mgrp sequence, to liberate residues 101-132 before being further cleaved at another such cleavage site to liberate MCH. The biological significance of the consequent release of the tridecapeptide Mgrp fragment (residues 101-113) is not yet known. In the chinook salmon, one of the MCH mRNA sequences has only a single Arg instead of the dibasic Arg Arg cleavage site prior Mgrp, and in this case, Mgrp may not be liberated (Minth *et al.*, 1989).

Recently, the MCH cDNA sequences of rats and humans have also been characterized (Nahon *et al*, 1989a; Presse *et al*, 1990). Both rat and salmon cDNA sequences are similar to human 7SL RNA which targets proteins to the endoplasmic reticulum but no functional significance for this has been suggested (Nahon *et al*, 1989b); it may reflect ancestral relatedness. The rat and human MCH precursors are very similar to one another but only show a 28% identity with salmonid preproMCH (equivalent to 54% nucleotide similarities). Rat and salmonid preproMCH sequences do, however, show several homologous regions, particularly towards the C-terminus which contains MCH. Upstream of MCH there are also two other putative peptides which could be cleaved from the molecule: NEI (neuropeptide (N) -glutamic acid (E) -isoleucine (I), residues 132-144) which is thought to be homologous to salmonid Mgrp, and NGE (neuropeptide (N) -glycine (G) -glutamic acid (E), residues 110-128). Rat and human preproMCH are identical between residues 114-165 (Fig. 1.2). These peptides, NEI and NGE, also share some sequence homology with α-MSH, CRF and GRF. It was suggested (Nahon *et al*, 1989a) that amidated NEI (or Mgrp) might be responsible for the cross-reactivity to α-MSH antisera that

is sometimes observed with MCH-positive neurones but not with antisera raised against other POMC-derived peptides. As α-MSH immunoreactivity is only observed in some MCH neurones, this would suggest that the fragments are processed differently within different neurones. The lack of any such reaction in the eel, trout or human may indicate a lack of amidation of this fragment.

1.4 The Pigmentary role of MCH in teleosts

Unlike other vertebrate classes, teleosts have adapted MCH for use as a melanotropin during adaptation to pale backgrounds. MCH is antagonistic to α-MSH action at the melanophore level and to α-MSH release at the pituitary level. Teleost melanophores from all species show pigment aggregation in response to MCH *in vitro* (Wilkes *et al.*, 1984a, b; Baker *et al.*, 1985a; Oshima *et al.*, 1985; Fujii and Oshima, 1986; Nagai *et al.*, 1986, Baker 1988a). Melanosome aggregation can normally be observed with MCH concentrations ranging from 10^{-9} to 10^{-12}M, although the concentration of MCH required to elicit half maximal pallor (melanophore index = 3), termed the EC_{50}, varies with different teleost species. For example, melanophores of both the grass carp, Ctenopharyngdon (EC_{50} = 6 x 10^{-11}M) (Baker *et al.*, 1985a), and the Amazonian eel, Synbranchus (EC_{50}= 1 x 10^{-11}M), are more sensitive to sMCH than those of rainbow trout, Salmo (EC_{50}= 3 x 10^{-10}M) (Baker, 1988a). In trout, the EC_{50} of melanophore response to sMCH *in vitro* (3 x 10^{-10}M) is higher than the concentration of circulating MCH (about 50pM = 5 x 10^{-11}M). Green and Baker (1989) have shown that in this species, MCH will synergise with norepinephrine (NE) *in vitro* to induce full pallor. They suggest that

this may occur under physiological conditions since denervated trout melanophores will not show melanosome aggregation in response to circulating MCH. Synergism between MCH and NE was not observed in the carp but this may be associated with the fact that their melanophores are more sensitive to MCH. The plasma MCH concentration in this species is not yet known.

The action of MCH on teleost melanophores *in vitro* is antagonistic to that of α-MSH (Fujii and Oshima, 1986; Baker, 1988b). This antagonism is mutual at concentrations of less than 10^{-9}M on scales of Salmo, Anguilla, and Ctenopharyngdon (Baker, 1988b) but the extent to which each hormone is involved in melanophore control also varies with species. Of these three species studied, trout showed a steeper dose-response curve to MCH than to α-MSH; in Chinese grass carp the opposite was true and in the eel, both curves were shallow. Hence, the hormone secretion strategy required in order to achieve certain colour changes must vary with the species involved. Structure activity studies (Baker *et al.*, 1985a; Hadley *et al.*, 1987; Kawazoe *et al.*, 1987a; Baker *et al.*, 1990) have shown that the presence of the disulphide bridge is important for maintaining high bioactivity. The three $Arg^{(4,9,12)}$ and the Tyr^{11} residues are also required for full biological activity (Figure 1.1), while modification of Trp^{15} or oxidation of Met^{9} or Met^{6} only slightly reduces biological activity in the Amazonian eel (Kawazoe *et al.*, 1987a). On the other hand, studies by Matsunaga and co-workers (1989) suggested that Trp^{15} was crucial for maintaining full bioactivity on Synbranchus melanophores. The same workers also claim that MCH_{5-15} is the minimal fragment with

full potency: modifications which change the ring shape, such as iodination of Tyr^{11} or replacement of Gly^{9} with Ala cause a marked depression of biological activity (Baker *et al.*, 1990).

Melanin-concentrating hormone probably acts through its own specific receptors on teleostean melanophores. *In vitro*, MCH will induce pallor in the presence of phentolamine (α-adrenergic receptor blocker) and/or propanolol (β-adrenoceptor blocker) showing that MCH does not act via either of these receptors (Rance and Baker, 1979). Moreover, unlike the α-MSH receptor (Sawyer *et al*, 1983; Fujii and Oshima, 1986), MCH-receptor activation is not calcium dependent (Oshima *et al*, 1986; Castrucci *et al*, 1989) and may even be enhanced in its absence. MCH receptor-activation is thought to stimulate the production of diacylglycerol (DAG) in Synbranchus (Abrao *et al.*, 1989), while α-MSH receptor-activation stimulates cAMP production (Eberle, 1988). Recent evidence suggests that α-MSH antagonises the action of MCH through such an increase in cAMP, since forskolin (a cyclic AMP stimulator) antagonises the response of teleost melanophores to MCH *in vitro* (Baker, 1988a).

There is some evidence that MCH may itself activate the α-MSH receptor even though these two peptides show no similarity in their primary structure. At pharmacological doses (10^{-7} to 10^{-5}M), MCH exhibits melanosome-dispersing properties on isolated Synbranchus melanophores (Castrucci *et al.*, 1987). This effect is similar to the weak α-MSH-like activity sometimes induced by MCH in tetrapod melanocytes (Wilkes *et al*, 1984b; Baker *et al.*, 1985a). Such 'auto-

antagonism' is, however, abolished in the absence of calcium (Castrucci *et al*, 1989; Lebl *et al*, 1989) which suggests that at supra-physiological doses, MCH interacts with α-MSH receptors.

In addition to its antagonistic action to α-MSH on teleostean melanophores, circulating MCH is able to depress the release of α-MSH from melanotrophs in the pituitary. This has been demonstrated both *in vivo* and *in vitro*. Implantation of slow release capsules containing MCH in black-adapted trout (Baker *et al.*, 1986) depressed circulating α-MSH concentrations. A reduced rate of α-MSH synthesis and release was confirmed through cytological observation of the melanotrophs in the pars intermedia of these fish. Chronic administration of MCH also resulted in reduced melanogenesis within the melanophores. Melanogenesis is generally associated with changes in α-MSH secretion (Wilson and Dodd, 1973; van Eys and Peters, 1981; Rodrigues and Sumpter, 1984) so its depression could be a reflection of reduced levels of circulating α-MSH rather than a direct effect of MCH on the melanophores. *In vitro*, MCH directly modulates the secretory activity of melanotrophs (Barber *et al*, 1987). Thus, when trout or eel neurointermediate lobes were incubated with MCH antiserum, the release of α-MSH was considerably enhanced, suggesting that MCH normally suppresses MSH release in these species, in conjunction with other agents such as dopamine. These paracrine actions of MCH are not unexpected in light of the close anatomical localization of the MCH neurosecretory nerve terminals and the melanotrophs of the pituitary (Naito *et al*, 1985).

1.5 The role of MCH as a modulator of stress responses

MCH fibres also terminate near corticotrophs in the pars distalis (Naito *et al.*, 1985; Powell and Baker, 1987) which release another bioactive POMC-derived peptide; ACTH, released under conditions of stress. MCH seems to modulate stress responses in teleosts and therefore may be of some importance from a practical, aquacultural viewpoint. Moreover, recent evidence suggests that the release of MCH may itself be modulated by stress so experiments must be designed with care.

The importance of MCH as a modulator of stress responses in teleosts was first noted by Baker and Rance (1981). They found that white-adapted fish (with high circulating levels of MCH) responded to chronic stress by secreting less cortisol than black-adapted fish. White-adapted fish also released significantly lower levels of cortisol in response to acute stress (Gilham and Baker, 1985). Although similar observations could not be repeated in another laboratory (Pickering *et al.*, 1986), this might reflect differences in the levels of stress used in the two studies. These observations imply that high plasma MCH concentrations reduce cortisol secretion, but direct application of MCH to trout interrenals *in vitro* does not alter cortisol release (Green *et al.*, 1991). The spontaneous release of bioactive ACTH following stress was also found to be lower in incubated trout pituitaries from white- than from black-adapted trout (Baker *et al.*, 1985b). In addition, ACTH from incubated trout pars distales was lower in fish implanted with MCH capsules (Baker *et al.*, 1986). These experiments therefore suggested that MCH can depress

ACTH release, acting either directly on the corticotrophs or indirectly by inhibiting CRF release from the hypothalamus. Baker and co-workers (1985b) proposed that MCH directly inhibited ACTH release, since ACTH could be inhibited *in vitro* in a dose-dependent manner with concentrations of MCH ranging from 10^{-10}M to 10^{-7}M. The same workers also showed that salmonid MCH will inhibit corticotrophin factor (CRF)-induced ACTH release from trout pituitaries *in vitro*. Recent work (Green *et al.*, 1991) suggests that MCH can also depress the release of CRF bioactive material from trout hypothalami in response to stress. Incubated hypothalami from white-reared fish released significantly more CRF-like bioactivity than those from black-reared fish when MCH antiserum was added to the incubation medium. *In vivo*, however, such apparent hypothalamo-corticotrophic modulation by MCH may only occur under conditions of stress. The question arises whether the effect of MCH on the hypothalamo-pituitary system is peculiar to teleosts or might apply to other vertebrates. Baker and co-workers (1985b) found that their preparation of salmonid MCH (sMCH) could depress the release of CRF-stimulated ACTH from isolated rat pituitary fragments, although significantly higher concentrations of salmonid MCH were required than in fish. This was initially thought to reflect species differences between the structure of MCH and its receptors. However, subsequent investigations have failed to show any modulatory effect of rat MCH on either CRF release from the rat hypothalamus, or ACTH release from the anterior pituitary (Navarra *et al.*, 1990). The involvement of MCH in the stress response of vertebrates generally remains enigmatic.

The development of a solid-phase radioimmunoassay for measuring plasma MCH concentrations in trout allowed Green and co-workers (1991) to investigate the relationship between plasma MCH titres and stress *in vivo*. Green (1990) found that acute stress, such as a single intra-peritoneal injection of saline, increased plasma cortisol concentrations but not MCH titres. However, when this stress was repeated over a number of days, plasma MCH concentrations increased two to nine-fold, while plasma cortisol titres remained high. In addition, Green showed that such increments in MCH concentrations are independent of the time of day at which the stress is applied. Trout reared on either white or black backgrounds also showed significantly reduced hypothalamic and pituitary hormone stores following both mild handling stress and repeated injection stress, which suggests that MCH is released into the circulation following stress and depressed by dexamethasone. Thus, the role of MCH as a modulator of the stress response may be fundamentally important in the negative feed-back control of MCH release.

1.6 Other possible actions of MCH in vertebrates

The widespread distribution of MCH within the brains of other vertebrates suggests that it may act as a neuromodulator in a number of different functions. It has been suggested (Zamir *et al.*, 1986) that in rats, MCH may be involved in regulating food and water intake and thus electrolyte balance, since rats given 2% saline instead of drinking water for five days showed significantly increased MCH concentrations in both the lateral hypothalamus and in the pituitary gland. In male rats, intravenous injections of salmonid MCH was also

shown to stimulate growth hormone release (Kawauchi *et al*, 1986). Behavioural responses are also modulated by MCH in the rat: intracranial MCH administration reduced the α-MSH-induced grooming response, but not ACTH-induced grooming behaviour (de Graan *et al*, unpublished; cf. Eberle, 1988). This suggests that these two types of grooming behaviour are regulated differently, and implies that MCH may antagonize some effects of neuronal α-MSH in rats.

In conclusion, little is yet known about the control of MCH release, either in terms of environmental conditions or neural/hormonal regulation. Since MCH is a general vertebrate neuropeptide, such an understanding is likely to be relevant to all other vertebrates. Fish have evolved MCH for use as a hormone so its release can be more easily monitored than can the release of MCH from neurones in the brains of other vertebrates, where it presumably has a neuromodulatory role. This does not, however, preclude the possibility of its involvement as a neuromodulator in the teleost brain. The present investigations arose from an interest in the influence of the retina and the pineal on MCH secretion.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 ANIMALS

Rainbow trout, Onchorhynchus mykiss (formerly Salmo gairdneri R.), weighing between 150-250g, were obtained from a local fish farm (Alderley Trout Ltd., Wotton-under-Edge, Glos.) where they were maintained on natural photoperiod in outdoor ponds. Once in the University aquarium, they were housed (in groups of 12 or less) in white or black coloured 250l tanks, with continuously flowing (100 ml/min), aerated tap water. Trout were acclimated to aquarium conditions under various lighting regimes, during which time they were not fed. The temperature was maintained at 10±1 °C.

Illumination

The aquarium was illuminated with fluorescent strip lighting (Osram L58W/23, Germany). Incident light intensity was measured in the aquarium, at the surface and up to 30 cm under the water of both white and black tanks, and outside.

Conditions	Incident Light	White tank	Black tank
Without lid	- at water surface	135 lux	135 lux
	- 5 cm below surface	130 lux	130 lux
	- 10 cm below surface	125 lux	95 lux
	- 30 cm below surface	125 lux	75 lux
	- reflected light	14 lux	4 lux

Photoperiod

In the aquarium, fish were acclimated to photoregimes which resembled those outside at the time of collection. Natural photoperiod was estimated from Whitaker's Almanack (1987-1990). Environmental light intensity, measured over the twilight periods for several days (Fig. 2.1), was less than 100 lux at sunrise and sunset.

Aquarium illumination differed from natural conditions in several respects:

1. Light intensity was constant throughout the photophase.
2. The wavelengths of light emitted did not change throughout the light period.
3. There was an instant on/off change in illumination which may have acted as a stressor (Reiter, 1988). Dimmer switches at light onset and offset would have been an improvement.
4. Once the photoperiod was set, the daylength was constant and did not show daily fluctuations.

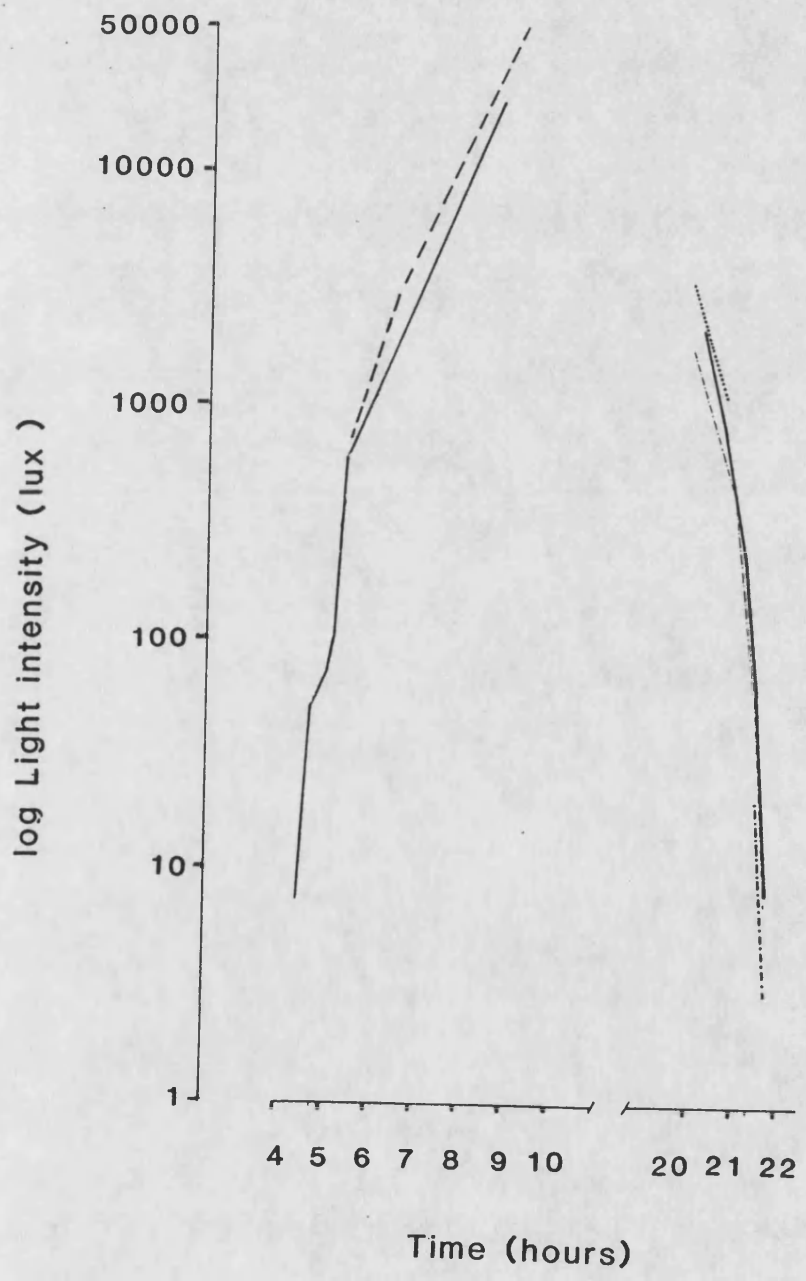
2.2 COLLECTION OF BLOOD

Fish taken from one tank were caught in a single sweep of the net and anaesthetised with a lethal dose of benzocaine (Ethyl-P-aminobenzoate, initially made up as a 2.8% solution in ethanol before diluting to a final concentration of 1:2000 w:v in water) or phenoxyethanol (1:1667 v:v). The type on anaesthetic used did not affect MCH release as fish were bled within 5 min. of anaesthesis (Green, 1990). Blood was collected from the caudal peduncle into chilled 4 ml polypropylene

FIGURE 2.1

ENVIRONMENTAL LIGHT INTENSITY (LUX) MEASURED OVER DAWN AND DUSK FOR SEVERAL DAYS IN SUMMER.

Sunrise was at 04:50 and sunset at 21:25 (Whitaker's Almanack, 1988). Days on which the measurements were taken: ___ (2 June); – – – (5 June); ..... (7 June); -.-.-.- (3 June); -------- (4 June)



tubes containing 50µl 0.2M Na_2EDTA as anticoagulant and 20-50 µl Aprotinin (equivalent to 5000 KIU) as a general protease inhibitor.

Blood samples were centrifuged at 2000g for 10 min at 4 °C. Plasma was aliquotted in appropriate volumes into 4 ml polypropylene tubes, snap frozen at -80 °C and stored at -20 °C. Samples were assayed for MCH, MSH and/or cortisol as soon as possible after death.

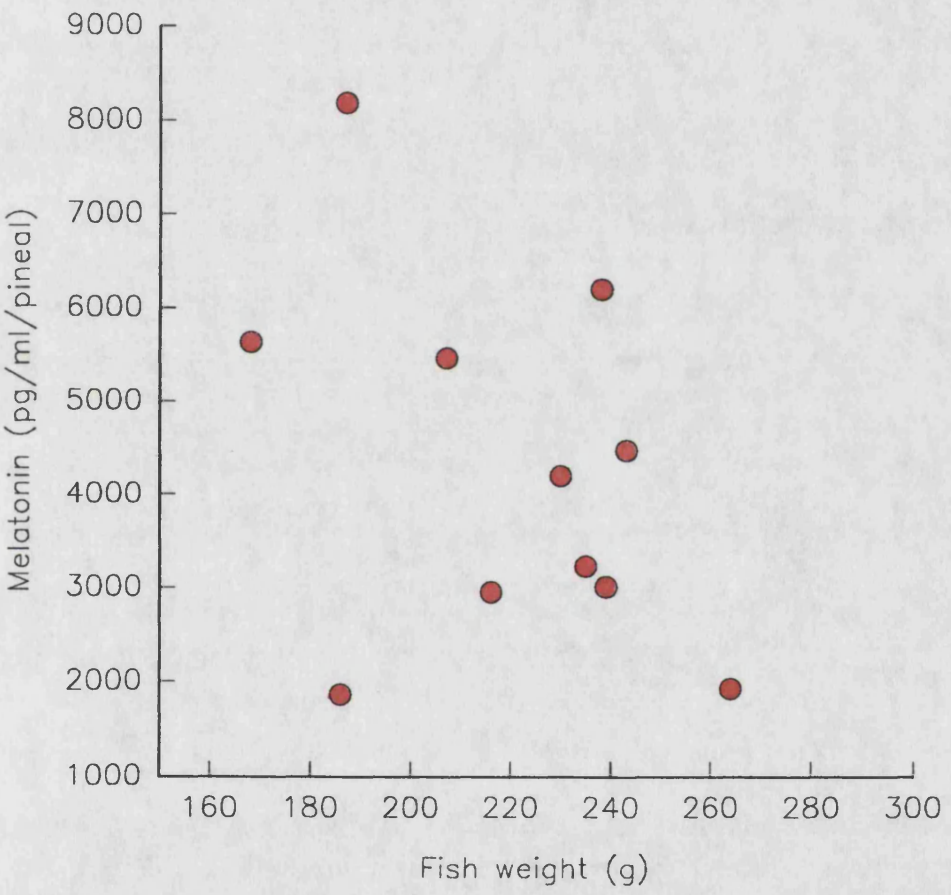
2.3 IN VITRO INCUBATION OF TROUT PINEALS

During the last half of the light period, small trout (each weighing about 150 g) were decapitated and their pineals dissected. Small fish were used because their pineals were easier to remove than those of larger trout. Furthermore, no correlation was found between fish weight and pineal activity (Fig 2.2). The pineals were placed individually in sterile well plates containing 1 ml Eagle's Minimum Essential Medium, modified with Hanks salts and 20 mM Hepes buffer without glutamine or sodium bicarbonate (MEM) (Flow Laboratories, Irvine, Scotland).

Incubations were done at 10-11 °C. Well plates were placed in sealed plastic bags, then gassed with oxygen and gently aggitated on a rotator. After pre-incubating for at least two hours in the light, the medium was replaced, and the pineals left to incubate for a further hour (basal incubation period). Addition of potential stimulators/inhibitors of melatonin secretion, with changes of medium in each subsequent hour, was done when pineals were incubated in the light or dark. During the dark period, pineal incubation and medium

FIGURE 2.2

NON-RELATIONSHIP BETWEEN FISH WEIGHT AND NOCTURNAL MELATONIN RELEASE FROM UNSTIMULATED TROUT PINEALS AFTER ONE HOUR *IN VITRO*.



Pineals from fish weighing between 160-270g body weight were pre-incubated for about four hours in the light before incubating for a further hour in the dark. Medium was changed hourly. Melatonin release was measured by RIA. Correlation co-efficient, r=-0.233

collection were performed under a red safety lamp since fish pineals *in vitro* are relatively unresponsive to these wavelengths (Dodt, 1963; Meissl and Ekstrom, 1988a). As melatonin degrades upon exposure to light, the collected medium was protected from incident light as much as possible. Medium was divided into small aliquots, snap frozen at -80 °C and stored at -40 °C to await assay for melatonin content.

2.4 RADIOIMMUNOASSAYS

Radioimmunoassay (RIA) has become an increasingly important technique for measuring hormone concentrations in plasma, tissue extracts, and tissue culture media. It is based on the principle that a known concentration of radiolabelled antigen will compete with either a known (standard curve) or an unknown (sample) amount of antigen for binding sites on a known concentration of specific antibody. The ratio of bound: unbound radiolabelled antigen is directly proportional to the ratio of radioactively labelled: unlabelled antigen. Hence, the amount of antigen in the test fractions can be calculated by comparing the percent of labelled antigen bound or displaced from antibody with those of the range of standards. Separation of the antibody-bound from the unbound fraction may be achieved, either by absorbing the unbound molecules onto dextran-coated activated charcoal, or by binding the antibody to a solid-phase matrix which can be isolated by centrifugation and retained for counting.

Many plasma hormones may have to be extracted prior to assay due to a phenomenon known as the 'plasma effect'. Interference from other proteins within the plasma may compete with antibody in binding the

hormones or may in some other way affect the shape of the standard curve so that direct hormone assay is not possible. Extraction proved necessary for plasma cortisol and α-MSH.

Detailed below are the RIA procedures used to measure MCH, Cortisol, α-MSH and melatonin in plasma, tissue extracts or tissue culture medium.

2.5 SOLID-PHASE RIA FOR PLASMA MCH

(As cited by Kishida *et al.*, 1989)

Buffer

Ice cold 0.05M Phosphate buffered saline (pH 7.6) containing 0.9 % NaCl, 0.1 % gelatine (PBSG), 0.01 % thimerosal (as antibiotic) and 1 % BSA.

Standards

MCH (Peninsula Laboratories) was dissolved in 0.1 % trifluoroacetic acid (TFA) to give a final concentration of 10^{-5}M (21 µg/ml). This stock solution A was diluted with solid-phase buffer to 4 ng/ml before being stored in aliquots of 500 µl at -40 °C.

Iodinated MCH

Radiolabelled MCH was prepared by the Chloramine T method (See Appendix 1). It was stored in 50% methanol, 50% 0.05M Phosphate buffer (pH 7.4) with 0.25% BSA at -20 °C.

Immunobeads

Antibody was coupled to immunobeads (Bio-Rad, Richmond, California)

according to the manufacturer's instructions. Details are given in Appendix 2.

Procedure

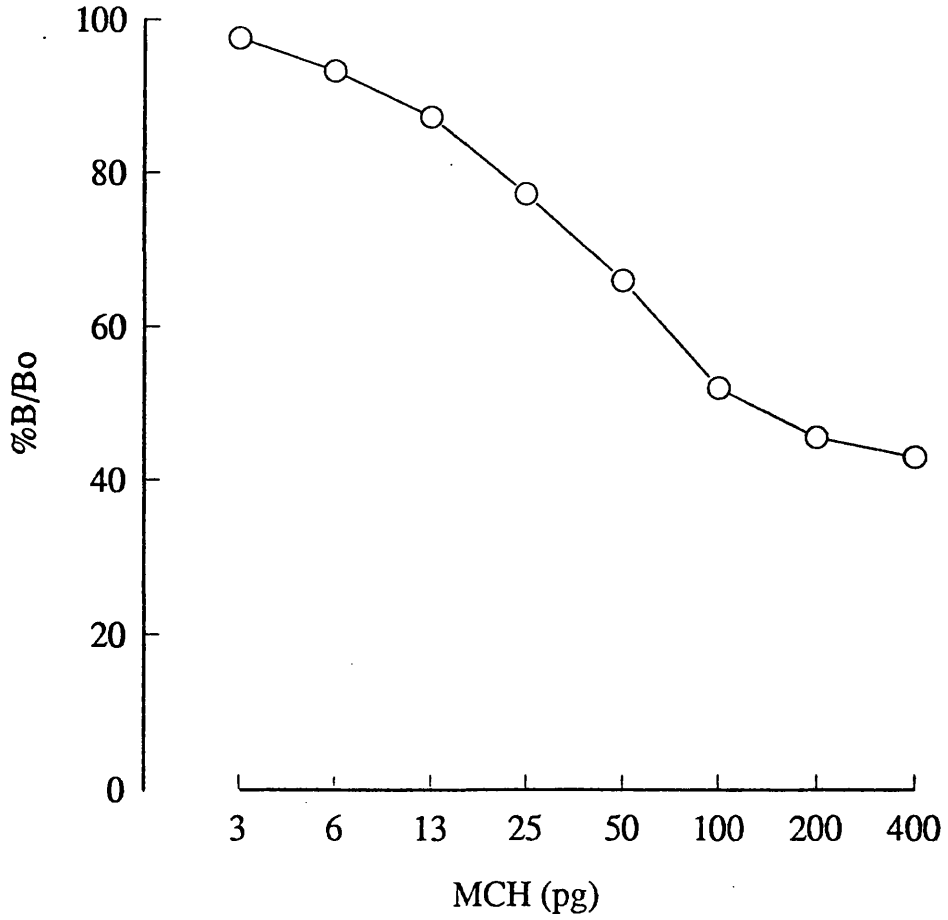
The RIA protocol is described briefly below. Non-specific binding, inter-assay variation and percent recovery were routinely determined for each assay.

The assay was performed on ice. Immunobeads were diluted 1:50 with Solid-phase buffer and 100 µl were added to all tubes in the RIA, except those measuring non-specific binding. The final volume in all tubes was made up to 1200 µl with assay buffer.

Addition	Standard Curve	Plasma Samples	Recovery Tubes	Recovery Controls
Standard MCH	100 µl	-	100 µl	-
Sample	-	500 µl	500 µl	500 µl
Antibody	100 µl	100 µl	100 µl	100 µl
Buffer	1000 µl	600 µl	500 µl	600 µl
Total	1200 µl	1200 µl	1200 µl	1200 µl

Tubes were centrifuged briefly (800 x g, 1 min) and then incubated at 4°C with gentle agitation for 24h. Next day, tubes were centrifuged for 15 min at 3500 x g and the supernatant decanted. The remaining pellet was washed with 1 ml cold Solid-phase buffer, centrifuged for 15 min at 3500g and the supernatant discarded as before.

Figure 2.3
A typical standard curve for the measurement of MCH in the solid-phase RIA



^{125}I-MCH, diluted to about 5000 cpm, was added to each tube in 50 µl, followed by 950 µl Solid-phase buffer. After brief centrifugation (800 x g for 1 min), the tubes were incubated with gentle aggitation at 4 °C for a further 24 h. Next day, 1 ml Solid-phase buffer was added to all tubes except those with tracer alone. The tubes were centrifuged for 15 min at 3500 x g, and the supernatant decanted. Remaining droplets were brought down by brief centrifugation before counting each tube on a γ-counter for 120s.

Comments

MCH antibody (kindly donated by Dr AN Eberle) is very specific for MCH (Eberle, 1988) and does not cross-react with opiomelanocortin peptides nor neurohypophysial hormones.

The sensitivity of this assay ranged from 6.25-400 pg. A typical standard curve is shown in Fig. 2.3. Non-specific binding was about 3% (n=23). Internal assay standards and plasma samples with added MCH were always run in conjunction with the assay and the results corrected accordingly. Recovery was 131.3 ± 8.9 % (n=23). Inter-assay variation was 15.6%.

One possible explanation for why the recovery from spiked plasma is greater than 100 % is that the MCH in assay buffer alone (in the standard curve) might become partially oxidised during the course of the assay. If the antibody used in this assay is less able to recognise oxidised MCH than the reduced form, then recoveries from

spiked plasma might appear greater than 100 %. Kishida and co-workers (1989) found that after correction, however, plasma values closely resembled those derived from another assay procedure: the Sep Pak method.

2.6 RADIOIMMUNOASSAY FOR PLASMA α-MSH

α-MSH was extracted from plasma by the Florisil method (Wilson and Morgan, 1979) and subsequently assayed using a modification of the specific radioimmunoassay described by Baker *et al.* (1984, 1986).

Buffer

Sodium phosphate buffer (0.05M) containing 0.9 % NaCl, 0.01 % thimerosal as antibiotic, and 0.1 % gelatine, adjusted to pH 7.6 and stored at 4°C.

Standards

A stock solution of 10^{-5}M α-MSH was prepared in 0.01M HCl containing 1 % BSA. This was diluted further in 0.05M PBSG to give a final concentration of 4 ng/ml. Aliquots of 500 µl were stored at -40 °C.

Iodination of α-MSH

α-MSH was iodinated by the Chloramine T method (Appendix 1) and stored in 50% methanol, 50% 0.05M Phosphate buffer, pH 7.4, containing 0.25% BSA.

Antibody

Antiserum (raised in this department by Dr T J Bowley, R6FB) was used at a final dilution of 1:60 000. This shows negligible cross-reactions with mammalian β-MSH (<0.03%), 1-24 ACTH (<0.04%), and other ACTH-

related fragments, but showed significant cross-reaction (70%) with des-acetylated α-MSH (Wilson and Morgan, 1979; Bowley *et al* , 1983).

Extraction of α-MSH from plasma

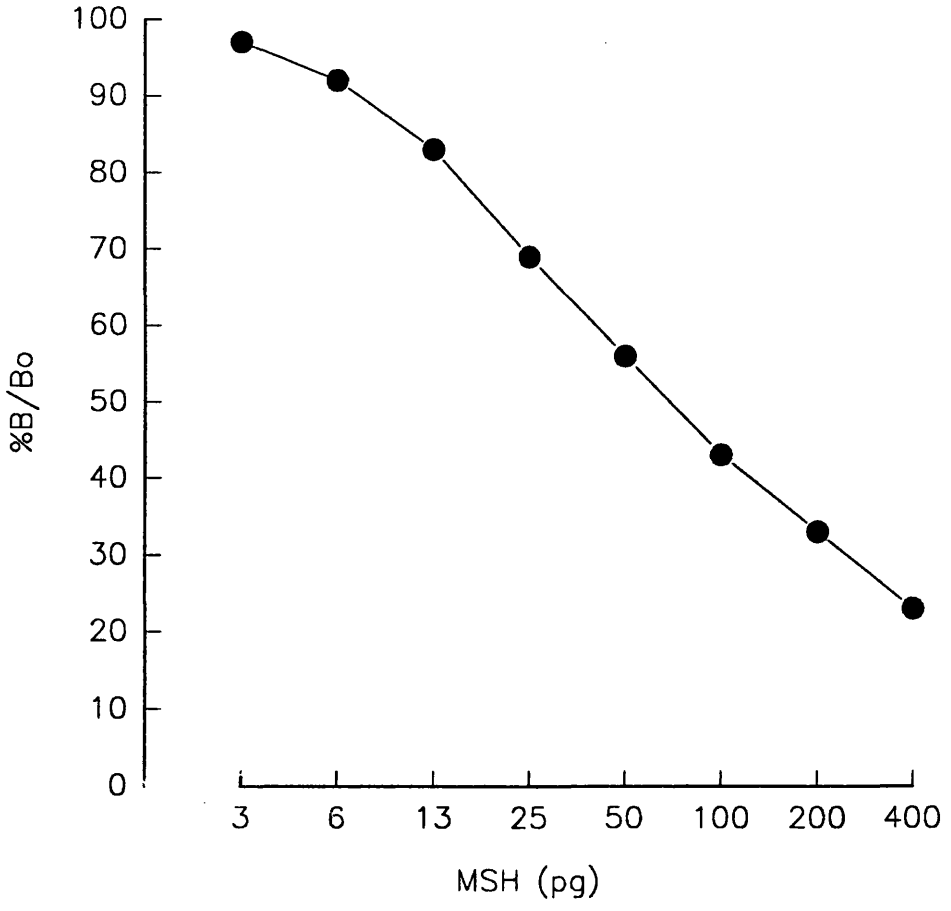
Plasma samples of 500 µl were acidified with 200 µl 0.4M HCl. A suspension of 50 mg Florisil (100-200 mesh: Sigma) in 1 ml distilled water was added to each sample and the tubes were tumbled on a Stuart rotator at 4 °C for 30 min. After centrifugation at 2000 x g for 10 min, the supernatant was aspirated. The pellet was washed with 2 ml 0.4M HCl, followed by 2 ml distilled water. The α-MSH was eluted with 1 ml ethanol/ammonium hydroxide solution (2:3 v:v) containing 2 % mercaptoethanol, by mixing at 4 °C for 30 min. After centrifugation at 1500 x g for 15 min, the supernatant was collected in tubes containing polypep (10 µg/10 µl) and dried under vacuum overnight. Pooled plasma samples containing added α-MSH or buffer, were also extracted and served as necessary controls.

RIA Protocol

Plasma α-MSH extracts were resuspended in 250 µl 0.05M PBSG, vortexed and allowed to stand for 1 h on ice. Volumes of 100 µl were transferred in duplicate to new 4 ml polypropylene tubes. Iodinated α-MSH (about 3000 cpm/50 µl PBSG) was added to each tube, followed by 50 µl antibody solution and 100 µl ice-cold PBSG.

After 24h incubation at 4 °C, 500 µl ice-cold dextran-coated charcoal (See Appendix 3) were rapidly added to adsorb unbound peptides. Tubes were left to incubate at 4 °C for 15 min before centrifugation at 2000

Figure 2.4
A typical standard curve for the measurement of MSH in the charcoal-separated RIA.



x g for 15 min at 4 °C. The supernatant was aspirated and the charcoal pellets were counted in a gamma-counter, each for 120s.

Comments

Recovery was 79.9 ± 2.7% (n=9). Occasionally, unextracted, diluted plasma was placed in the RIA. This method sometimes gave the same results as extracted values but at other times gave excessively high α-MSH values in plasma from both black- and white-adapted fish. It was therefore decided that this latter assay procedure was unreliable, and was no longer used.

2.7 TISSUE CONTENT ASSAYS

Pituitaries and hypothalami were removed rapidly and individually placed in 500µl 0.01M HCl on ice. They were sonicated for 30s and allowed to extract for one hour at 4°C before centrifuging at 3000 x g. The supernatant was stored in aliquots at -20°C until assay. Extracts were appropriately diluted before use (see below) and the pH was adjusted to about 7.6 with 10% Tris where necessary.

Charcoal-separated RIA for pituitary α-MSH.

(As cited in Bowley *et al.*, 1983)

Pituitary extracts were diluted 1:10 000 with PBSG (as used in plasma α-MSH and cortisol assays). Immunoreactive α-MSH was determined in 100µl aliquots of this diluent using the same RIA protocol as for extracted plasma α-MSH.

Charcoal-separated RIA for hypothalamic and pituitary MCH

(As cited by Barber *et al.*, 1987)

Hypothalamic extracts were diluted 1:200 in PBSG (as above), while pituitary extracts were diluted 1:400 with PBSG, before use. Antibody against MCH (Eberle) was used at a dilution of 1:60 000, and immunoreactive MCH was determined in 100 µl aliquots using the same RIA protocol as for extracted plasma α-MSH.

2.8. RADIOIMMUNOASSAY FOR PLASMA CORTISOL

(As cited by Baker and Rance, 1981).

Buffer (PBSG)

Sodium phosphate buffer (0.05M) containing 0.9 % NaCl, 0.01 % thimerosal as antibiotic, and 0.1 % gelatine, adjusted to pH 7.6 and stored at 4°C.

Standards

A stock solution of 10 µg/10 ml ethanol was diluted with absolute ethanol to give standards ranging from 160 ng/ml to 1.25 ng/ml. These were stored at -20 °C in tightly sealed vessels. A standard curve, using 10 µl volumes of the above standards, was run in triplicate, in parallel with the assay (Figure 2.5). The sensitivity of this assay ranged from 1.25-20 ng/ml plasma.

Labelled Cortisol

Labelled cortisol, 1,2,6,7,^{3}H-cortisol (Amersham) was diluted with Analar-grade ethanol to give a stock solution of 1 µCi/100 µl and stored below -20 °C. A 100 µl volume of this solution was evaporated to dryness and resuspended in 20 ml PBSG.

Antibody

Antiserum (R5; raised by Dr I D Gilham in this department) shows relative cross-reactivities: cortisol 100%; corticosterone 2.09%; prednisolone 84.62%; progesterone 0.0085%; cortisone 0.26%; deoxycortisone 0.44%; 11-deoxycortisol 3.33%; 17α-OH progesterone 1.25%; and testosterone 0.008%. It was diluted 1:10 with PBSG and stored at -40 °C in 1 ml aliquots. This was then diluted 200-fold in the tritiated cortisol solution (above) to give a final dilution of 1:2000.

Extraction of cortisol from plasma

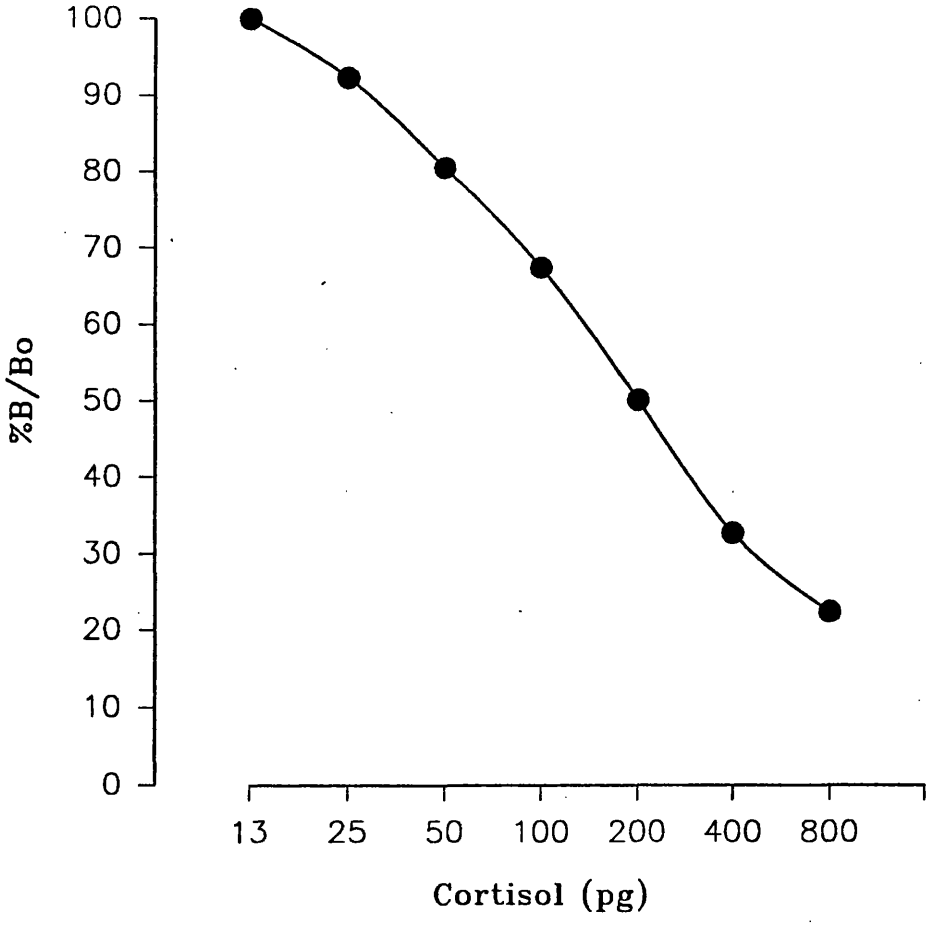
Cortisol was extracted by adding 250 µl absolute ethanol to 50 µl plasma, then vortexing. A further 250 µl ethanol was then added and the suspension mixed. Tubes were centrifuged at 2000 x g for 10 min (4 °C). A 400 µl volume of the resulting supernatant was transferred to a 4 ml polypropylene tube and the alcohol evaporated in a vacuum centrifuge.

RIA Procedure

Ethanolic standards and extracted cortisol samples were evaporated to dryness and resuspended in 200 µl of the labelled cortisol solution containing antibody. Tubes were vortexed, centrifuged briefly (800 x g for 1 min), and then incubated overnight at 4 °C.

The following day, 500 µl of ice-cold dextran-coated charcoal were added to all tubes (Appendix 3). These were left for 15 min before centrifuging at 2000 x g for 15 min. The supernatant, containing the antibody-bound fraction, was tipped into 6 ml scintillant (Optiphase

Figure 2.5
A typical standard curve for the measurement of cortisol by RIA.



Safe, LKB Scintillation Products). The vials were shaken well and left to stand for about 1h before counting in an LKB Rack-Beta scintillation counter with quench correction.

Comments

The separation of free from bound ligand was achieved by the addition of ice-cold dextran-coated activated charcoal. This avidly adsorbs unbound ligand in a time-dependant manner. A long delay between the addition of charcoal to the first and last assay tubes will cause significant assay drift, so it is important that this step is done as rapidly as possible. Similarly, if the incubation is allowed to proceed for too long, the charcoal will also strip ligand off the antibody. During a normal assay charcoal addition took under three minutes to complete using an Eppendorf multipetter (4780). Standard curves placed at both the beginning and end of the assay showed that during this time there was no significant assay drift. Furthermore, a fixed incubation period (15 min after addition of charcoal to the first tube) was used.

2.9 RADIOIMMUNOASSAY FOR MELATONIN

Protocol modified from that recommended by Guildhay Antisera Ltd (Fraser *et al.*, 1983).

Assay Buffer

0.1M Tricine (Sigma) with 0.9 % NaCl and 0.1 % gelatine (BDH), adjusted to pH 5.3 with NaOH. This was made up each week in 250 ml volumes using HPLC-grade, freshly distilled water and stored at 4 °C.

Standards

Melatonin was dissolved in Analar-grade ethanol (10 mg/500 µl) and further diluted with water to give a final concentration of 1 mg/ml. This solution was stable at 4°C for at least three months in the dark. A working stock of 1 µg/ml was prepared in water and could be stored at 4°C for a month. This stock was further diluted with Eagles modified Minimum Essential Medium with Hepes (MEM) plus assay buffer (1:4 v:v) to give standards ranging from 2 - 1000 pg/200 µl.

Radiolabelled Melatonin

Tritiated Melatonin (Amersham) (80 Ci/mMol) was diluted 1:100 with ethanol and stored at -40 °C. This working stock was diluted to give approximately 8500 dpm/100 µl by evaporating a known amount of tritiated melatonin to dryness and resuspending in assay buffer.

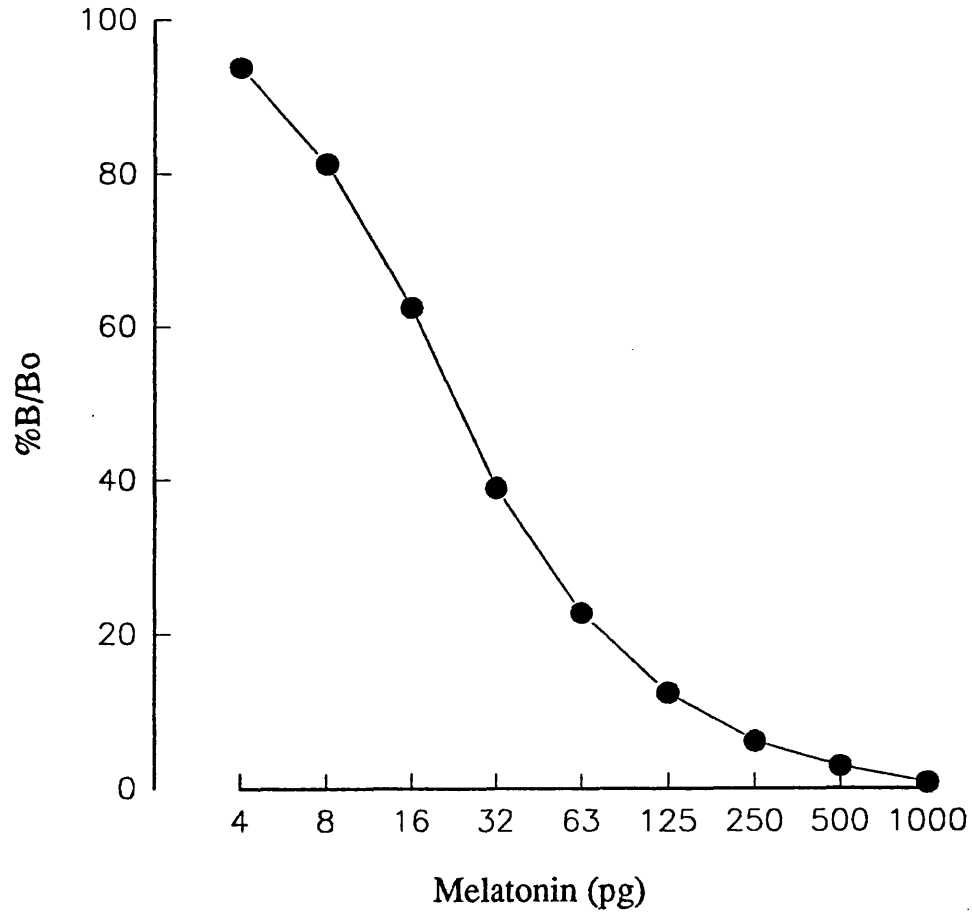
Antibody

Freeze-dried sheep anti-melatonin antiserum, raised against N-acetyl-5-methoxytryptophan/bovine thyroglobulin (Guildhay: Batch 704/8483), showed relative cross-reactivities with melatonin (100%), N-acetyl tryptamine (0.9%), 6-hydroxy melatonin (0.33%), N-acetyl tryptophan (0.22%), and all other indole related compounds (<0.06%). It was dissolved in 2 ml water to give an intermediate dilution of 1:10. This was stored in 100 µl volumes. Before assay, this intermediate solution was diluted 1:300 to give a working solution with an initial dilution of 1:3000.

RIA Protocol

Culture media collected in the light and dark were treated differently. Medium collected from pineals incubated in the light were

Figure 2.6
A typical standard curve for the measurement of melatonin by RIA



diluted 1:5 in assay buffer whereas those collected in the dark were diluted 1:10 in MEM before being further diluted 1:5 in assay buffer.

Samples and standards (200 µl) were assayed in duplicate in 4 ml polypropylene tubes on ice. Assays were performed away from direct sunlight to reduce degradation by light. Antibody was added in 100 µl volumes and the mixture allowed to incubate for 30 min on ice, afterwhich an equal volume of ^{3}H-MT was added. Tubes were briefly centrifuged and left to incubate overnight at 4°C.

Next day, unbound melatonin was adsorbed with 500 µl dextran-coated charcoal suspension in tricine buffer. Tubes were centrifuged (2000 x g, 15 min, 4 °C) after 15 min incubation. The supernatant was then decanted into 4 ml scintillant and left to stand for an hour before counting in an LKB Rack-Beta Scintillation counter, with quench correction.

Comments

As melatonin is light-sensitive and the radioimmunoassay may be capricious, precautions taken are listed below:

1. All solutions were made up with fresh double-distilled HPLC-grade quality water every week. In the event of loss or lowering of binding or of contamination, all solutions (except standards) were discarded and made up fresh.
2 The assay was carried out in an area uncontaminated by indoles.
3. The assay was performed away from direct sunlight. Samples and

standards were kept in darkened containers.

4. Care was taken to ensure that pipette tips were clean and not handled by their tips.

Inter- and intra-assay variation was determined using pooled pineal culture medium of known concentration, stored at -20°C. Inter-assay variation was about 15 % and intra-assay variation was about 11.2 % (n=19). Assay drift was apparent in assays of more than 130 tubes so all assays were restricted to less than 100 tubes. Recovery was 94.9 ± 13.7, n=19.

2.10 IODINATION OF MELATONIN

(As detailed by Vakkuri *et al.*, 1984)

Iodogen (Pierce Chemical Co.) was dissolved in chloroform to give a final concentration of 0.1 mg/ml. Iodogen coated vials were prepared by drying down a 10 µl aliquot of this solution in an Eppendorf under a stream of nitrogen gas. The vials were capped, sealed with parafilm and stored at room temperature in a dessicator in the dark.

A stock solution of melatonin was prepared in methanol (1 mg/ml) and stored at -20 °C until use. Care was taken to limit the exposure of melatonin to light at all times. Before iodination, 100 µl stock melatonin was dried down under nitrogen gas and resuspended in the same volume of 0.05M sodium phosphate buffer. It was vortexed well and left to extract overnight at 4 °C.

A 10µl volume of melatonin in buffer was placed into a vial containing iodogen, together with 200 µCi Na^{125}I. The vial was vortexed and the reaction allowed to proceed for one minute before the reaction was terminated by the addition of 100 µl chloroform. The solution was mixed well and the chloroform fraction removed to a new vial. Again 100 µl chloroform was added to the original reaction mixture, the vial vortexed, and the chloroform fraction added to the previous chloroform extract. This procedure was repeated once more before the pooled fractions were run on a thin layer chromatography (TLC) plate to separate di-iodinated melatonin from iodine and unlabelled melatonin.

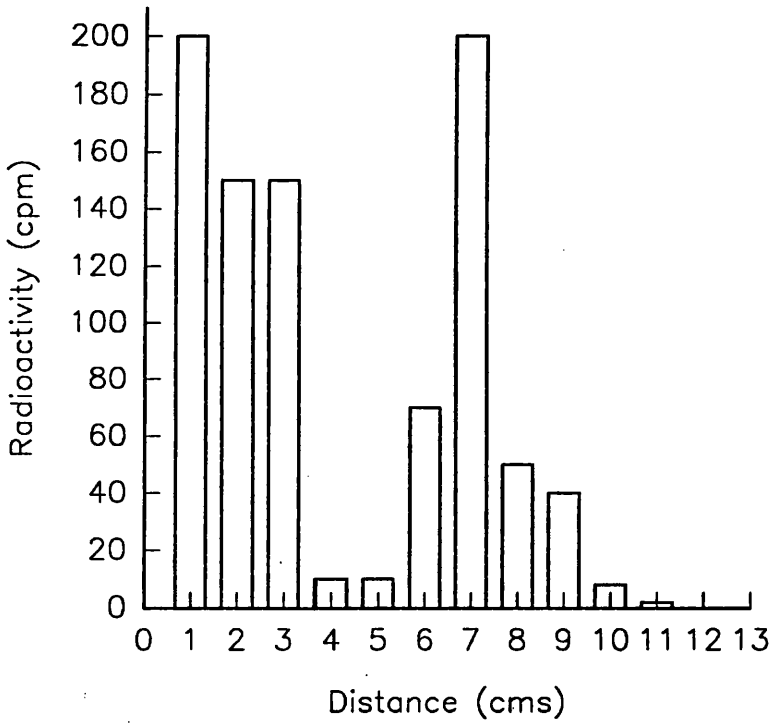
A TLC plate (20 x 20 cm silica gel, Kodak Ltd.) was dried at 80 °C for 15-30 min to dehydrate it before use. Under a safe light in the dark, the iodinated melatonin was spotted onto the cooled TLC plate which was then placed into a fully saturated tank containing mobile phase (Chloroform: methanol: Glacial acetic acid; 93:7:1). It was left in the dark to run for about an hour until the solvent front was near the top of the chromatogram. The chromatogram was then cut into 1 cm horizontal strips and each was counted with a Geiger-counter. The Rf for iodinated melatonin varied between 0.5-0.6. The strip with the highest radioactivity was placed in a vial containing 2 ml methanol and allowed to extract for several hours.

A second purification was done. The extracted methanol fraction was dried down under nitrogen and resuspended in 400 µl chloroform before spotting onto another TLC plate, and running as before. The final methanol solution containing di-iodinated melatonin was stored at

FIGURE 2.7

PRIMARY PURIFICATION OF IODINATED MELATONIN BY TLC.

Radioactivty (cpm) of horizontal one centimetre strips of the chromatogram, from the origin (0).



Iodinated melatonin was spotted onto a TLC plate at the origin (0) under a red safety lamp, and the chromatogram left to run for an hour. It was then cut into 1cm strips and their radioactivity (cpm) was counted with a hand-held Geiger conunter. Iodinated melatonin came off at an Rf of 0.5- 0.6 (strip 7). This strip was then extracted for two hours in methanol before being re-purified by TLC.

-20 °C. A 5 µl volume was counted on the day of purification. This reading was used to calculate the specific activity and the molarity of the di-iodinated melatonin.

$$\frac{\delta N}{\delta t} = \lambda N$$

Where $\frac{\delta N}{\delta t}$ = decay per second

λ = decay constant (negative)

N = number of atoms per mole.

Assume that every molecule of melatonin has been di-iodinated and that no melatonin has been lost during purification. (The percentage of lost melatonin can be calculated by comparing the radioactivity (cpm) in the selected band after the second purification with that in the first.)

This radiolabelled ligand could be stored for two months before the specific activity fell below an acceptable level.

2.11 HISTOLOGY

Preparation of Tissue

Trout brain and retina were dissected from decapitated fish. The tissues were rinsed in 0.01M Phosphate buffered saline (PBS), pH 7.6, containing 15 % sucrose before being frozen in hexane at -30 °C. Frozen material was wrapped in foil and stored with dessicant at -80°C.

Sections were cut to 16 µm on a cryostat and thaw mounted onto poly-L-lysine (1 mg/ml)-coated slides. All slides were kept in a dessicator at 4 °C overnight. They were then stored with dessicant at -80 °C until required.

Dorsal trout scales were removed and teased apart in MEM. Care was taken not to damage them during this operation. Scales were placed on poly-L-lysine coated slides so that their dorsal surface was uppermost. These were left to dry overnight at 4°C before being frozen at -80°C with tissue sections.

2.12 AUTORADIOGRAPHY

(As detailed by Laitinen *et al.*, 1989)

Frozen sections of brain and retina together with frozen melanophores, were brought to room temperature in a dessicator for at least five hours. They were then pre-incubated in 0.05M Tris/HCl buffer containing 4mM $CaCl_2$ for 15 min at room temperature.

An appropriate volume of di-iodinated melatonin in methanol was dried under nitrogen gas and resuspended in 0.05M Tris/HCl containing $CaCl_2$ to give a final concentration of about 70 pM 2-[^{125}-I]-melatonin (322000 cpm/ml buffer). Cold melatonin with a final concentration of 1µM was also prepared from stock (1mg/ml) melatonin in methanol.

Sections were incubated for one hour in the dark, each covered with a drop of radiolabelled melatonin to allow binding of this tracer to receptors. Non-specific binding was determined by the addition of excess melatonin (1µM) to the radioactive solution.

After incubation, the slides were rinsed 3 x 5 min. in ice cold 0.05M Tris/HCl buffer, pH 7.4, before rinsing in ice cold water for 30s. The

sections were then drained and dried vertically in a fume cupboard in the dark, before being apposed to Hyperfilm ^{3}H (R70; Kodak Ltd.) for about a week. The length of exposure varied according to the activity of the 2-[^{125}I]-melatonin. During this period, the slides were kept at 4°C in a light-proof bag inserted in a lead cassette.

At the end of the exposure, films were developed in standard Kodak X-ray developer. Finally, the sections were stained in haematoxylin for 5 min, counter-stained in Eosin for 2 min, dehydrated rapidly in alcohol, cleared in xylene and mounted.

2.13 STATISTICS

Statistical comparisons of means were made with the pooled Student's t test. Data were log transformed (standard deviation proportional to mean) or arc sine, square-root transformed (percentages) when appropriate. One-way analysis of variance (ANOVA) was used to compare daily changes in plasma MCH concentrations.

CHAPTER THREE

THE INFLUENCE OF THE EYE ON MCH RELEASE

3.1 GENERAL INTRODUCTION

Melanin-concentrating hormone (MCH) causes melanosome aggregation in the dermal melanophores of teleost fish and thus acts as a paling hormone during adaptation to white backgrounds (Rance and Baker, 1979; Oshima *et al.*, 1985; Baker, 1988a, b). Immunocytochemical studies (Naito *et al.*, 1985; Bird *et al.*, 1989) have shown that MCH is a neurohypophyseal hormone with the majority of perikarya located in the lateral nucleus tuberis of the ventral hypothalamus. Most MCH axons project to the pituitary, whence the hormone is released into the blood stream, but a few axons may also be traced to the brain, particularly the pretectal area (Naito *et al.*, 1985), which also receives fibres from the retina and pineal.

Measurement of both hypothalamic and pituitary tissue MCH contents by radioimmunoassay (Baker, 1988b), suggested that the hormone was released when fish were placed on a white background. It has recently become possible to measure plasma concentrations of MCH in trout (Eberle *et al.*, 1989; Kishida *et al.*, 1989). Fish adapted to white backgrounds or transferred from black to white tanks had higher plasma MCH levels than black-adapted fish (Kishida *et al.*, 1989). The speed of this response was quite rapid, with nearly maximal levels being reached within two hours after transferral from black to white

backgrounds. However, chromatic adaptation to a white background starts within a few minutes (Rodrigues and Sumpter, 1984) as a result of direct innervation of melanophores by branches from the sympathetic nervous system. Severance of these local nerves in hypophysectomised fish prevents melanin concentration (Baker and Ball, 1975) despite being maintained on a white background.

The lateral eyes are the principal receptors for colour change responses but it is not yet known how information about the environment is transduced from the teleostean retina to effect changes in plasma concentrations of MCH and/or its antagonistic counterpart, α-MSH. In the past, background adaptation responses have been related to the regions of retina illuminated and the relative intensities of light reaching these different retinal areas. Experiments on Fundulus heteroclitus (Butcher and Adelmann, 1937) involving eye rotation and selective covering of the eyes, showed that stimulating the dorsal region of the retina was associated with pallor, while ventral retinal stimulation was associated with darkening. Further investigation (Butcher, 1938) involving eye-capping experiments confirmed this regional specificity and revealed that the anatomical structure of the dorsal and ventral regions of retina were different. In the dorsal region, ie. that located dorsal to the optic nerve (about 70% of the total retinal area), rods, single cones and double cones were found; while the lower region had a specialised crescentric ridge containing a greater density of double cones and rods than in any other part of the retina, but no single cones. It was, therefore, possible that this different distribution of retinal photoreceptors and their central

nervous connections might be involved in colour change responses. Butcher also suggested that the ratio of direct: reflected light entering the eye influenced body coloration. Unfortunately, however, no quantitative measurement of body colour was made. Hogben and Landgrebe (1940) similarly showed that specific retinal regions appear to be associated with different background adaptation responses in the teleost Gasterosteus aculeatus. Once again, the photoreceptors apparently concerned with black background responses were located in the ventral retina, below the optic nerve, while white background receptors appeared to be restricted to a central region of retina, situated above and below the origin of the optic nerve. Although Gentle (1972) could not find any anatomical differences in dorsal and ventral regions of the minnow, Phoxinus phoxinus retina, he suggested that the dorsal retina was important for maximal paling, while the ventral region was necessary for maximal darkening. Following removal of the ventral retina, the fish paled, but not maximally, however, and they still retained some ability to darken. It is likely that the concept of two clear-cut antagonistic regions in the eye controlling colour change is an over-simplification.

The following experiments attempted firstly, to determine plasma MCH titres in white-adapted or black-adapted trout and those kept in the dark and secondly, to determine whether selective stimulation of different areas of trout retina will result in changes of plasma MCH concentrations. For example, is stimulation of the dorsal retina important for initiating elevated circulating MCH titres?

3.2 MATERIALS AND METHODS

Experiment One: Twenty four sexually immature rainbow trout, 150-200g body weight, were collected and initially placed in white 250l tanks with continuously flowing aerated fresh water under constant illumination (incident light intensity 135 lux, measured at water level). They were left to acclimate to aquarium conditions for two weeks, after which eight fish were transferred to:

a) an illuminated 250l black tank,

b) an illuminated 250l white tank,

c) a covered 250l black (dark) tank.

They were maintained under these conditions for a further week before measuring plasma MCH, MSH and cortisol concentrations, as detailed in Chapter Two.

Experiment Two: Twenty four sexually immature rainbow trout, 150-200g body weight, were held in black or white 250l tanks. Fish in one group of black and white tanks were placed under constant bright illumination (1500 lux, measured at the water surface) while those in a second were held under constant dim illumination (75 lux, incident light at water surface). All fish were left for two weeks before measuring plasma MCH levels at 14:00h (See Chapter Two for details).

Experiment Three: Sexually immature rainbow trout, 150-200g body weight were collected and initially placed in white 250l tanks with continuously flowing aerated fresh water under constant illumination (light intensity 135 lux, measured at water level). They were left to acclimate to aquarium conditions for two weeks, after which trout were serially transferred in groups of six to smaller (100l) experimental glass tanks for a 24h period:

a) **W:** a tank with white base and sides and overhead illumination (for total retinal illumination)

b) **Bb:** a tank with black sides and a black lid, illuminated from below (for illumination of dorsal retina only)

c) **Bw:** a tank with black sides and a white lid, illuminated from below (for dorsal and ventral but no illumination of lateral retina)

d) **B:** a tank with black base and sides and overhead illumination (for ventral retinal illumination only).

Reflectance off the sides and base of black tanks was kept to a minimum by lining their inner surface with matt-black adhesive paper. The tank lids were also submerged 2cm below the water level to prevent reflection from the air-water interface. Incident light intensity, measured 1cm from the water surface, was 350-450 lux for illumination sited above the tanks, and 450 lux for illumination sited below the tanks, while reflected light intensity from white base and sides ranged from 100-120 lux.

All fish were killed at 14:00h. The experiment was performed in two groups: Bb and Bw first, followed by W and B. The two experimental groups Bb and Bw were repeated on a separate occasion.

3.3 RESULTS

Experiment One: the aim of this experiment was to see how plasma concentrations of MCH and α-MSH differ when fish are adapted to white or black backgrounds or maintained in the dark. White-adapted (W) trout maintained in constant light for 21 days were very pale in colour, while those transferred to a black background (B) for 7 days

in constant light were very dark and difficult to see against the black background. Fish kept in constant darkness (D), however, were intermediate in colour and readily visible on an illuminated black background.

White-adapted fish had significantly higher plasma MCH levels (214.2 ± 34 pg/ml, n=8) than B-trout (55.4 ± 5.8 pg/ml) (Fig 3.1a.). Fish maintained in the dark, however, had significantly lower plasma MCH concentrations (27.9 ± 3.4 pg/l) than illuminated black-adapted trout. Hypothalamic MCH contents (Table 3.1) in white-adapted fish were significantly lower (P<0.01) than black- or dark-adapted fish. Pituitary MCH concentrations in trout maintained in the dark were significantly higher than those of fish adapted to either black or white illuminated tanks.

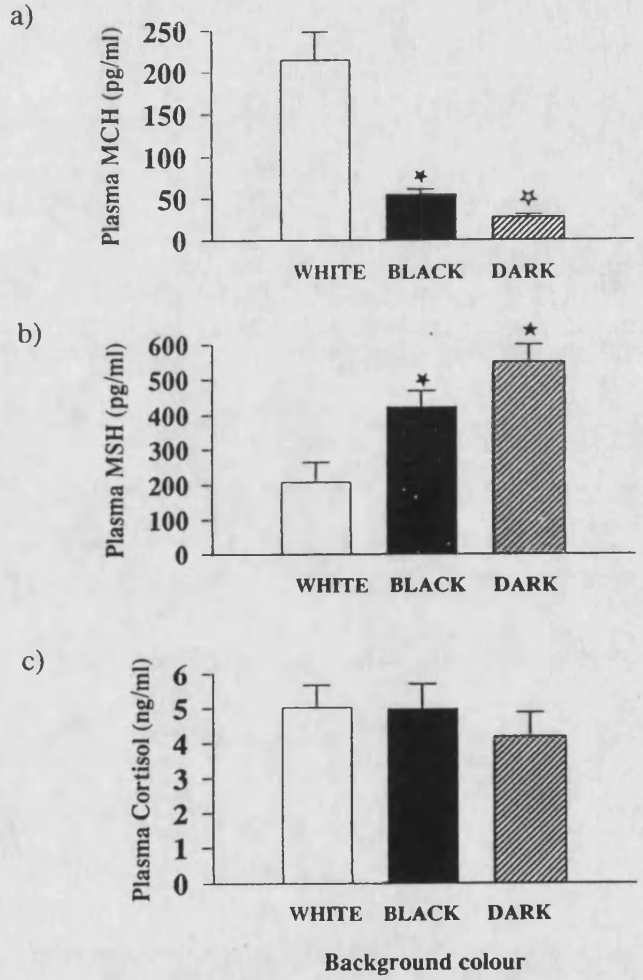
Table 3.1 TISSUE CONTENT OF HORMONES IN TROUT KEPT IN ILLUMINATED BLACK OR WHITE TANKS OR IN THE DARK (ng ± SEM; n=8).

	HYPOTHALAMIC MCH	PITUITARY MCH	PITUITARY α-MSH
White-adapted trout	20,8 ± 3	269,3 ± 79	2473 ± 270
Black-adapted trout	69,4 ± 11 *	353,0 ± 49	1767 ± 332
Dark-adapted trout	55,9 ± 7 *	617,9 ± 80 *	2056 ± 175

Note: * P<0,01 compared with White-adapted trout.

Figure 3.1

Plasma MCH, MSH and cortisol concentrations from trout maintained in illuminated black or white tanks, or in constant darkness for one week.



Note: Values are given as mean ± SEM (n=8). * $P < 0.05$ compared with fish maintained in continuously illuminated white tanks. ✡ $P < 0.05$ compared with fish maintained in continuously illuminated black tanks. Plasma MCH concentrations in black adapted trout were significantly lower than those in white tanks but higher than those in dark-maintained trout. Concentrations of the antagonistic hormone, MSH, were significantly higher in both black- and dark-maintained fish. Plasma cortisol levels were not statistically different: values were low (less than 6ng/ml) in all groups of fish.

Plasma MSH concentrations showed the opposite trend to MCH (Fig. 3.1b). Black-adapted fish and those maintained in the dark, had significantly higher α-MSH levels than those on illuminated white backgrounds. There were no differences between pituitary α-MSH concentrations in any of the groups.

Circulating cortisol levels were measured to see whether fish were stressed by their holding conditions. Plasma cortisol levels were low, ranging from 4.2 - 5.0 ng/ml, with no difference between any of the groups.

Experiment Two: The aim of this experiment was to investigate the importance of light intensity in determining plasma MCH concentrations in trout kept in black or white tanks. The intensity of incident or reflected illumination under which fish on the same background colour were maintained, did not alter plasma MCH concentrations. Fish held in white tanks under intense (1500 lux) or dim (75 lux) illumination had significantly higher ($P>0.001$) plasma MCH concentrations than either group of illuminated black-adapted fish (Table 3.2). However, the ratio of incident to reflected light intensity (I/R) in all conditions appears to be inversely proportional to plasma MCH concentrations. White tanks under both bright and dim illumination have a lower I/R ratio with correspondingly high plasma MCH levels, while the I/R ratio of either illuminated black tanks was about four times higher but the concentrations of plasma MCH in these fish were about 10-fold lower.

Table 3,2 PLASMA MCH CONCENTRATIONS IN BLACK- AND WHITE-ADAPTED TROUT UNDER DIM AND BRIGHT OVERHEAD ILLUMINATION

Tank Colour	MCH (pg/mℓ)	Incident light (lux)	Reflected light (lux)	Incident / Reflected
2A White	121 ± 44	1500	200	7,5
2B White	140 ± 14	75	14	5,4
2C Black	18 ± 5,2 *	1500	50	30,0
2D Black	11 ± 1,6 *	75	4	20,0

Note: * P<0,01 compared with white-adapted fish

Experiment Three: The aim of this experiment was to determine whether illumination of specific areas of the retina could result in changes of plasma MCH titres similar to those that occur during background colour adaptation. The ventral surface of trout retina will receive incident light when fish are placed in tanks with overhead illumination. If the tank is white, light will be reflected off its base and sides which will also illuminate the dorsal and lateral regions of the retina. Conversely, when the source of illumination is below the tank, incident light reaches the dorsal retina. A white lid will reflect some of this light onto the ventral retina as well.

These theoretical predictions were disrupted by the behaviour of the fish placed in tanks with black sides and lid, illuminated from below (Bb). Instead of swimming horizontally, they swam nose downwards so that they were facing the source of illumination. In this case, therefore, it is likely that they not only received dorsal retinal

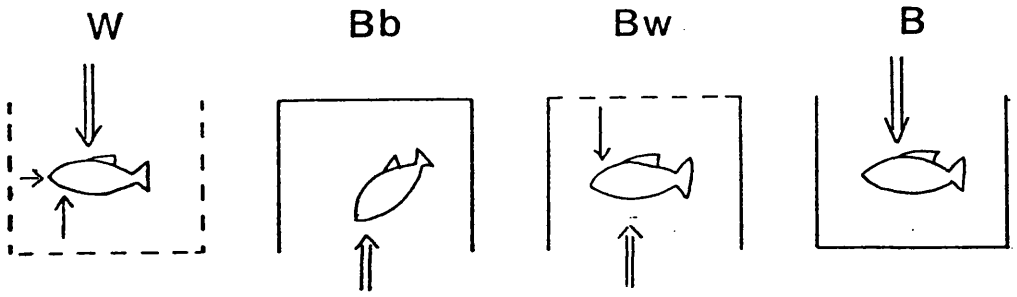
illumination (as intended) but also considerable lateral stimulation. This behaviour was not observed in any other group of fish.

Levels of plasma MCH varied greatly within each experimental group so that few statistical differences could be shown with Student's T test. Nevertheless, there was a striking similarity between plasma MCH concentrations in groups W and Bb, and groups B and Bw (Fig. 3.2). Titres of MCH in W (102.1 ± 19 pg/ml) and Bb fish (94.2 ± 20 pg/ml) were about twice those of B (48.6 ± 22 pg/ml) and Bw fish (48.3 ± 12 pg/ml). Values are significantly higher in W than Bw trout ($P<0.05$). In a second experiment values in Bb fish (37.2 ± 4.9 pg/ml, n=6) were again twice as high as those in Bw fish (19.17 ± 1.6 pg/ml, n=6), and the differences were significant.

The solid-phase plasma MCH assay sometimes gave plasma recoveries ranging from 100-200 or more percent, while those with either inter-assay variation standards or oxidised MCH approximated 100%. Hence, the addition of plasma was not considered to give a reliable indication of percent recovery. When possible, non-corrected values from repeated assays were meaned, and these values thought to better reflect hormone levels. Intra-assay variation was low (9.7%) making it possible to compare MCH values from different groups within one assay but the absolute plasma hormone values may not be accurate, and it may be less helpful to compare hormone concentrations between assays. Hormone levels from all individuals within one experiment were therefore assayed together.

FIGURE 3.2

Twenty-four hour illumination of dorsal and ventral retina in trout: Effect on plasma MCH.



Region of retinal Illumination	Dorsal Lateral Ventral	Dorsal Lateral	Dorsal Ventral	Ventral
Plasma MCH (pg/ml)	100.2 ± 19	94.2 ± 20	48.3 ± 12 *	48.6 ± 22
Plasma Cortisol (ng/ml)	7.5 ± 5.4	20.3 ±4.7	3.4 ± 0.9	36.3 ± 9

Trout were illuminated from above and below for twenty-four hours. Incident light (double arrow) and reflected light (single arrow) from a white background (dashed line) illuminated different regions of trout retina. Plasma MCH and cortisol concentrations (mean ± SEM, n=6) were measured by RIA.
* $P<0.05$ compared with fish housed on a white background with overhead illumination (W).

Cortisol levels were generally higher in all groups of fish in this experiment than in experiment one. Black-adapted trout had the highest plasma cortisol levels (36.3 ± 9 ng/ml), significantly greater than their white-adapted counter-parts (3.4 ± 0.9 ng/ml). Cortisol concentrations in Bb and Bw fish were ranged from 7.5 to 20.3 ng/ml.

3.4 DISCUSSION

The results of experiment one confirm that MCH is released into the blood stream of teleosts in response to pale background colour (Kishida *et al.*, 1989). The high level of release in white-adapted fish is associated with lowered pituitary and hypothalamic MCH tissue concentrations. Furthermore, this experiment shows that illumination is important for determining the colour-change response, since fish maintained in the dark have significantly lower plasma MCH concentrations than those on illuminated backgrounds of either colour. The question of why fish were pale even though MCH titres were low and α-MSH concentrations were high is discussed later.

The eyes must be the primary photoreceptors of colour-change responses. Many lower vertebrates, including trout, will become pale on a white background, yet remain an intermediate shade in the dark. Early researchers therefore postulated that the intermediate colour was a resting state, and that the colour change responses of paling or darkening were related to the intensity of incident or reflected light perceived by the eyes. Observations of the fish in experiment two show that this is clearly not the case. Increasing the incident light intensity from 75 to 1500 lux did not modify circulating MCH

concentrations in either black- or white-adapted fish (Table 3.2). Moreover, white-adapted fish under dim illumination (75 lux) had significantly higher plasma MCH concentrations than black-adapted fish under intense illumination (1500 lux). Measurement of reflected light intensities from the base and sides of the tanks showed that about four times less light is reflected off black than white tanks. However, it is unlikely that reflected light intensities alone determine colour change responses, since brightly illuminated black tanks reflected more light (50 lux) than dimly illuminated white tanks (14 lux) yet fish in the former had significantly lower plasma MCH concentrations. However, the results of experiment two do suggest that the ratio of incident to reflected light (I/R) might influence colour-change responses in the trout. A low I/R ratio (white tanks) is associated with high plasma MCH levels, while a high I/R ratio (black tanks) is associated with low circulating MCH values. Under normal conditions, incident light will be received onto the ventral surface of trout retina, while reflected light will stimulate lateral and dorsal retinal areas.

This led to the idea that it was the region of retina stimulated by light that determined the colour of the fish. Former experiments on teleosts involving eye-capping, eye-rotation and retinal ablation (Butcher and Adelmann, 1937; Butcher, 1938; Hogben and Landgrebe, 1940; Gentle, 1972) suggested that this was the case. In all species studied, stimulation of the ventral retina (ie. retina ventral to the optic nerve) was associated with a dark body colour. This may also be the case in trout, since incident illumination of the ventral retinae

(fish in black tanks with overhead illumination) was associated with low plasma MCH, and high α-MSH concentrations, in all experiments. Conversely, the area of retina associated with body pallor seems to vary with species and might be related to the shape of the eye. In the minnow, Phoxinus phoxinus L, (Gentle, 1972), and the killifish, Fundulus heteroclitus (Butcher and Adelmann, 1937; Butcher, 1938), the dorsal retina is important in inducing pallor. Removal of the dorsal retina in P. phoxinus prevented white background adaptation (Osborne Index 6 - 7.5, where 1= maximal pallor and 9= maximal darkening), while removal of the ventral retina prevented maximal darkening (O.I.: 4 - 6.5) (Gentle, 1972). In the three-spined stickleback, Gasterosteus aculeatus (Hogben and Landgrebe, 1940) the lateral area appears to be involved - the extreme dorsal retina being uninvolved in colour change responses. Interpretation of the results of experiment three suggest that illumination of particular regions of trout retina are important for the secretion of MCH. It is tentatively suggested that stimulation of the lateral retina of trout is required for elevated plasma MCH concentrations, and thus pallor. In those experiments when the lateral retinae of fish were illuminated (W and Bb), plasma MCH levels were higher than when lateral illumination was minimized (B and Bw), irrespective of whether ventral (B) or dorsal (Bw) retinae were illuminated with incident light. Hence, unlike P. phoxinus and F. heteroclitus, stimulation of the dorsal retina of O. mykiss does not seem to be involved in the paling response. Plasma MCH concentrations in Bw fish were low despite dorsal retinal illumination with incident light, together with some reflected light projecting onto the ventral retina. High plasma MCH concentrations were only found when the

lateral retina was stimulated, either by reflection from the side walls, or because fish faced into the direction of the light source, as in Bb. The present experiments therefore show that during colour-change responses, the eyes of trout function as integrated units. MCH release may be determined by the relative illumination of different retinal areas. In this way, information about the I/R ratio may be gained (ratio of ventral stimulation to lateral plus dorsal stimulation), with stimulation of the lateral retina also being important for the release of the paling hormone: MCH. A similar system may exist in P. phoxinus (Gentle, 1972). Total ablation of the ventral retina prevents the minnow from achieving maximum pallor, while removal of the dorsal retina prevents maximal darkening. Without these critical retinal areas, it may have been impossible for the fish to accurately determine the appropriate ratio of incident to reflected light entering the eye, and therefore fish did not respond as expected.

The design of experiment three was not ideal: fish plasma cortisol levels suggest that they were chronically stressed, probably as a result of crowding in the small experimental tanks. This crowding may have partly masked the background colour of the tank in which they were housed. Moreover, fish were only kept in the experimental tank for one day to ensure a reasonable standard of water quality, and it was not possible to perform all four experiments on the same day. By housing fish individually in small perspex tanks with continuously flowing aerated fresh water, competition between individuals could be removed. If the tanks were small enough to prevent the fish from

turning around or orienting themselves vertically in the tank (as they did in condition Bb) they would have the added advantage of ensuring that only the intended areas of retina were illuminated. In this way the influence of the dorsal, lateral, and ventral retinae on plasma MCH and αMSH levels over a several day period could be investigated. Alternatively, further eye-capping experiments could be performed in the larger 250l tanks, although it would be difficult to determine the intensity of light reaching the different retinal areas in this way.

The second problem arising from the first experiment was why fish were an intermediate colour in the dark, despite having significantly lower circulating MCH levels than, and similar circulating α-MSH levels to, fish maintained on an illuminated black background. This difference in body coloration cannot be therefore accounted for simply by MCH or α-MSH levels. Such nocturnal body lightening has been reported in a number of vertebrates, including Anolis carolinensis lizards (Underwood, 1985); Xenopus laevis tadpoles (Bagnara, 1963); the ammocoete larvae of a few species of lamprey (Young, 1935; Eddy and Strahan, 1968; Joss, 1973), as well as several teleosts: the upside-down catfish, Synodontis nigriventis (Nagaishi and Oshima, 1989), the winter flounder, Pseudopleuronectes americanus (Burton, 1987), and trout, Salmo gairdneri R. (Hafeez and Quay, 1970; Owens *et al.*, 1978). In many cases, this dark-induced pallor has been attributed to melatonin release from the pineal at night. Melatonin is a powerful melanosome aggregating agent in amphibia (Lerner *et al.*, 1958), but in the pencil fish, it may produce both pallor and darkening, depending on the sensitivies of the melanophores (Reed, 1968). Diurnal colour

changes are widely observed in early developmental stages of lower vertebrates, such as primary stage tadpoles, and are therefore considered to be a primitive characteristic. Evidence for the involvement of melatonin in nocturnal pallor in adult trout was provided by Hafeez and Quay (1970). They found that blind trout, although dark in colour during periods of illumination, became paler at night but after pinealectomy, they no longer showed this nocturnal paling. Furthermore, Hafeez (1970) showed that pharmacological doses of melatonin administered through intra-peritoneal injection, or via the ambient water were able to induce pallor in trout. Later work on trout (Owens *et al.*, 1978) suggested that under natural photoperiodic conditions, trout would not normally show nocturnal pallor. However, it is possible that in experiment one, high plasma melatonin titres were responsible for the intermediate shade of the fish maintained in continuous darkness for a week. Iodinated melatonin binds to trout scales (Chapter Five, II), suggesting the presence of putative melatonin receptors, yet it is difficult to induce melanosome aggregation with exogenous melatonin in trout melanophores *in vitro* (Rance and Baker, 1979).

Recently, Burton (1991), working on the winter flounder, Pseudopleuronectes , showed that surgical denervation of melanophores, by severing the spinal chord, or local injection of phentolamine, an α-adrenergic blocker, abolishes this nocturnal paling response. It was therefore suggested that local nervous stimulation of melanophores (rather than hormonal control) causes nocturnal pallor, at least in this species. Little is known about the activity of the sympathetic

neurones which innervate trout melanophores, and how this varies day and night. It is possible that the local sympathetic neurones fire under both illuminated and dark conditions but that firing is inhibited on a black background (when the ventral retina is preferentially illuminated) so that the fish appear dark in colour rather than an intermediate shade. On an illuminated white background fish are very pale. Such pallor may result from a combination of melanophore innervation and MCH release. Green and Baker (1989) have shown that exogenous norepinephrine will synergise with MCH at the melanophore level *in vitro* and this may also occur *in vivo*.

CHAPTER FOUR

A DIURNAL RHYTHM OF PLASMA MCH LEVELS IN TROUT

4.1 INTRODUCTION

In teleosts, chromatic adaptation to pale backgrounds is achieved partly by MCH (Rance and Baker, 1979; Oshima *et al.*, 1985; Baker, 1988a). Plasma MCH concentrations in white-adapted trout are significantly higher than those of black-adapted counterparts (Eberle *et al.*, 1989; Kishida *et al.*, 1989; Green *et al.*, 1991; present work, Chapter Three). MCH acts directly at the melanophore level, causing aggregation of melanin granules within melanophores (Oshima *et al.*, 1985; Nagai *et al.*, 1986; Baker, 1988b; Green and Baker, 1989) so that the fish appears pale in colour. High circulating concentrations of MCH have also been shown to inhibit the release of the antagonistic melanotropin: α-MSH (which causes body darkening) and possibly thereby reduce melanogenesis (Baker *et al.*, 1986). MCH will also inhibit the release of α-MSH from teleost pituitaries *in vitro* (Barber *et al.*, 1987).

MCH perikarya and fibres have been immunocytochemically demonstrated in the hypothalamus and pituitary of a variety of teleost species, including rainbow trout (Naito *et al.*, 1985; Powell and Baker, 1987, 1988; Batten and Baker, 1988; Bird and Baker, 1989; Bird *et al.*, 1989). The majority of immunoreactive MCH fibres terminate in the neural lobe of the pituitary (Naito *et al.*, 1985; Powell and Baker,

1987, 1988; Batten and Baker, 1988), where the hormone is stored prior to release. Transferral of fish from white to black backgrounds results in an accumulation of MCH in the pituitary as hormone release is suppressed on a black background but synthesis continues (Rance and Baker, 1979; Barber *et al.*, 1987). That hormone is released into the blood of fish on a white background was later confirmed (Kishida *et al.*, 1989). Plasma MCH concentrations in trout transferred from black to white tanks rose to peak values within two hours (Kishida *et al.*, 1989), suggesting that MCH is released directly in response to background colour.

MCH release is not, however, controlled solely by background colour. Trout maintained in the dark have significantly lower plasma MCH values than those on an illuminated black background (Kishida *et al.*, 1989; present work; Chapter Three) suggesting that in the absence of light, MCH release is inhibited (or not stimulated). It was therefore decided to investigate whether the plasma concentrations of MCH varied over a 24 hour period in fish adapted to long or short daylengths. Long periods of retinal illumination, as in the summer, might be expected to enhance MCH release, and shorter periods to curtail its release. Manipulation of environmental lighting conditions may thus increase understanding about the control of MCH release.

4.2 MATERIALS AND METHODS

SHORT DAYLENGTH

In December, 150 fish were collected and placed in white 250l tanks in

groups of 12. They were acclimated for two weeks under a short photoperiod regime resembling natural photoperiodic conditions, viz. 9L:15D, with lights on at 07.30 and off at 16.30 (GMT). Tanks were illuminated with overhead fluorescent lights. The intensity of incident light measured at the water surface was about 170 lux. Although lighting levels were significantly lower than those observed outside (Chapter Two; Fig. 2.1), such light intensities were considered sufficient to entrain trout photoperiodic rhythms (Bromage, per. com.). Blood was collected from six fish every three hours (Chapter Two) giving each tank a 24 hour interval between disturbances. During the dark period, sampling was done under a red safety light.

The effect of extending the photoperiod on trout plasma MCH levels was investigated by either a) advancing the time of light onset by six hours, or b) by delaying dark onset by six hours. In both experiments, 48 fish were first sampled over a 24h period under the normal light regime. This served as the control. More fish were then sampled immediately under the new light regime, while others were sampled after a week's acclimation to the new photoperiod.

LONG DAYLENGTH

Fish were collected in June and placed in white 250l tanks in groups of 12. They were acclimated to a 18L:6D photoperiod regime for two weeks with lights on at 04:30 and off at 22:30 (BST). Fish were collected under a dim red light during the dark periods.

Two experiments were carried out to investigate a) the effect of delaying light onset by six hours, and b) the effect of continuous light on trout plasma MCH concentrations. In each case, 48 fish were first sampled over a 24h period under the normal light regime (control) and sampling continued immediately after changing the photoperiod. Plasma MCH levels were also measured over a 24h period in fish which had acclimated to constant light for 10 days.

PLASMA MCH CONCENTRATIONS IN TROUT ADAPTED TO A BLACK BACKGROUND

In May, fish were placed in black 250l tanks in groups of 12 and acclimated to a 18L:6D photoregime for two weeks. Lights came on at 04:30 and went off at 22:30. Sampling began at 22:00 and continued until 22:00 the following evening.

STATISTICS

One way analysis of variance (ANOVA) was used to compare daily changes in plasma MCH concentrations.

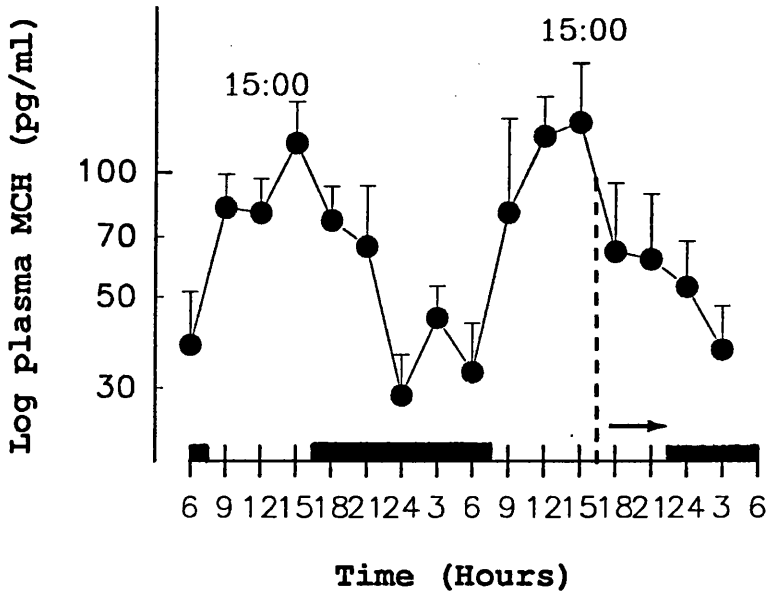
4.3 RESULTS

In two experiments in which trout were adapted to short daylengths, plasma MCH concentrations varied diurnally with low levels during the night (scotophase) and higher levels during the day (photophase). The pattern of change in plasma MCH concentrations over the 24h period varied slightly in the two experiments, however. In experiment one (Fig 4.1a), titres rose gradually after light onset with peak MCH concentrations being recorded 7.5h after light onset (peak values:- 107.9 ± 29.2 and 127.7 ± 49.1 pg/ml). After dark onset, MCH concen-

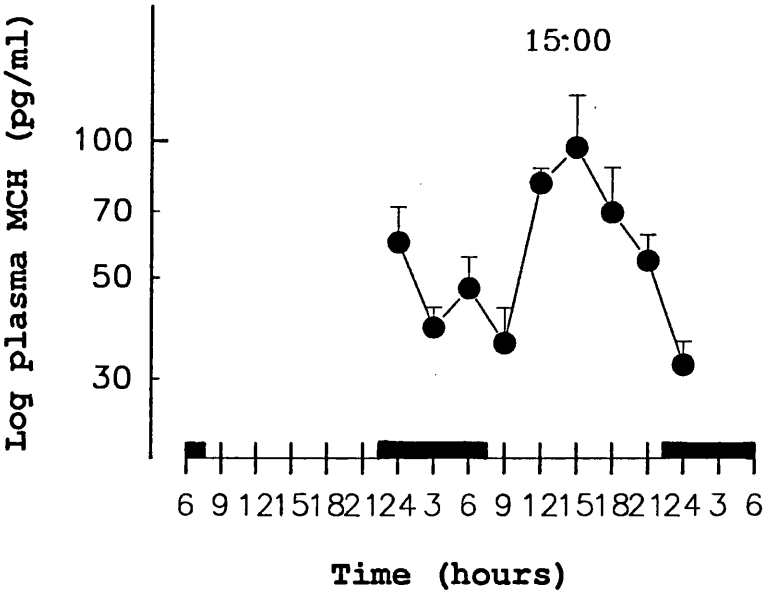
Figure 4.1

Plasma MCH concentrations in trout adapted to short daylength (9L:15D)

a) Effect of delayed dark onset.



b) Seven days later.



Blood was collected every three hours and MCH concentrations measured by solid-phase RIA. Values given as mean ± SEM, n=6. Dark bar represents scotophase.

trations then fell gradually, with minimum values recorded around midnight (27.7 ± 7.2 pg/ml, n=6) and remained low throughout the remaining scotophase. Similarly, in experiment two (Fig. 4.2a), plasma MCH concentrations rose gradually with light onset, but in this case, peak MCH concentrations (101.6 ± 10.3 pg/ml, n=6) were recorded 4.5h after light onset, three hours earlier than those in fish the previous year. Plasma MCH concentrations fell significantly 1.5h prior to dark onset. Hence, at 15:00 the levels of plasma MCH measured in trout acclimated under essentially the same conditions, varied significantly between years.

a. Effect of delayed dark onset on plasma MCH concentrations in trout

The results of experiment one (Fig. 4.1a) suggested that MCH titres decreased in response to dark. However, when the onset of dark was delayed by six hours, one and a half hours after presumptive dusk, levels of plasma MCH fell despite prolonged photophase, resembling the pattern seen in the acclimated fish. Much intra-group variance was evident in plasma MCH concentrations from all groups of fish throughout the six hour extended photophase, making the decline in plasma MCH titres at 18:00 insignificant. With dark onset, the variance between plasma MCH concentrations in individual fish declined and levels continued to fall 4.5h after new light offset (10.5h after previous dusk).

After one week's adaptation to the new 15L:9D photoregime (Fig. 4.1b), peak plasma MCH values were seen 7.5h after light onset, as before,

and values again declined prior to dark onset. Variance in plasma MCH titres from all groups of fish was smaller. Peak (96.8 ± 29.4 pg/ml) and nadir (38.9 ± 4.2 pg/ml) plasma MCH values were not significantly different from those of fish adapted to a short daylength photoregime (9L: 15D). That is, plasma MCH values in trout adapted for one week to a 15L: 9D photoregime were essentially the same as those adapted to a 9L: 15D photoregime.

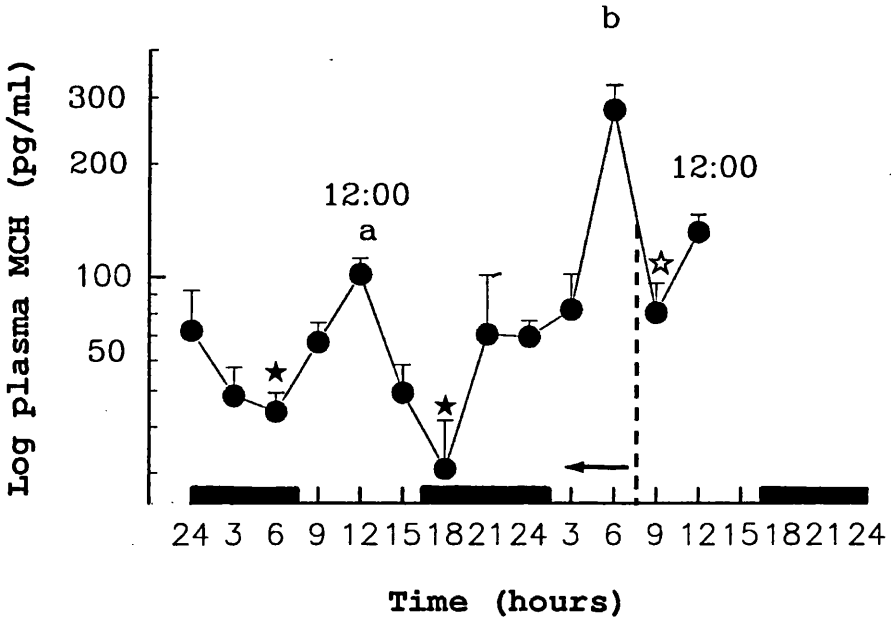
b. Effect of advanced light onset on plasma MCH concentrations

When the lights came on six hours earlier than normal at 01: 30 instead of 07: 30, plasma MCH concentrations rose to peak values 4.5h after light onset. This resembled the response to light onset for fully acclimated fish, monitored on the previous day, except on that occasion the peak occurred at 12: 00 noon (Fig. 4.2a). Unlike the previous day, however, peak MCH titres were three-fold higher than before. By 09.00, 7.5h after light onset, levels of MCH had declined significantly, as on the previous day, but instead of continuing to decline, plasma MCH concentrations rose again at 12.00 noon, as though returning to the normal diurnal pattern of secretion. Unfortunately, sampling was discontinued at this point, so it is not known whether the hormone would have declined in the normal pattern or whether the hormone levels would have continued to fluctuate.

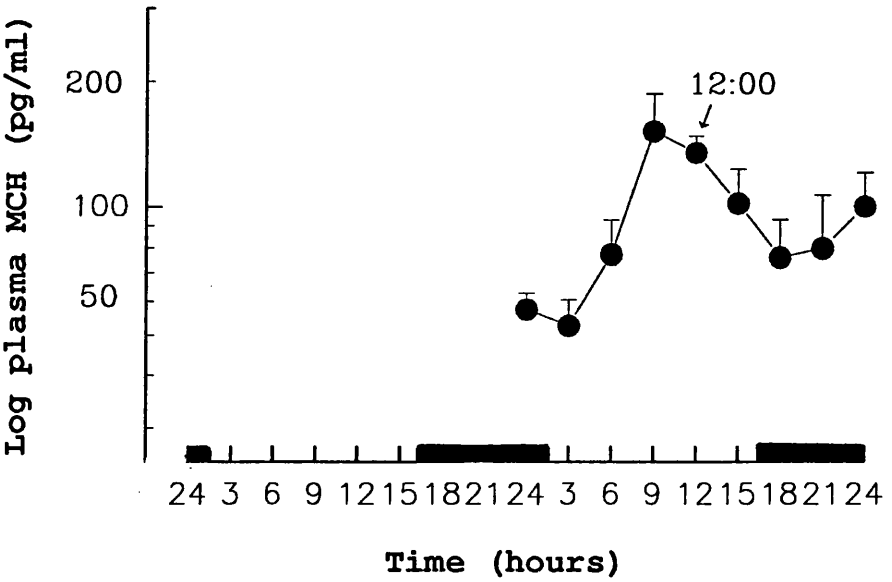
After one week's acclimation to the new 15L: 9D photoregime (Fig. 4.2b), peak MCH concentrations had shifted by three hours to coincide with the mid-photophase, 7.5h after light onset. Plasma MCH levels then gradually declined before dark onset.

Figure 4.2
Plasma MCH concentrations in trout adapted to short daylength (9L:15). Experiment Two

a) Effect of advanced light onset



b) Seven days later



Blood was collected every three hours and MCH titres measured by solid-phase RIA. Values are given as mean ± SEM, n=6. Solid horizontal bar represents scotophase.

★P < 0.05 compared with a
☆P < 0.05 compared with b

In conclusion, trout MCH concentrations in fish adapted to a short day-length photoregime show about a three-fold change in light and dark. Plasma levels rise in response to light onset but do not remain high throughout the photophase; MCH titres fall in anticipation of dark.

LONG DAYLENGTH

As in winter, trout plasma MCH concentrations varied diurnally with low levels during the night and high levels during the day (Fig. 4.3). Peak plasma MCH concentrations in fish adapted to 18L:6D photoregimes, however, were about three-fold higher than those recorded in fish adapted to short daylength regimes (9L:15D). These fish also showed a larger intra-group variation during the photophase, with some individuals appearing more responsive to long daylength than others.

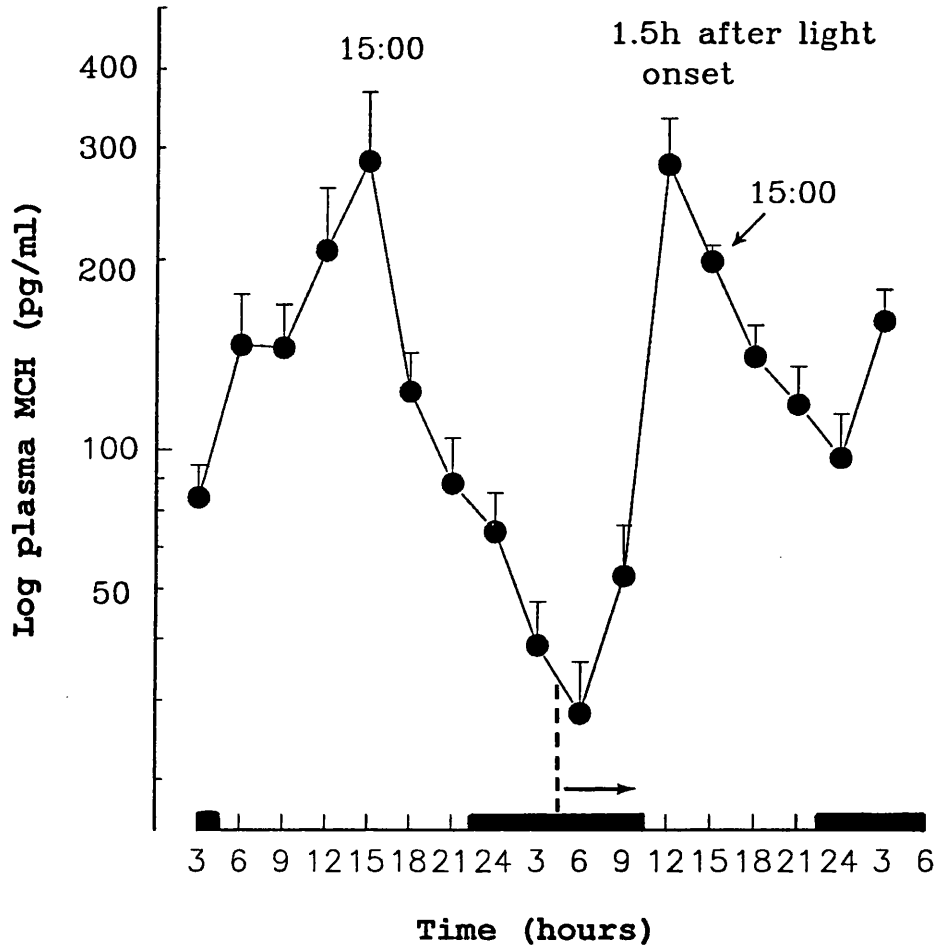
Plasma MCH concentrations rose gradually after light onset. Peak values were recorded 10.5h after light onset and then declined gradually prior to dark onset. Plasma MCH concentrations measured during the scotophase were the same regardless of the photoregime to which fish were adapted.

a. Effect of delayed light onset on plasma MCH concentrations in trout

Delaying light onset by six hours (and thereby extending the dark phase) prevented the predicted rise in trout plasma MCH concentrations at 06:00 (Fig. 4.3). MCH levels remained low (less than 63 pg/ml) during the prolonged darkness. One and a half hours after light onset,

Figure 4.3
PLasma MCH concentrations in trout adapted to long daylength (18L:6D)

Effect of delayed light onset



Blood was collected from trout adapted to an 18l:6D photoregime every three hours and MCH concentrations were measured by solid-phase RIA. Values are given as mean ± SEM, n=6. Solid bar represents scotophase. Light onset (04:30) is delayed by six hours on the second day. MCH values remained low during the extended darkness but reached normal peak values 1.5h after delayed light onset.

however, levels had risen significantly (P<0.01) to similar peak levels as the day before. Plasma MCH titres then declined prior to dark onset, in an almost parallel pattern to those the previous day. Yet, at 03:00 (1.5h after lights off) MCH titres appeared to increase (160.2 ± 19.5 pg/ml). This increase in circulating MCH was not expected at this time, the value being significantly different from that recorded at 03:00 the previous morning.

After one week's adaptation to the new photoregime, plasma MCH concentrations were measured only between 09:00 to 18:00 inclusive. Values were high for dark values, resembling those seen at 09:00 in the light on a long photoperiod. MCH titres rose progressively in the light. Although sampling ceased at this point, it is clear that peak MCH values had shifted by at least 7.5h under the new 12L:12D photoregime.

In conclusion, MCH concentrations in trout acclimated to a long day-length photoregime, increase by about 10-fold from dark to light. There is no rise in plasma MCH titres during the dark phase in anticipation of light onset. Plasma MCH concentrations remain low when the dark period is extended, but then rise more rapidly than usual when light goes on, to reach peak values at the usual time of day.

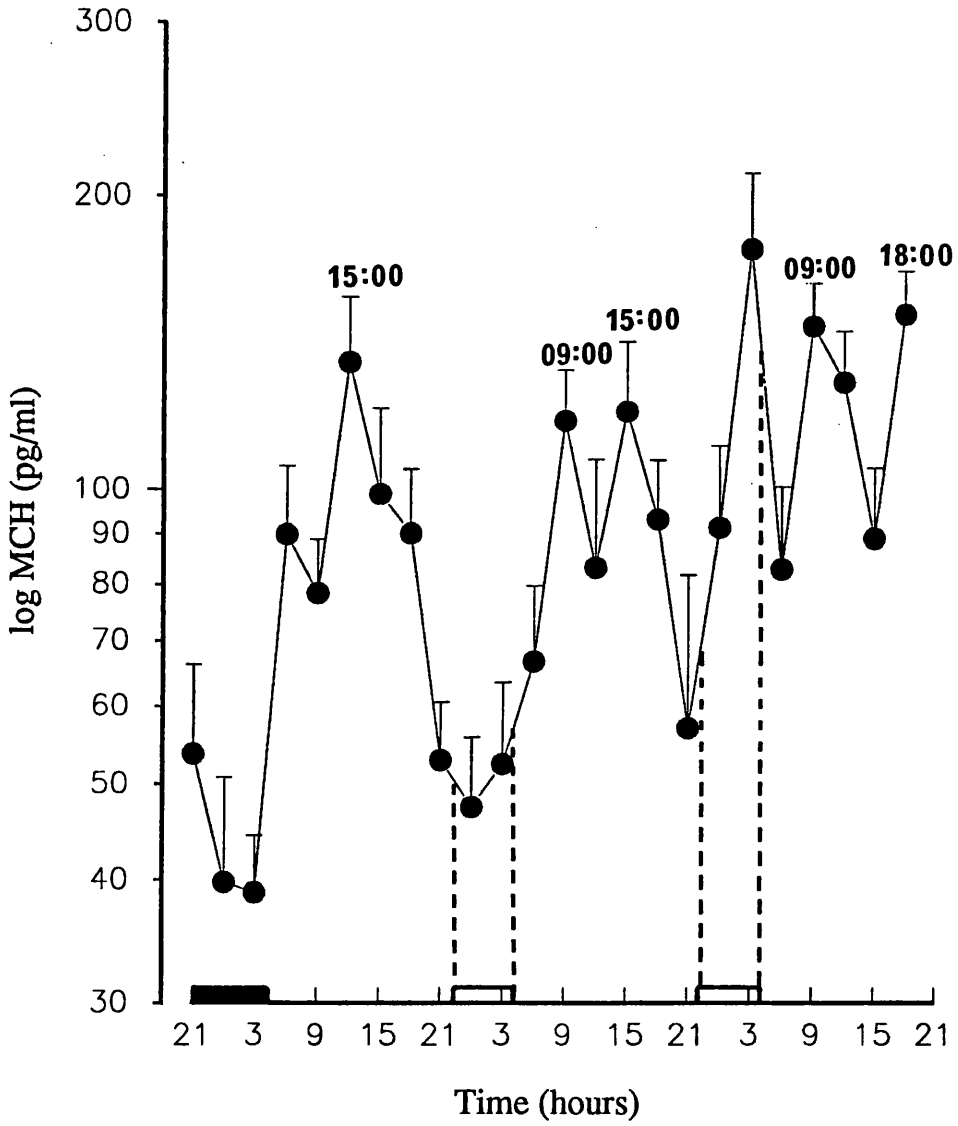
EFFECT OF CONSTANT ILLUMINATION ON PLASMA MCH CONCENTRATIONS IN TROUT

a. Over three days

Plasma MCH concentrations were measured every three hours in fish over a period of three days under constant light. During this experiment

Figure 4.4

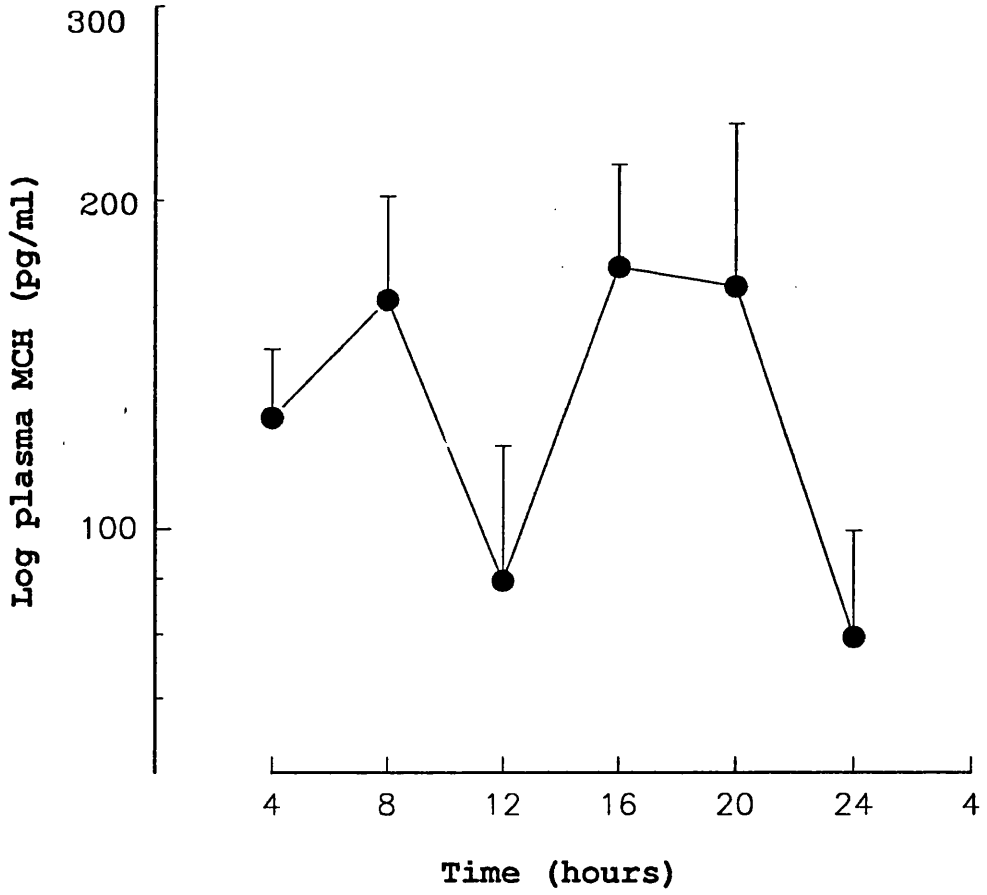
Changes in plasma MCH concentrations when white-adapted trout maintained under a long photoperiod (18L:6D) are placed under constant illumination for three days.



Blood was collected every three hours, starting at 21:00 during the last dark period. MCH concentrations were measured by solid-phase RIA. Values are given as mean ± SEM, n=6. Light onset was at 04:30. The solid horizontal bar represents the scotophase.

Figure 4.5

Plasma MCH concentrations in trout maintained in white tanks under constant illumination for one week.



Blood was collected every four hours throughout the 24 hour period and MCH concentrations measured by solid-phase RIA. Values are given as mean ± SEM, n=6.

the MCH solid-phase RIA gave technical problems, for reasons which are not yet clear. Samples collected over each 24h period were assayed together. Although the intra-assay variation was low, repeated assays of the same samples gave different absolute values in different assays. The means of the corrected values for each assay have therefore been calculated and are plotted on Fig. 4.4.

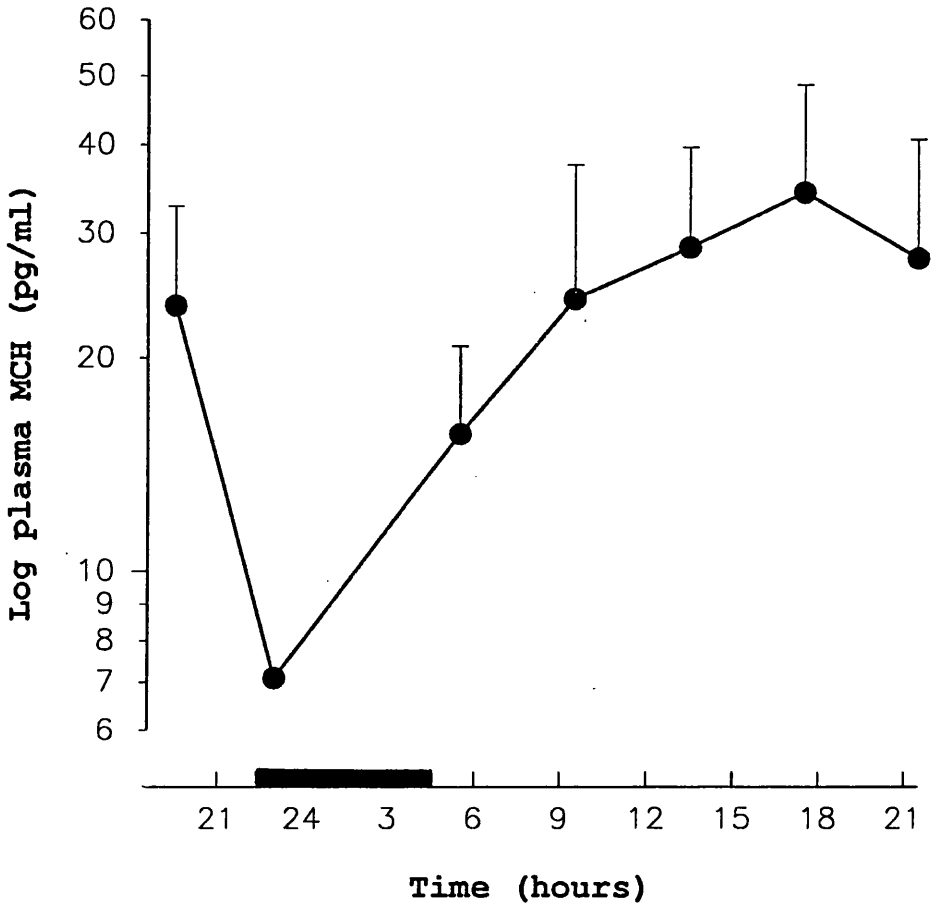
Over the first 24h, plasma MCH concentrations varied in a similar way to those of fish normally adapted to long photoregimes (18L:6D). Plasma MCH concentrations rose gradually with light onset to reach peak levels at 15:00, ie. 10.5h after dawn. Thereafter levels declined to near normal nocturnal values, and remained low despite the absence of dark. At 06:00 on the second day, 1.5h after the expected light onset, plasma MCH concentrations were the same as at this time the previous day, but then rose to premature peak values at 09:00, and fell to nocturnal values at noon, and rose once again to peak values at 15:00. Plasma concentrations then declined gradually prior to expected dark. Throughout this period and over the next 24h, peak plasma MCH concentrations were observed every 7.5h.

b. After one week

Trout maintained under constant light for one week had plasma MCH concentrations ranging from 79.6 to 173.7 pg/ml and the variation within each time group was high (Fig. 4.5). Two apparent peaks and two nadirs of MCH concentration were observed but none of the mean values were statistically different from each other. Sampling was only

Figure 4.6

Plasma MCH concentrations in trout maintained in black tanks under a 18L:6D photoregime. (mean ± SEM, n=6).



Blood samples were collected throughout the dark and light periods at regular intervals and MCH was measured by RIA. The solid horizontal bar represents scotophase.

performed every fourth hour for 24h, hence some of the resolution of the rhythm is expected to be lost.

In conclusion, although MCH secretion was suppressed during the first illuminated night-time period, and secretion increased at the normal time on the second day, the rhythm thereafter rapidly became disrupted. Autocorrelation analysis showed that the cyclical nature of trout plasma MCH concentrations was not evident under periods of constant illumination.

PLASMA MCH CONCENTRATIONS IN TROUT ADAPTED TO A BLACK BACKGROUND

Plasma MCH concentrations in fish adapted to a black background under a long day-length photoregime for two weeks (Fig. 4.7) also varied diurnally, with nadir at 24:00 (7.1 ± 0.13 pg/ml) and peak at 18:00 (34.2 ± 14.2 pg/ml) some 13.5h after light onset. Plasma MCH concentrations gradually declined prior to dusk, whereupon levels fell rapidly (from 21:00 to 24:00).

4.4 DISCUSSION

The results of these experiments show that MCH release is not solely controlled by retinal illumination, (as could be inferred from the comparison of plasma MCH titres in fish adapted to white or black backgrounds or kept in the dark: Chapter Three), but is also released on a diurnal basis. Plasma levels of MCH increased after light onset and reached maximum values around mid-photophase. In all but the first experiment (Fig. 4.1a), plasma MCH concentrations started to decline prior to the onset of darkness, rather than in response to it. This

suggests that the release of MCH is modulated by some time-keeping mechanism which can assess daylength and adjust MCH secretion accordingly so that peak values are achieved at mid-photophase. An extension of the photophase (delayed dark onset) did not, therefore, stimulate more MCH release - as one would have expected if MCH was released directly in response to retinal illumination. Moreover, fish seemed able to show some adaptation to new photoregimes so that after only one week, peak plasma MCH concentrations were shifted towards mid-photophase. Many rhythms require 8-14 days to entrain to new light/dark cycles (Binkley, 1988) and this may have proved more satisfactory in trout.

Light onset seems to act as an important cue for enhanced MCH release. Advancing the dawn by six hours in fish adapted to a short daylength regime (Fig. 4.2a) advanced MCH release such that peak levels were observed 4.5h after light onset, as on the previous day. Levels fell significantly over the next three hours, again as on the day before, but plasma MCH titres had risen again by 12:00. This noon-time increase in plasma MCH concentration was statistically insignificant. Had sampling continued for a longer period of time it would have been evident whether MCH values were returning to the normal diurnal pattern of secretion with a peak at midday or whether plasma levels were fluctuating randomly. Such a peak might suggest that MCH release is coupled to a pacemaker. Further support for the idea that MCH release is linked to a pacemaker is derived from the experiment involving fish adapted to a long photoperiod. Delaying light onset for six hours suppressed MCH release but when the light was eventually

switched on, a significant and rapid release of MCH was observed so that in only 1.5h, plasma MCH concentrations were almost identical to peak values of the previous day. Normally these values are reached only 10.5h after light onset. This suggests that the prolonged scotophase did not suppress MCH synthesis but only its release: the synthesis of MCH may be coupled to a pacemaker. Hence, the prolonged dark period may have suspended the normal post-dawn depolarization of MCH neurones to release small amounts of MCH into the blood. When light onset finally occurred, six hours late, all the MCH neurones may have depolarized simultaneously, creating a peak of plasma MCH. Under such conditions, one might expect that fish maintained in the dark for a week (Chapter Three) would have very low plasma MCH levels but high pituitary and hypothalamic stores, since MCH synthesis would be expected to continue on a diurnal basis without being released. Pituitary stores of MCH were indeed found to be significantly higher in dark-adapted than illuminated trout in either black or white tanks (Chapter Three) and hypothalamic MCH stores in the same fish were significantly higher than those in white-adapted trout.

In the aquarium, mid-day levels of MCH observed in summer were about three times higher than those observed in winter. However, the total MCH release (area under the curve) during the day in summer was twice that of fish in the first winter experiment, and five times that in the second winter experiment. Green (1990) measured plasma MCH levels throughout the year in trout kept at the fish farm under natural lighting conditions, but no significant seasonal differences were observed in plasma MCH titres from fish sampled in the field in the

morning. It should be noted, however, that trout maintained under these fish-farm conditions have very low plasma MCH levels: similar to black-adapted aquarium-housed fish, so differences between summer and winter values would have been difficult to detect. Although a diurnal rhythm of plasma MCH concentrations was observed in black-adapted trout maintained under aquarium conditions (Fig. 4.6), seasonal variation of plasma MCH levels in black-adapted fish was not investigated.

Many hormones are released diurnally. The possible interaction between MCH and the pineal, however, prompted the investigation of whether MCH release was circadian rather than diurnal in nature. Most circdian rhythms which are controlled by the pineal will free-run in conditions of constant darkness (DD). Under such conditions, however, MCH release is not stimulated (or is inhibited) (see Chapter Three, Fig. 3.1). Plasma MCH concentrations were therefore measured over three days in constant light to see whether the rhythm would be self-sustaining and continue despite constant conditions. Trout plasma MCH titres showed a characteristically diurnal pattern over the first 24 hours (Fig. 4.4), but by the third day levels fluctuated with a phase of about 7.5h. Thus, the MCH rhythm did not free-run in constant light and so may not be circadian in nature. Interestingly, similarities were observed between the pattern of MCH release during the second day under constant illumination, and that observed in fish after a six hour advance of dawn ie., a premature peak of MCH secretion and a subsequent peak at the normal time. The significance of this is uncertain but it may be that the absence of the six hour nightly

darkness was treated as a six hour advance in photophase. After a week of continuous illumination (Fig. 4.5), trout plasma MCH concentrations may have been biphasic with peak concentrations every 10h. Intra-group variation of plasma MCH titres was very high, perhaps indicating that as MCH release in individual fish was no longer entrained by the light/dark cycle, fish no longer secreted in synchrony. Alternatively, the biphasic pattern of MCH release under conditions of constant light might also suggest the presence of more than one oscillator governing this rhythm. The circadian rhythms of many multicellular organisms are governed by several pacemakers. These may be linked in series (laterally) or may each be independently entrainable by the light-dark cycle. Typically under constant light (LL) the rhythm becomes bimodal, or 'splits'. For example, when the arctic rodent Sperophilus undulatus is maintained for extended periods in LL, the activity rhythm splits spontaneously before rejoining as one activity band (Pittendrigh, 1960). Similar phenomena have been observed in the starling, Sturnus vulgaris, (Gwinner, 1974) and a lizard, Sceloporus olivaceus, (Underwood, 1977). Hence, the biphasic variation in trout plasma MCH concentrations may indicate the presence of a multiple pacemaker system but this idea needs further investigation.

The adaptive significance of this diurnal release of MCH is not known at present. In teleosts, MCH is primarily involved in maintaining pallor and one would therefore expect it to be released directly in response to pale background colour, regardless of the daylength to which the fish were adapted. MCH has, however, been implicated in a number of other physiological functions, such as stress, and sexual

maturity, and its diurnal release may be related to these. One of the most important of these is the relationship between stress and MCH release. Under chronic stress, white-adapted trout (with high plasma MCH concentrations) have been shown to secrete about four times less cortisol than black-adapted fish (Baker and Rance, 1981). They also secrete significantly lower levels of cortisol in response to acute stress, and this response is more easily suppressible with dexamethasone, than in black-adapted fish (Gilham and Baker, 1985). During a 24h period there also appears to be a reciprocal relationship between plasma MCH and cortisol. Continual measurement of plasma cortisol in fish adapted to low-level chronic stress (Pickering and Pottinger, 1983; Rance *et al.*, 1982) showed that values also vary diurnally. Peak concentrations were observed around midnight, when MCH release was depressed, and levels tended to be low throughout the photophase. Plasma cortisol concentrations measured in trout during the present experiments were always low (less than 6ng/ml) and no overt diurnal rhythm was observed. This shows that these trout were maintained in a relatively stress-free environment throughout the experimental period, so it is unlikely that the interaction between plasma cortisol concentrations and MCH secretion is responsible for the diurnal variations of MCH concentrations. It is possible, however, that the fluctuations in plasma MCH values observed during the scotophase may have been partly due to handling stress under experimental conditions.

The age or sexual maturity of the fish used may also affect plasma MCH concentrations. These factors are known to affect both the cortisol

and MCH values. Several investigators (Barton *et al.*, 1980; Pickering and Christie, 1981; Rance *et al.*, 1982) have shown that the age of trout may influence plasma cortisol concentrations: younger fish have significantly lower circulating cortisol than older fish. Plasma MCH levels in sexually mature males are also significantly lower than those of female trout (Baker, per. obs.) which suggests that MCH might also have a reproductive role in trout. For this reason, all the present experiments were performed on sexually immmature female trout.

CHAPTER FIVE

A POSSIBLE INTERACTION BETWEEN MELATONIN AND MCH CONCENTRATIONS IN TROUT

Melanin-concentrating hormone was so-named because of its ability to cause melanosome aggregation within teleostean melanophores, so that fish appear pale in colour. It is released into the circulation in response to pale background colour (Baker, 1988a, b; Barber *et al.*, 1987; Kishida *et al.*, 1989) and this response is thought to be primarily mediated by the eyes (present study, Chapter Three). In trout, plasma MCH concentrations rise to maximal values within two hours after transferral in the middle of the day from black to white tanks (Kishida *et al.*, 1989). Under normal photoperiodic conditions, plasma MCH concentrations in white-adapted trout also vary diurnally (present study, Chapter Four). During the dark, MCH values are low; concentrations rise slowly with light onset to reach a peak at around midday, and then gradually decline towards dark onset. Hence, the rise in plasma MCH concentrations after exposure to dark is much slower than after adaptation to an illuminated black background. It is theoretically possible that some factor released in the dark might inhibit MCH release. One such agent is melatonin, which is released by the pineal at night. Several pieces of circumstantial evidence suggest that the release of MCH might be linked to that of melatonin. Firstly, the pineal (like the eyes) is directly photoreceptive (Dodt, 1963; Ekstrom and Meissl, 1988; Meissl and Ekstrom, 1988a, b) and therefore

able to distinguish between light and dark, though not background colour. Secondly, neurones from the pineal terminate in the pretectal area of trout brain (Hafeez and Zerihun, 1974) a region also innervated by MCH fibres (Naito *et al.*, 1985) and retinal fibres (Pinganaud and Clairambault, 1979) which suggests that some communication between all three neurone types is possible. Thirdly, melatonin has been shown to modulate a number of other vertebrate hormones. Moreover, both melatonin (Hafeez, 1970) and MCH (cf Eberle, 1988) are able to cause melanosome aggregation in trout melanophores, although melatonin is not thought to be involved in background adaptation in trout (Owens *et al.*, 1978). This chapter therefore considers how MCH and melatonin might influence each other's release in trout. The effects of melatonin administration on trout plasma MCH concentrations were not investigated due to the absence of an assay for plasma melatonin and difficulties with melatonin administration. Preliminary investigations in which scilastic tubing packed with melatonin were implanted intra-peritoneally proved unsuccessful, as did intraperitoneal injection of a melatonin solution since the circulating concentration of melatonin at the time of the experiment could not be measured. Hence, the two main experimental approaches used were: firstly, the effect of exogenous MCH (and other agents) on melatonin release from isolated pineals *in vitro*, and secondly, autoradiographical investigations of the location of melatonin binding sites in the brain and pineal of trout. For ease of description, this chapter has therefore been sub-divided into two main sections.

5.1 SECTION ONE: REGULATION OF THE TROUT PINEAL *IN VITRO* : EFFECT OF MCH.

INTRODUCTION

The vertebrate pineal organ is thought to synchronize a number of biochemical, physiological and behavioural processes to the light/dark cycle through the synthesis and secretion of melatonin at night. Daily rhythms (such as activity cycles) are synchronized by elevated melatonin titres at night, while seasonal events (such as seasonal breeding) are believed to be regulated by the changing duration of elevated plasma titres throughout the year as a consequence of changing daylength (cf. Binkley, 1988).

Unlike most transmitter and hormone systems, melatonin is not stored prior to release (Gern *et al.*, 1978), nor does it seem to be released at specific sites. Being a small, lipophilic molecule, melatonin apparently diffuses out of the cell after synthesis into the general circulation and cerebrospinal fluid. In all vertebrates, melatonin release occurs during the dark phase of the photoperiod (scotophase) regardless of whether they are active during the day or night.

In trout, melatonin release measured *in vivo* (Duston and Bromage, 1986a) or *in vitro* (Gern and Greenhouse, 1988) rises immediately after dark onset. Levels remain high throughout the scotophase and decline again in response to light onset. Factors governing the circadian release of melatonin in trout are not yet understood. Like that of other non-mammalian vertebrates, the trout pineal is directly

photosensitive (Dodt, 1963; Meissl and Ekstrom, 1988a, b). It is situated under a transparent window of cartilage on the dorsal surface of the skull that allows between 1-50% incident light to penetrate it (Morita, 1966). Photoreceptive pinealocytes, with various morphologies, have characteristics of both rods and cones of the retina (Collin *et al.*, 1986) but unlike the eyes, they are unable to detect movement or contrast (Ekstrom and Meissl, 1989). Like retinal photoreceptors, pinealocytes are spontaneously active in the dark, but become hyperpolarised in response to light. The degree of hyperpolarization is inversely proportional to the intensity of illumination to which they are exposed. In trout, some pineal photoreceptors are most sensitive to blue/green light with wavelengths ranging between 520-530nm (Dodt, 1963; Meissl and Ekstrom, 1988a, b). However, since plasma melatonin concentrations are the same in fish on black or white backgrounds, Owens and co-workers (1978) suggested that the pineal is not involved in assessing the colour of the background to which fish adapt.

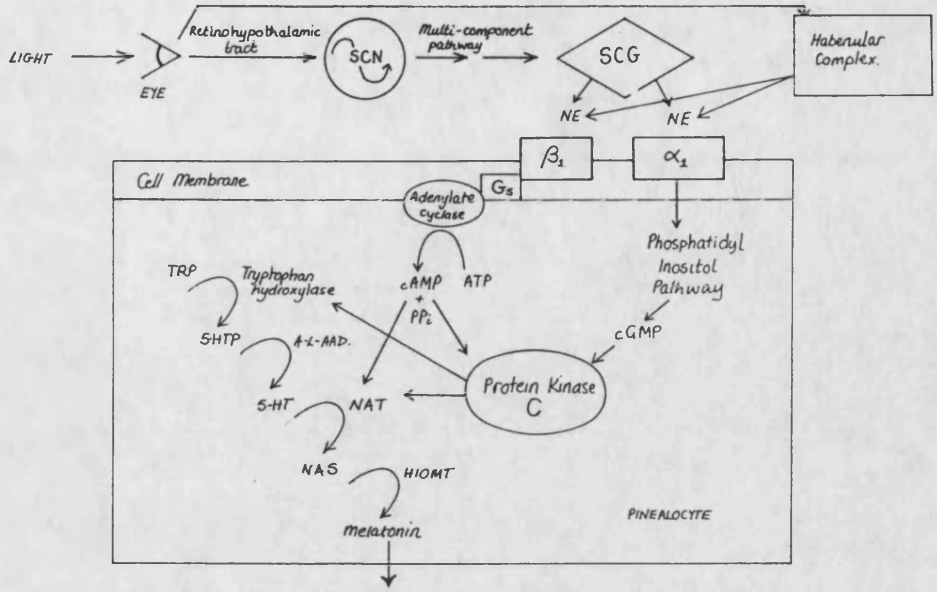
The mammalian pineal is not directly photosensitive. Information about environmental lighting conditions is conveyed from the retina along the retino-hypothalamic pathway to the suprachiasmatic nuclei (SCN) of the hypothalamus (Klein and Moore, 1979). These nuclei are thought to contain an endogenous rhythm generator or pacemaker which then drives the circadian rhythm of melatonin synthesis in the pineal (cf. Armstrong, 1989). Hence, the mammalian pineal is not a rhythm generator, it is an oscillator driven by the SCN. Information from the pacemaker is transmitted either via the habenular complex (Ronnekleiv

et al., 1980; Semm *et al.*, 1981; Moller and Korf, 1987) or via the superior cervical ganglia (SCG) along a complex multi-component pathway to the pineal. Sympathetic innervation provides the major regulatory input to the pineal. In the rat, norepinephrine stimulates melatonin synthesis by activating β_1- and α_1-adrenergic receptors (Yuwiler, 1987; Chik *et al.*, 1988a). Pinealocyte β_1-adrenoceptors activate adenylate cyclase (Chik *et al.*, 1988b) and so stimulate cAMP production. An increase in intracellular cAMP activates the rate-limiting enzyme for melatonin synthesis, N-acetyltransferase (NAT). Meanwhile pinealocyte α_1-adrenoceptors potentiate the β_1 response by activating the phosphatidyl inositol pathway, and probably protein kinase C (Klein *et al.*, 1983; Sugden *et al.*, 1985). Sympathetic fibres innervating the mammalian pineal have been shown to contain neuropeptide Y (Shiotani *et al.*, 1986; Moller *et al.*, 1987) which will also enhance melatonin production (Reuss and Schroeder, 1987; Vacas *et al.*, 1987). Many other neurotransmitters, including vasointestinal polypeptide (VIP), dopamine (DA) and γ-amino butyric acid (GABA) have also been implicated in modulating pineal melatonin production (Kaneko *et al.*, 1980) in mammals.

In birds, the system is more complex than in mammals, as the pineal itself is directly photoreceptive (cf. Binkley, 1988). Isolated chick pineals *in vitro* will rhythmically release melatonin into the medium when maintained under a normal light/dark cycle or under conditions of continuous darkness (Deguchi, 1979a, 1981; Takahashi *et al.*, 1980; Wainwright and Wainwright, 1980, Hamm *et al.*, 1983; Takahashi and Menaker, 1984; Robertson and Takahashi, 1988), or continuous red light

Figure 5.1: ADRENERGIC REGULATION OF MELATONIN SYNTHESIS

a) IN MAMMALS



b) IN BIRDS

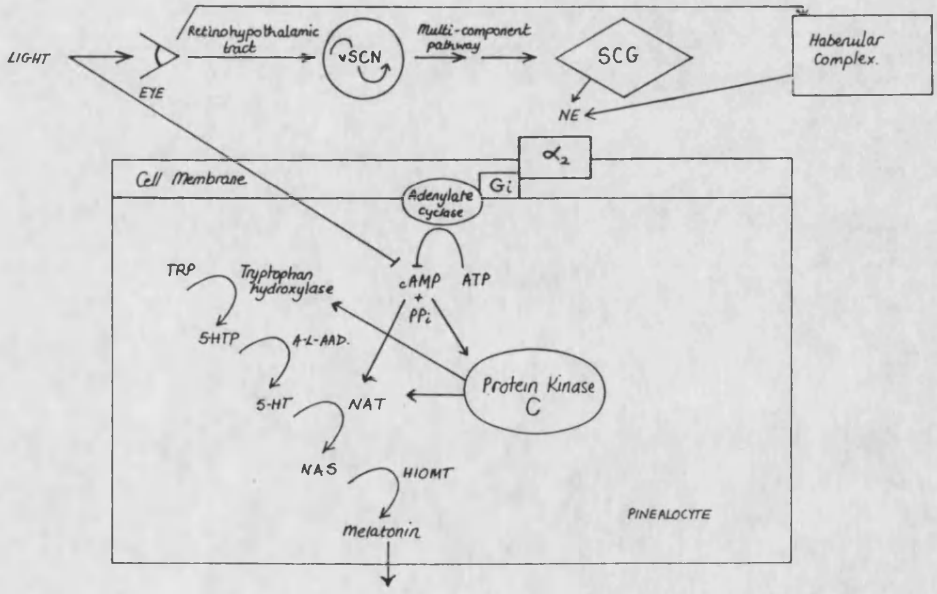


Diagram representing subcellular mechanisms controlling melatonin production after adrenergic stimulation of the mammalian and avian pineal gland. SCN; suprachiasmatic nucleus; vSCN; avian SCN-equivalent; SCG; superior cervival ganglion; NE; norepinephrine; Gs; G-stimulatory protein; Gi; G-inhibitory protein; cAMP; cyclic adenosine mono-phosphate; ATP; adenosine tri-phosphate; cGMP; cyclic guanine mono-phosphate; TRP; tryptophan; 5-HTP; 5-hydroxytryptophan; A-L-AAD; aromatic L-aminoacid decarboxylase; 5-HT; serotonin; NAT; N-acetyltransferase; NAS; N-acetylserotonin; HIOMT; hydroxyindole-O-methyltransferase; arrow; stimulation; blunt arrow; inhibition.

(Zatz *et al.*, 1988). Indeed, even dissociated chick pineal cells will express a circadian oscillation of melatonin production (Robertson and Takahashi, 1988) which is entrainable by light. Cells *in vitro* show an anticipatory rise in melatonin synthesis prior to lights off, and a decrease in melatonin production before light onset, which also suggests that the rhythm is not driven by lighting levels but contains a self-sustaining oscillator. Some information about photoperiod may still be relayed from the eyes along the retinohypothalamic pathway to the SCN-equivalent, and thence to the pineal via sympathetic innervation of the SCG. However, lesions in the SCG of blind birds do not abolish rhythmical melatonin synthesis within the chick pineal (Hamm *et al.*, 1983). Two explanations for this have been put forward: that of extraocular, non-pineal brain photoreceptors (cf. Cassone and Menaker, 1984) and /or pineal innervation from other central nervous projections (Korf *et al.*, 1982). Like mammals, the avian melatonin rhythm is inhibited by acute light exposure during the dark phase (Hamm *et al.*, 1983) and stimulated by agents which increase intracellular levels of cAMP (Deguchi, 1979b), and cGMP (Wainwright, 1980). But in contrast to mammals, the presence of adrenergic agents, such as norepinephrine (Zatz and Mullen, 1988), which is released from pinealopetal sympathetic nerves during the day, inhibits pineal melatonin synthesis (Cassone *et al.*, 1986a). This inhibition is thought to be mediated by postsynaptic α_2-adrenoceptors (Pratt and Takahashi, 1987; Voisin and Collin, 1986; Voisin *et al.*, 1987, Voisin *et al.*, 1990) which inhibit cAMP production via the Gi adenylate cyclase regulatory protein (Voisin *et al.*, 1990; Pratt and Takahashi, 1989). In birds, this effect can be blocked by phentolamine, a general

α-adrenergic antagonist. Hence, it is generally believed that during the day, visual information about lighting conditions is transmitted from the eyes to the SCN and SCG and then the pineal, to inhibit melatonin production, but that at night, melatonin release from the pineal feeds back to inhibit the SCN (cf. Cassone and Menaker, 1984). Although β-adrenoceptors have been identified in chick pineal cells (Bylund *et al.*, 1988) at a 10-fold lower receptor density than α_2-receptors, stimulation of β-receptors does not appear able to elevate cAMP levels for long enough to activate NAT (Voisin *et al.*, 1990). It may be that the β-receptors are located in cells that do not synthesise melatonin. Other agents known to affect chick melatonin release include forskolin and other cAMP-stimulating agents (Binkley *et al.*, 1978); VIP (Zatz *et al.*, 1990); and L-dopa (Wight, 1971).

TABLE 5.1

SUMMARY OF COMPOUNDS THAT AFFECT NAT ACTIVITY OR MELATONIN PRODUCTION IN MAMMALIAN AND AVIAN PINEALS *IN VITRO* AND *IN VIVO*.

COMPOUND	MAMMALS	BIRDS
Forskolin	+	+
Norepinephrine	+	-
L-Dopa	+	?
VIP	+	+
Carbachol	-	?
Acetylcholine	-	?
αMSH	-*	?

Note: Stimulatory (+); Inhibitory (-); Unknown (?); Depresses norepinehrine-induced stimulation (-*).

In comparison to mammals and birds, little is known about factors affecting the control of melatonin production in teleosts. It is well established that the teleostean pineal is photosensitive but it is not known whether the synthesis of melatonin is driven by an endogenous

circadian oscillator within the pineal, or whether this rhythm is driven by another pacemaker centre in the brain. As in other vertebrates, NAT (rather than HIOMT) acts as the rate-limiting enzyme in melatonin synthesis in teleosts (Birks and Ewing, 1981, 1986; Falcon *et al.*, 1987; Morton and Forbes, 1988). Melatonin is produced rhythmically when isolated pineals from pike (Falcon *et al.*, 1987) and goldfish (Kezuka *et al.*, 1988) are maintained in continuous darkness, but this is apparently not the case for trout pineals. Indeed, Gern and Greenhouse (1988) found that melatonin secretion from trout pineals *in vitro* rapidly dampened in continuous darkness with no depression at the time of natural day, but they commented that this did not preclude the presence of an endogenous pacemaker.

Given that circulating MCH concentrations fluctuate diurnally with high levels during the day and low levels at night, it was theoretically possible that high concentrations of plasma MCH might modulate pineal activity to reduce melatonin secretion. Moreover, the presence of both MCH (Naito *et al.*, 1985) and pineal (Hafeez and Zerihun, 1974) innervation in the pretectal area of trout brain suggested that there might be some communication between these two cell types. It was therefore decided to investigate a variety of peptides (including MCH) and neuromodulators that might affect melatonin secretion from trout pineals *in vitro*. Since norepinephrine is known to synergise with MCH at the melanophore level (Green and Baker, 1989) particular attention was paid to the effect of norepinephrine.

5.2 MATERIALS AND METHODS

The following experiments involve pineal incubations *in vitro* as described in Chapter 2.3. In the aquarium, trout were maintained under a photoregime which approximated the environmental photoperiod. Pineals were dissected from trout in the morning. They were incubated in 1ml Eagle's Minimum Essential Medium containing Hank's salts and Hepes buffer (Flow Laboratories, Scotland), either in the light, when basal melatonin was low, or in the dark, when melatonin secretion was enhanced. Agents were tested for their ability to enhance or depress melatonin secretion under both lighting conditions. After preliminary trials, the following regimes were adopted: illuminated pineals were pre-incubated for a period varying from 1-1.5h, after which the pre-incubation medium was discarded and subsequently medium was changed hourly. The first hour established the level of melatonin secretion in the light (basal level of secretion). Secretagogues were added during the following period when pineals were either maintained in the light or placed in the dark, fresh medium and secretagogues being added hourly.

The following agents were tested on melatonin secretion:

a. Forskolin (Sigma): (10^{-7}M, 10^{-6}M). Stock solution prepared in absolute ethanol and stored at -20°C before diluting appropriately in MEM.

b. Norepinephrine (Sigma): (10^{-6}M, 10^{-5}M, 10^{-4}M, 10^{-3}M)

c. General α-adrenergic agonist: Phenylephrine (Sigma) (10^{-4}M)

d. General β-adrenergic agonist: Isoproterenol (Sigma) (10^{-4}M)

e. General α-adrenergic antagonist: Phentolamine (Sigma) (10^{-4}M)

f. General β-adrenergic antagonist: Propranolol (Sigma) (10^{-4}M)

g. Dopamine (Sigma): (10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-5}M)

h. Dopaminergic antagonist, Haloperidol (Sigma) (10^{-4}M)

i. Acetylcholine (Sigma): (10^{-5}M, 10^{-6}M)

j. Acetylcholine agonist: Carbachol (Sigma) (10^{-4}M)

k. GABA (Sigma) (10^{-4}M)

l. MCH (Peninsula Laboratories) (10^{-8} M, 10^{-9}M)

m. α-MSH (Sigma) (10^{-8} M)

n. Vasointestinal Polypeptide (VIP) (Sigma): (10^{-4}M)

Stock solutions of agents b-h were dissolved immediately prior to use in 0.01M HCl containing 1mg/ml ascorbic acid, while stock solutions of agents i-l were dissolved in 0.01M HCl. All additives were diluted 1:100 in the tissue culture well to give the appropriate dilutions stated above.

Melatonin released into the incubation medium was collected each hour and measured by RIA (Chapter 2.8). Each pineal served as its own control. Hourly melatonin release from each pineal was divided by the corresponding basal release rate to give a factor of change from the basal, where basal release = 1. The mean factor of change for each experimental group was then plotted on a log scale. Data was arcsine squareroot transformed and then analysed using a two-tailed Student's t test.

5.3 RESULTS

Influence of light and dark on melatonin release from isolated trout pineals *in vitro*

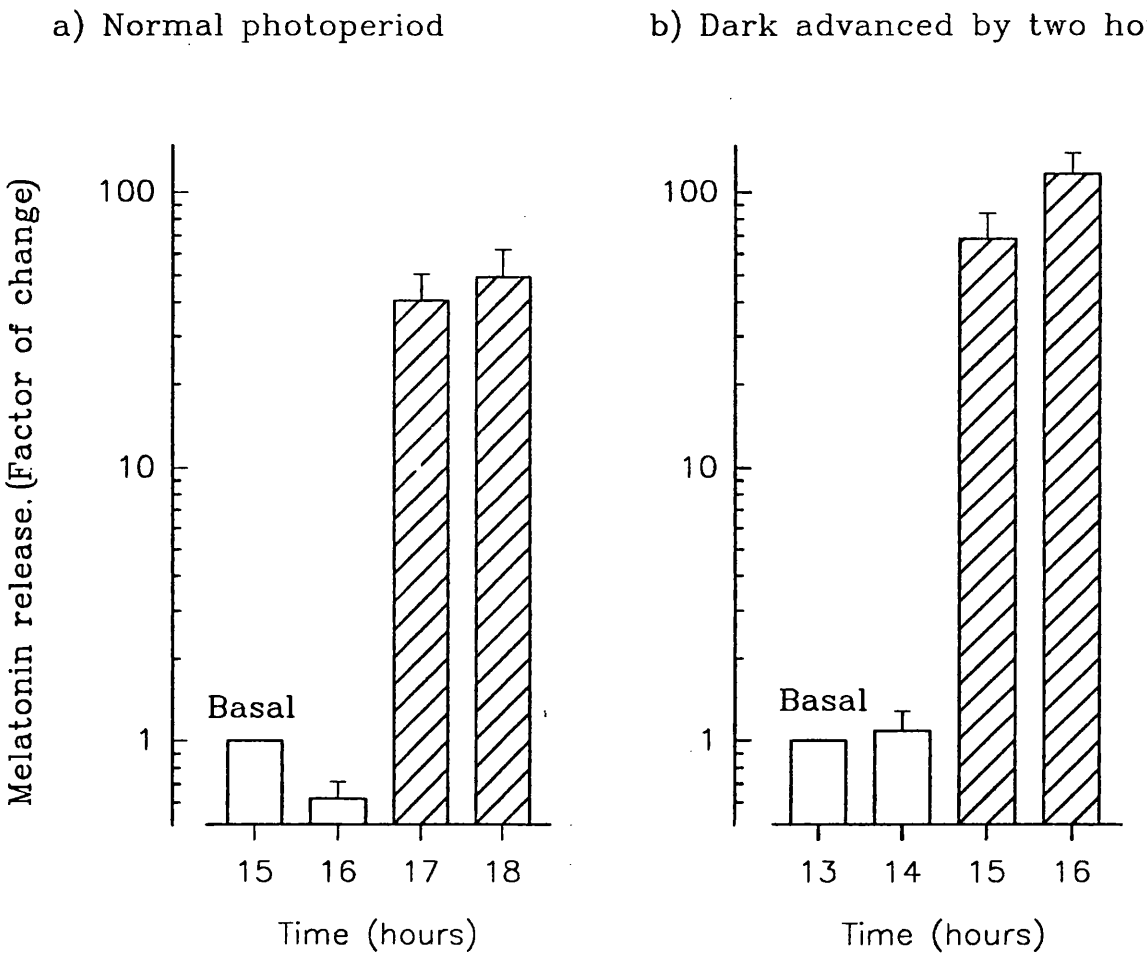
Melatonin release from individual pineals under illumination was low (0.2 -0.4ng/h) (Table 5.2). Some slight but insignificant variation in melatonin release over successive hours was noted (Fig. 5.2). After one hour in the dark, melatonin release had increased to over 10ng/h. Further darkness usually resulted in slightly elevated release rates (between 16-30 ng/h, depending on the individual [Table 5.2]) but maximum rates of release were generally reached after two hours of darkness and then remained high throughout the remaining scotophase (Fig. 5.6, Fig. 5.7). The same level of melatonin release was seen when the dark phase was advanced by two hours (Fig. 5.2), showing that isolated pineals release melatonin in direct response to darkness. Although melatonin secretion seemed higher after advancing the dark phase, the large individual variation between fish make such differences insignificant. Variation is reduced by comparing change in secretion of each pineal compared with its basal release rate (Fig. 5.2).

TABLE 5.2 HOURLY RELEASE OF MELATONIN FROM ISOLATED TROUT PINEALS IN STATIC ORGAN CULTURE IN LIGHT AND DARK; EFFECT OF A TWO HOUR DARK-ADVANCE. (ng/h ± SEM, n=6)

Dark onset	Light	Light	Dark	Dark
Coincident with normal dark onset	0,4±0,09	0,2±0,03	11,9±1,1	16,3±5,4
Dark advanced 2h	0,3±0,06	0,3±0,07	17,5±2,4	29,3±2,9

Note; Melatonin release in the dark is significantly higher than that in the light in both cases ($P<0,05$)

Figure 5.2
Melatonin release from isolated trout pineals in the light and dark : Effect of a two hour dark–advance



Pineals were placed in vitro at 11:30h. Melatonin release per hour (mean ± SEM) is expressed as a factor of change from basal release (basal=1) and plotted on a log scale against time in hours. Open bars represent melatonin release in the light. Hatched bars represent melatonin release during the dark. Normal dark onset is 16:00h.

Influence of forskolin on melatonin release from trout pineals *in vitro*

The addition of forskolin to induce cAMP production in trout pineals *in vitro*, resulted in a dose-dependent increase in the rate of melatonin release (Fig. 5.3). Doses of 10^{-6}M forskolin caused a 10 to 20-fold increase of melatonin release in the light. Transfer to the dark with forskolin caused a further 10-fold increase in melatonin synthesis in the first hour with no further increase thereafter. After two hours in the dark, the forskolin treated pineals still secreted more melatonin than control pineals in the dark. A lower concentration of forskolin (10^{-7}M) only doubled the rate of melatonin release.

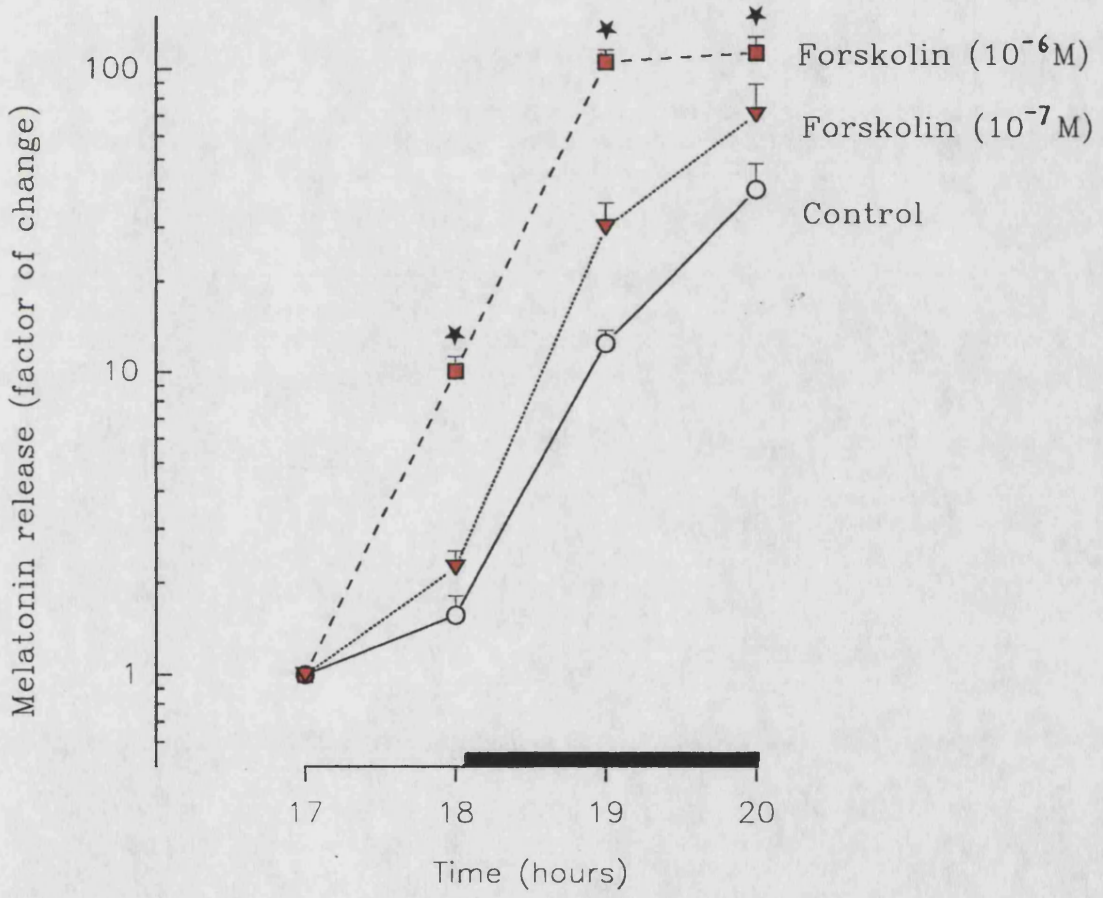
The effect of circulating titres of MCH (10^{-8}M) (Kishida *et al.*, 1989) on melatonin release from control and forskolin-stimulated trout pineals was also investigated. The addition of MCH alone did not alter melatonin release from incubated trout pineals in either the light or the dark (Fig 5.4a). In two experiments, 10^{-8}M MCH depressed melatonin release from forskolin-stimulated trout pineals under illumination, in comparison to those stimulated with 10^{-6}M forskolin alone (Fig. 5.4b). This could not, however, be repeated using a new, unoxidised preparation of MCH.

Influence of norepinephrine on melatonin release from trout pineals *in vitro*

a. In the light

Illuminated pineals showed a dose-dependent increase in melatonin release on hour after the addition of norepinephrine (NE) (Fig.

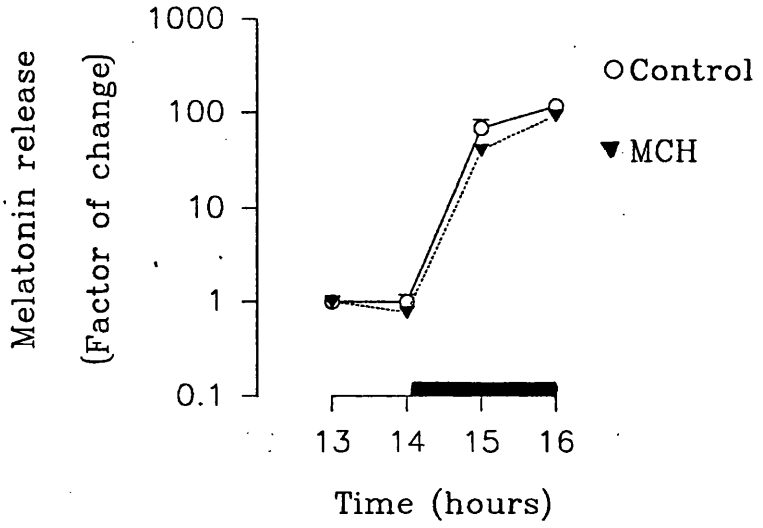
Figure 5.3
Effect of two doses of forskolin on melatonin release from isolated trout pineals in vitro



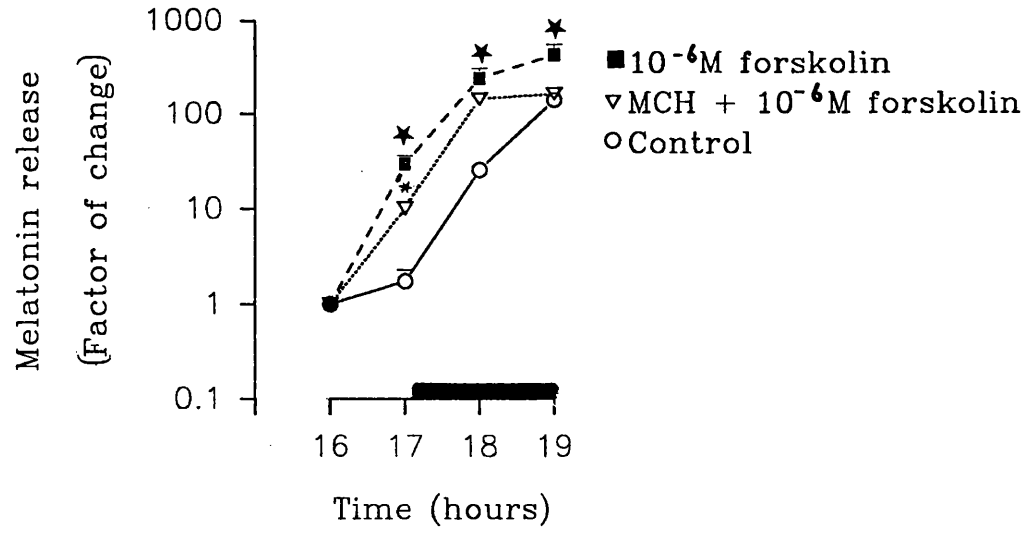
Melatonin release per hour (mean ± SEM, n=6) is expressed as a factor of change from basal (basal=1) and plotted on a log scale. Dark onset at 18:00, horizontal bar represents scotophase.
* P <0.01 compared with controls.

Figure 5.4
Effect of 10^{-8}M MCH on pineal melatonin release from isolated trout pineals in vitro in the presence or absence of forskolin

a) MCH alone



b) MCH with 10^{-6}M forskolin



Melatonin release per hour (mean ± SEM, n=6) is expressed as factor of change from basal (basal=1) and plotted on a log scale. Bar indicates scotophase. * $P < 0.05$ compared with forskolin-stimulated pineals, ✶ $P < 0.05$ compared with control.

5.5a). The addition of 10^{-3}M NE resulted in a 12-fold increase, and 10^{-4}M norepinephrine in a six-fold increase in melatonin release which was significantly higher than control values. Concentrations of 10^{-5}M or less were ineffective during the first hour but after a second hour of stimulation (Fig. 5.5b), melatonin release from pineals stimulated with 10^{-5}M NE were also now significantly higher than release from unstimulated pineals. The response to 10^{-3}M NE declined during the second hour.

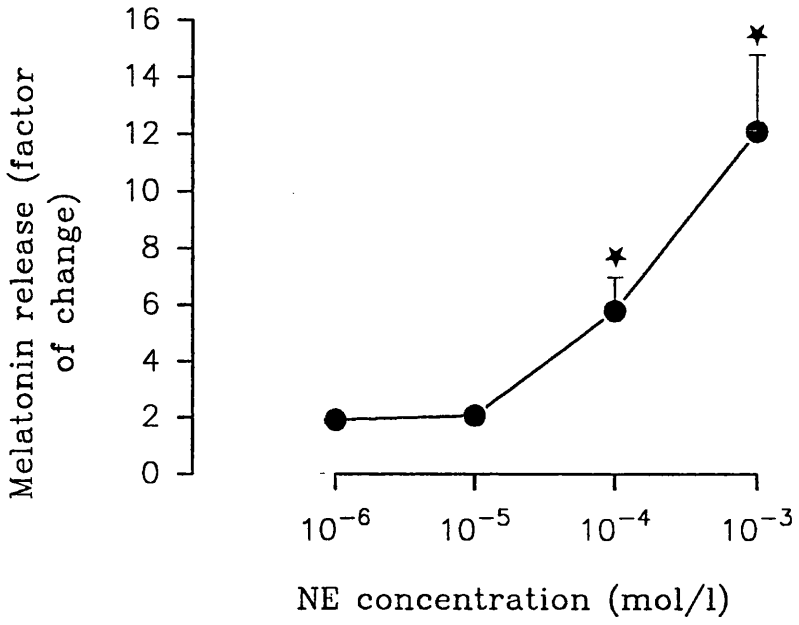
When illuminated pineals were incubated with both 10^{-3}M NE and 10^{-6}M forskolin together, the effect was apparently additive (Table 5.3) but large individual variation between fish mean that the values are not significantly different. After two hours stimulation, however, pineals stimulated with both forskolin and norepinephrine released significantly more melatonin (16.74 ± 0.8, n=5) than pineals stimulated with forskolin alone (8.2 ± 1.1, n=5).

TABLE 5.3 EFFECT OF 10^{-6}M FORSKOLIN PLUS 10^{-3}M NE ON MELATONIN RELEASE FROM ILLUMINATED TROUT PINEALS AFTER ONE HOUR *IN VITRO*

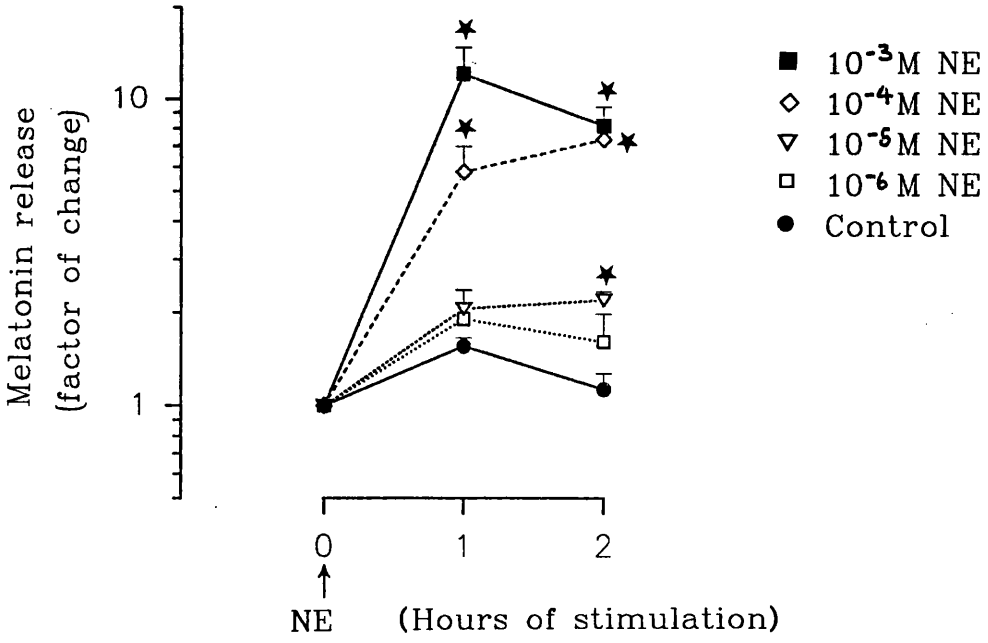
Treatment	Melatonin release (ng/h, n=5)		Factor of change
	pre-stimulation	post-stimulation	from basal
10^{-6}M Forskolin	0,3 ± 0,06	3,8 ± 1,1	19,9 ± 6,9
10^{-3}M NE plus 10^{-6}M Forskolin	0,2 ± 0,03	7,1 ± 0,7	40,7 ± 5,7

Figure 5.5

a) Dose response to norepinephrine of illuminated trout pineals after one hour in vitro

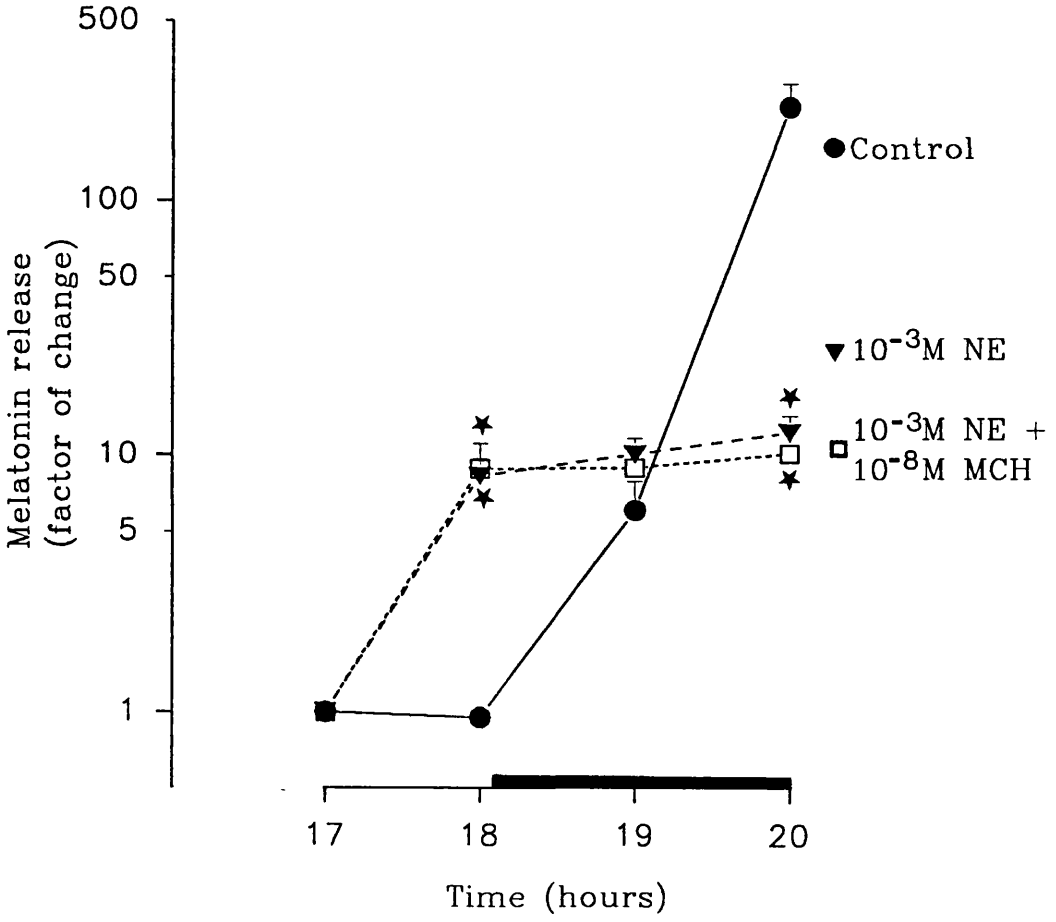


b) Effect of different doses of NE on melatonin release from illuminated trout pineals in vitro.



Melatonin release per hour (mean ± SEM, n=6) expressed as a factor of change from basal (basal=1) and plotted on a log scale. ✶ $P<0.01$ compared with the controls

Figure 5.6
Effect of Norepinephrine, alone or with MCH, on melatonin release from isolated trout pineals in vitro



Melatonin release per hour (mean ± SEM, n=6) is expressed as factor of change from basal (basal=1) and plotted on a log scale. Bar indicates scotophase. * $P < 0.05$ compared with control.

Stimulation of pineals with 10^{-3}M NE plus 10^{-8}M MCH did not alter melatonin release compared with those stimulated with NE alone (Fig. 5.6).

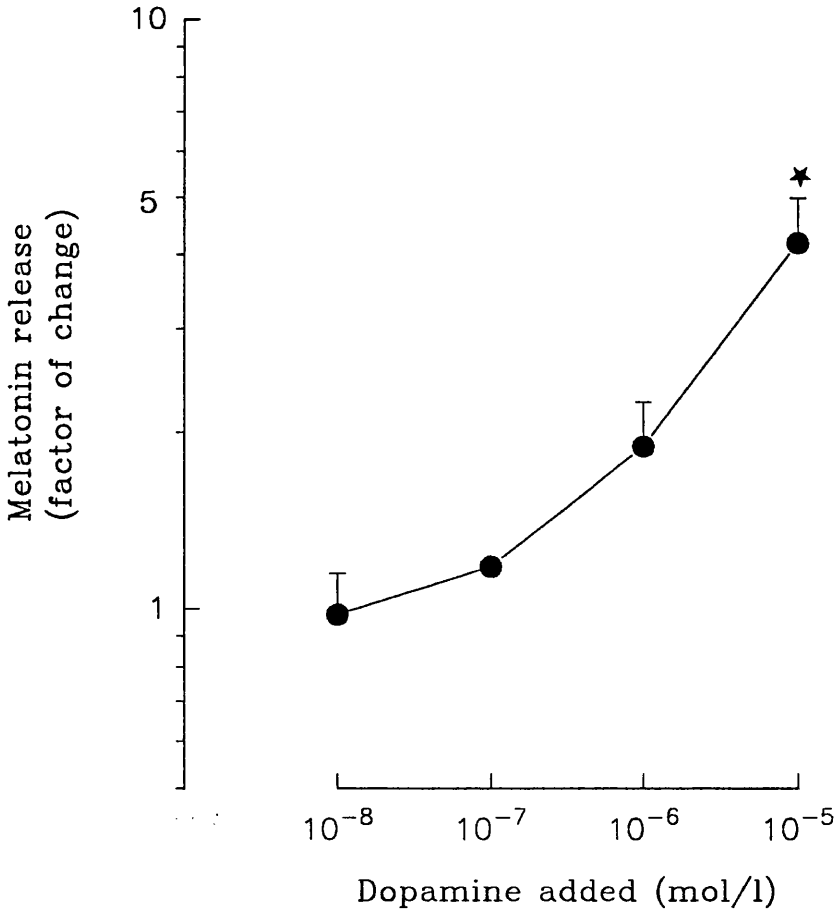
b. In the dark

To test the effect of norepinephrine on pineals placed in the dark, glands were pre-incubated in the light for one hour (basal secretion). NE was then added and the glands placed simultaneously in the dark. Stimulation of trout pineals *in vitro* for one hour with concentrations of NE ranging from 10^{-5}M to 10^{-3}M did not enhance the amount of melatonin released, compared with darkness alone. Continued stimulation with NE, however, caused a dose-dependent decline in melatonin release. The reduction in melatonin release from pineals treated with 10^{-3}M and 10^{-4}M NE was significantly reduced after both three and four hour incubations (Fig.5.7). Melatonin release was measured every 20 min. throughout the first hour in the dark to test if an initial increase in melatonin synthesis was observable. The rate of release from pineals treated with NE was the same as that from unstimulated pineals during the first hour in the dark (Fig. 5.6). No significant depressive action of NE was observed during this period.

The ultimately depressive effect of high concentrations of NE was confirmed in a further experiment in which pineals were treated in the light with 10^{-4}M NE for two hours and then transferred to dark and incubated overnight with 10^{-3}M NE (Fig. 5.8). In spite of the initial stimulatory effect of NE in the light, melatonin release after

Figure 5.7

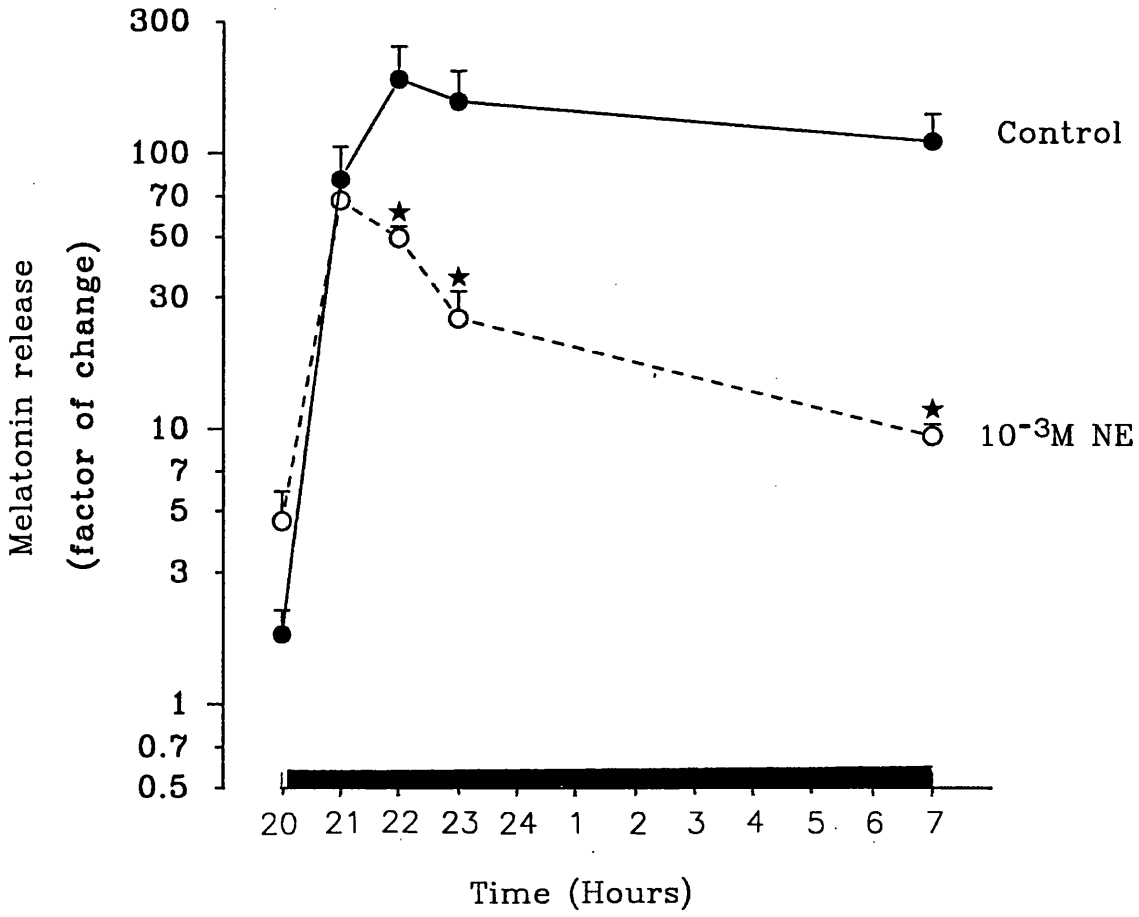
Dose response to dopamine of illuminated trout pineals after one hour in vitro.



Melatonin release per hour (mean ± SEM, n=6) is expressed as factor of change from pre-stimulated values and plotted on a log scale. * P <0.05 compared with conrol (unstimulated) values.

Figure 5.8

Effect of high concentrations (10^{-3}M) norepinephrine on melatonin release from isolated trout pineals in vitro.



Pineals were pre-stimulated with 10^{-4}M NE for two hours in the light before being transferred to the dark overnight and stimulated with 10^{-3}M NE. Solid bar represents scotophase. Melatonin release (mean ± SEM, n=6) is given as a factor of change from basal (1) where basal is pre-stimulated value. ★ $P < 0.05$ compared with control. Control pineals were unstimulated throughout the experiment.

the fourth hour of treatment was 3.5 times lower than that of the unstimulated controls (Fig. 5.8). Persistent stimulation with 10^{-5}M NE in the dark resulted in a progressive decline of melatonin release so that three hours after dark onset, the rate of melatonin release from NE-treated pineals was six times lower than that of controls, and after 11 hours it was 10 times lower than release from controls.

Influence of adrenergic agonists and antagonists on melatonin release from trout pineals *in vitro*

In the light, neither the general α-adrenergic agonist, phenylephrine (10^{-4}M), nor the general β-adrenergic agonist, isoproterenol (10^{-4}M) were able to mimic the effects of 10^{-4}M NE on trout pineals *in vitro* when added either alone or together (Table 5.4). However, the ability of NE (10^{-4}M) to stimulate melatonin synthesis by inducing a 3-4-fold increase in melatonin release from illuminated pineals was confirmed in these *in vitro* experiments (Table 5.4).

TABLE 5.4 INFLUENCE OF ADRENERGIC RECEPTOR AGONISTS ON MELATONIN RELEASE FROM ILLUMINATED TROUT PINEALS *IN VITRO*

Treatment	Factor of change after one hour	Factor of change after two hours
Control	1,3 ± 0,2	1,8 ± 0,4
10^{-4}M Norepinephrine	3,4 ± 0,5 *	4,6 ± 1,3 *
10^{-4}M Phenylephrine	1,3 ± 0,1	1,6 ± 0,2
10^{-4}M Isoproterenol	1,5 ± 0,2	1,5 ± 0,3
Both 10^{-4}M Phenylephrine + 10^{-4}M Isoproterenol	1,3 ± 0,2	1,7 ± 0,6

Melatonin release (mean ± SEM, n=6) expressed as factor of change from basal (where basal = 1). * P<0,05 compared with control.

The addition of 10^{-4}M concentrations of either the general α-adrenergic antagonist, phentolamine, or the general β-antagonist, propranolol to trout pineals *in vitro* did not alone significantly alter melatonin release in either light or dark (Table 5.5). Although melatonin release in the presence of phentolamine was apparently depressed in the dark, there was considerable variation between glands and the differences from the controls were not statistically significant. Neither were 10^{-4}M phentolamine nor 10^{-4}M propranolol able to suppress norepinephrine-stimulated release from illuminated trout pineals *in vitro* (Table 5.6).

TABLE 5.5 EFFECT OF ADRENERGIC ANTAGONISTS ON MELATONIN RELEASE FROM TROUT PINEALS *IN VITRO*

Treatment	2nd hour light + drug	3rd hour light + drug	1st hour dark + drug	2nd hour dark + drug
Control	0,6±0,06	1,0±0,2	31,6±8,4	99,9±28,5
10^{-4}M Phentolamine	0,7±0,2	0,6±1,2	11,1±4,6	37,4±16,6
10^{-4}M Propranolol	1,0±0,3	0,6±0,1	21,3±2,6	50,9± 6,5

Melatonin release (mean ± SEM, n=6) given as factor of change from basal, where basal=1 (The first hour of medium collection after pre-incubation).

TABLE 5.6 EFFECT OF α- AND β-ADRENOCEPTOR BLOCKERS ON NOREPINEPHRINE-STIMULATED MELATONIN RELEASE FROM ILLUMINATED TROUT PINEALS *IN VITRO*

Treatment	Melatonin Release (ng/h) Pre-stimulation	Post-stimulation	Factor of change from basal
Control	0,5 ± 0,08 (6)	0,3 ± 0,06 (6)	0,7 ± 0,03
10^{-4}M NE	0,4 ± 0,05 (5)	2,7 ± 0,16 (5) *	7,1 ± 1,0 *
10^{-4}M NE+ 10^{-4}M Phentolamine	0,4 ± 0,04 (6)	2,1 ± 0,18 (6) *	6,0 ± 0,6 *
10^{-4}M NE+ 10^{-4}M Propanolol	0,3 ± 0,06 (6)	2,4 ± 0,16 (6) *	9,1 ± 1,4 *

Melatonin release (mean ± SEM), Number of individuals in each experimental group given in parentheses, * $P<0,05$ compared with control.

Influence of dopamine and haloperidol on the ability of norepinephrine to stimulate melatonin release from trout pineals *in vitro*

Haloperidol (10^{-4}M), a specific dopaminergic receptor antagonist, suppressed NE-stimulated melatonin release from illuminated trout pineals *in vitro* (Table 5.7) which suggests that NE might be acting via the dopamine receptor. In this experiment, haloperidol did not affect melatonin secretion when added alone. Addition of 10^{-4}M haloperidol to pineals in the dark, however, did significantly reduce melatonin secretion (Table 5.8) which suggests that dopamine receptors might normally be activated during the dark.

TABLE 5.7 INFLUENCE OF 10^{-4}M HALOPERIDOL ON NOREPINEPHRINE-STIMULATED MELATONIN RELEASE FROM ILLUMINATED TROUT PINEALS *IN VITRO*

Treatment	Melatonin release (ng/h) Pre-stimulation	Post-stimulation	Factor of change from basal
Control	0,3 ± 0,05 (5)	0,6 ± 0,3 (5)	1,5 ± 0,4 (5)
10^{-4}M NE	0,5 ± 0,12 (5)	2,6 ± 0,5 (5) *	5,3 ± 0,8 (5) *
10^{-4}M Haloperidol	0,3 ± 0,11 (5)	0,4 ± 0,1 (5)	1,4 ± 0,1 (5)
10^{-4}M NE+ 10^{-4}M Haloperidol	0,3 ± 0,03 (4)	0,5 ± 0,1 (4) #	1,8 ± 0,2 (5) #

Melatonin release (mean ± SEM), number of individuals per group given in parentheses, * P<0,05 compared with control, # P<0,05 compared with NE-stimulated pineals alone.

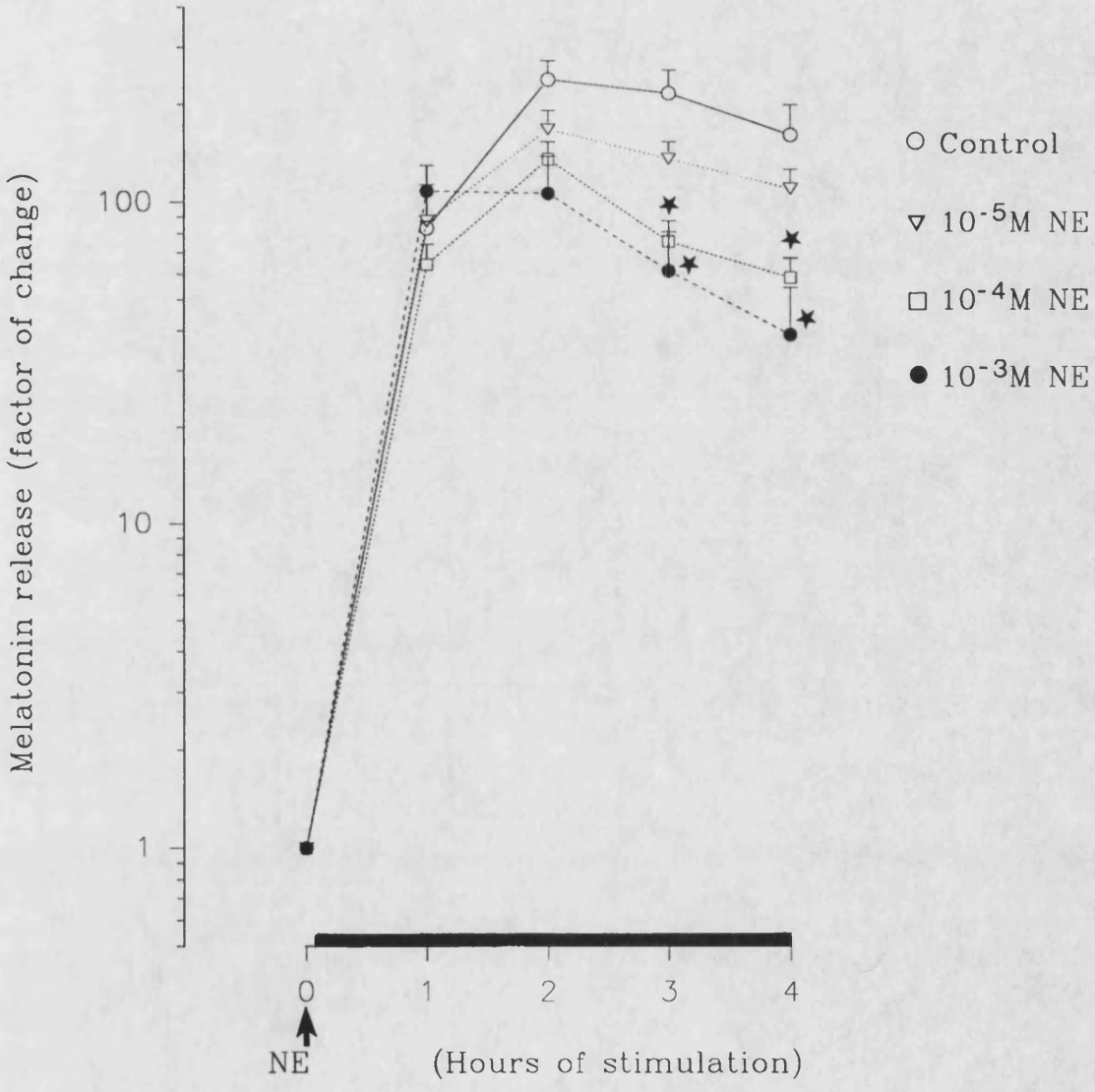
TABLE 5.8 INFLUENCE OF 10^{-4}M HALOPERIDOL ON MELATONIN RELEASE FROM ILLUMINATED TROUT PINEALS TRANSFERRED TO THE DARK

Treatment	Melatonin release (ng/h) in the dark	Factor of change from basal
Control	58,5 ± 9,0 (6)	71,6 ± 10,6 (6)
10^{-4}M Haloperidol	26,2 ± 5,4 (6) *	30,3 ± 5,2 (6) *

Melatonin release (mean ± SEM), number of individuals per group given in parentheses, * P<0,05 compared with control.

Figure 5.9

a) Effect of different doses of NE on melatonin release from trout pineals in vitro in the dark



Melatonin release per hour (mean ± SEM, n=6) expressed as factor of change from pre-stimulated values in the light. Solid bar represents scotophase. ✶ $P<0.05$ compared with control.

Melatonin release from illuminated pineals *in vitro* was increased by about four-fold with the addition of 10^{-5}M dopamine (Fig. 5.9). The effect of dopamine was blocked by the addition of haloperidol (Table 5.9).

TABLE 5.9 STIMULATORY EFFECT OF DOPAMINE ON MELATONIN RELEASE.

Treatment	Factor of change from basal
Control	0,96 ± 0,05 (6)
10^{-8}M Dopamine	0,98 ± 0,17 (6)
10^{-7}M Dopamine	1,18 ± 0,04 (4)
10^{-6}M Dopamine	1,89 ± 0,36 (5)
10^{-5}M Dopamine	4,18 ± 0,8 (6) *
10^{-5}M Dopamine + 10^{-4}M Haloperidol	0,89 ± 0,12 (6) #

Melatonin release (mean ± SEM), number of individuals in each experimental group given in parentheses, * P<0,05 compared with control, # P<0,05 compared with dopamine-stimulated pineals alone.

Influence of acetylcholine (ACh) and carbachol on melatonin release from trout pineals *in vitro*

Administration of either acetylcholine (10^{-6}M, 10^{-5}M) or the specific agonist for nicotinic receptors: carbachol (10^{-4}M), to trout pineals *in vitro* did not significantly alter melatonin release in either the light or the dark (Table 5.10).

TABLE 5.10 INFLUENCE OF ACETYLCHOLINE AND CARBACHOL ON MELATONIN RELEASE FROM ILLUMINATED TROUT PINEALS *IN VITRO*

Treatment	Melatonin release (ng/h)		Factor of change from basal	
	Light	Dark	Light	Dark
Control	0,73 ± 0,17	28,7 ± 2,1	0,96 ± 0,4	47,7 ± 8,0
10^{-5}M ACh	0,64 ± 0,14	31,1 ± 4,0	0,81 ± 0,3	56,4 ± 10,0
10^{-6}M ACh	1,16 ± 0,44	35,8 ± 1,7	0,66 ± 0,01	44,1 ± 7,6
Control	0,55 ± 0,07	58,5 ± 9,0	0,68 ± 0,05	71,6 ± 10,6
10^{-4}M Carbachol	0,47 ± 0,16	58,3 ± 12,1	0,65 ± 0,09	88,3 ± 2,4

Melatonin release (mean ± SEM, n=6)

Influence of MCH, α-MSH, VIP and GABA on melatonin release from trout pineals *in vitro*

a. MCH

Addition of 10^{-8}M salmonid MCH to isolated trout pineals *in vitro* did not alone alter melatonin release rates in the light or the dark (Fig. 5.4a). The importance of high circulating titres of MCH on melatonin secretion from trout pineals was also examined using fish reared under a long photoperiod in black and white tanks. Fish reared on white backgrounds have very high hypothalamic and pituitary MCH contents (Green *et al.*, 1991), while those reared on black backgrounds have significantly lower MCH stores. Pineals from both groups of fish released similar levels of melatonin in both light and dark conditions (Table 5.11). The trout in this experiment were about 60g in body weight. They released lower basal amounts of melatonin in the light but showed a more rapid, intense response to dark than the larger, farm-reared fish.

TABLE 5.11 EFFECT OF REARING CONDITIONS AND SMALL SIZE ON MELATONIN SECRETION FROM TROUT PINEALS *IN VITRO* (Values are ng/h ± SEM, n=6)

Rearing condition	Weight (g)	Melatonin release Light	Melatonin release Dark	Factor of change (D/L)
Black	57.5 ± 3	0.12 ± 0.03	8.15 ± 0.64	102.2 ± 30.5
White	60.7 ± 4	0.11 ± 0.03	9.38 ± 1.76	99.1 ± 13.6

b. α-MSH

Addition of 10^{-8}M α-MSH had no effect on melatonin secretion in either the dark or light (Table 5.12). Moreover it was unable to alter forskolin-stimulated melatonin release from trout pineals *in vitro*.

c. VIP

Melatonin secretion from trout pineals *in vitro* was not changed in either light or dark after the addition of 10^{-4}M VIP (Table 5.12).

TABLE 5.12 EFFECTS OF MSH, VIP AND GABA ON MELATONIN RELEASE FROM TROUT PINEALS *IN VITRO*

Treatment	Post-stimulation (ng/h) Light	Post-stimulation (ng/h) Dark	Factor of change from basal Light	Factor of change from basal Dark
Control	0.2 ± 0.03	11.9 ± 1.1	0.6 ± 0.1	40.5 ± 10.1
10^{-8}M α-MSH	0.3 ± 0.03	12.8 ± 2.0	1.0 ± 0.1	55.1 ± 6.9
Control	0.2 ± 0.03	12.4 ± 1.4	1.0 ± 0.2	31.6 ± 8.4
10^{-4}M VIP	0.1 ± 0.01	8.2 ± 1.7	0.8 ± 0.1	24.6 ± 4.7
Control	0.3 ± 0.07	11.4 ± 1.3	1.0 ± 0.6	31.3 ± 5.4
10^{-4}M GABA	0.1 ± 0.01	5.8 ± 1.7	0.2 ± 0.05 *	20.7 ± 2.0

Melatonin release per hour (mean ± SEM) expressed as factor of change from pre-stimulated basal release (basal=1). Number of individuals in each group given in parentheses. * $P<0.05$ compared with control.

d. GABA

The presence of 10^{-4}M GABA significantly depressed melatonin release from illuminated pineals but not from those in the dark (Table 5.12)

5.4 DISCUSSION

The results show that trout pineals maintained *in vitro* will respond directly to darkness by increasing melatonin release. This confirms the presence of photoreceptive cells within the pineal (Meissl and Ekstrom, 1988a, b). Under illumination, trout pineals release melatonin at a low rate (<1ng/h) (present study; Gern and Greenhouse, 1988) but upon exposure to darkness, this rate immediately increased to between 10-20 ng/h. In the present work, the average melatonin release rates in the dark were often double those recorded by Gern and Greenhouse (1988). There is no obvious reason why these fish behaved differently; perhaps it was due to differences between the strains of fish used. Moreover, isolated trout pineals *in vitro* responded to dark by increasing melatonin release by 50-100-fold, but plasma melatonin concentrations *in vivo* generally only show a 10-fold increase in the dark. There was no direct correlation between size of fish and melatonin release within trout of a similar weight group but smaller fish (60g) generally released slightly less melatonin in both light and dark. Fish of about 150g were generally used, as their pineals were easily removed. Individual pineal glands show much variation in their responsiveness to light and dark, perhaps reflecting slight damage upon removal or due to natural individual variation. Experiments were therefore designed such that each pineal served as its own control and changes in melatonin release rates were expressed

as a factor of change from basal (illuminated) levels. It has been suggested that melatonin release from trout pineals *in vitro* may be pulsatile (unpublished results, cited by Gern and Greenhouse, 1988). Although a slightly fluctuating level of basal secretion was observed in the present work, the incubation system used would not reflect pulsatility.

In five experiments in which transfer to the dark coincided with onset of dark phase *in vivo*, individual pineals did not show any anticipatory rise in melatonin secretion, nor was melatonin release in the dark diminished when dark onset was advanced two hours. Pineal incubations were generally performed during mid-late photophase and early scotophase. Hence, for the purposes of the present experiments, it was assumed that the time of day during which experiments were conducted would not materially alter pineal responsiveness. The control of melatonin release by trout pineal glands thus seems to be very different from those of other teleosts. Unlike either the pike (Falcon *et al.*, 1987) or the goldfish pineal (Kezuka *et al.*, 1988), trout pineals *in vitro* are unable to maintain rhythmic secretion of melatonin under conditions of continuous darkness (Gern and Greenhouse, 1988). In interpreting these observations one may suggest three possibilities: a) the trout pineal does not contain an endogenous oscillator which drives the circadian rhythm of melatonin synthesis and release but is, instead, modulated by a pacemaker centre somewhere in the brain; b) the pineal contains an endogenous pacemaker but is unable to express circadian rhythmicity in culture conditions,

or c) the rhythm of melatonin release is not of a circadian nature but is driven directly by light falling on the pineal.

Melatonin production by trout pineals *in vitro* seems to be mediated by high levels of intracellular cAMP, since the addition of forskolin significantly increased *in vitro* melatonin release in both light and dark. Forskolin also significantly enhances melatonin production in superfused pike pineals (Falcon and Collin, 1989). Cyclic AMP has also been implicated in melatonin synthesis in both birds and mammals, and is believed to activate NAT, the rate-limiting enzyme in melatonin synthesis (Binkley, 1988). Intracellular cAMP levels are controlled by the adrenergic system in birds and mammals. In mammals, β_1-adrenoceptor stimulation results in an increase in cAMP levels (Chik *et al.*, 1988b) and this response is potentiated by α_1-adrenoceptors (Yuwiler, 1987; Chik *et al.*, 1988a). In birds, α_2-adrenoceptor-stimulation inhibits adenylate cyclase and the release of intracellular cAMP (Zatz *et al.*, 1990). In some mammals, sensitivity to β-adrenergic stimulation may vary during the light-dark cycle. For example, melatonin production by the Syrian hamster pineal cannot be induced by β-adrenergic agonists after some hours of darkness (Reiter *et al.*, 1987), and it is thought that changes in β-receptor density over the 24-hour cycle may be responsible for this refractory period (cf. Pangerl *et al.*, 1990), since hamster pineal glands show a nightly decline in β-adrenoceptor numbers. Indeed, recent evidence (Patel and Demaine, 1990) suggests that in hamsters, NE may not act directly on the pinealocytes as previously supposed. Instead, NE released from sympathetic nerves via the SCG may act to disinhibit normally

suppressed units and thereby permit melatonin secretion. They also suggested that a directly stimulatory neurotransmitter may exist in mammals and postulate that neuropeptide Y is a likely candidate.

Norepinephrine is known to be involved in pineal melatonin production in both mammals and birds, so one might anticipate that it is also involved in pineal melatonin production in the trout. The present work, however, suggests that this is not the case. Although Moller and van Veen (1982) originally suggested that the teleostean pineal is unlikely to be directly innervated by aminergic neurones, Ekstrom and Meissl (1989) have recently traced noradrenergic fibres from the habenular area of the trout brain to the pineal using a highly specific antiserum, In the present work, norepinephrine was shown to stimulate melatonin release from illuminated trout pineals *in vitro* but only at pharmacological doses. Moreover, equally high doses of either specific α- or β-adrenergic agonists were unable to mimic its effect. Enhanced norepinephrine-induced melatonin release could not be depressed by the addition of either general α-, or β-agonists but it was totally blocked by the addition of haloperidol, a specific receptor blocker (Table 5.6). Since dopamine also stimulates melatonin release, it seems likely that norepinephrine was interacting *in vitro* with dopamine receptors to increase melatonin production. Norepinephrine has been shown to interact non-specifically with dopamine receptors in the rat hypothalamus (Tiligada and Wilson, 1990) to inhibit β-endorphin release, an effect that is partially antagonised by haloperidol (Vermes *et al.*, 1985). It is unknown, however, whether dopamine stimulates melatonin production in trout

pineals *in vivo*. The presence of dopamine neurones in the trout pineal has not yet been investigated but such neurones have been found in the rat pineal gland (Ebadi, 1984) in which L-Dopa stimulates NAT, the enzyme responsible for melatonin synthesis (cf. Binkley, 1988). It may be significant that dopamine receptors have been reported at peripheral sites in mammals where dopaminergic innervation is lacking (cf. Kruk and Pycock, 1983). Dopamine, as well as adrenaline and noradrenaline, is released from the adrenal medulla. If circulating dopamine were responsible for stimulating peripheral sites such as the trout pineal, one would expect it to act at lower concentrations (2 x 10^{-8}M unstressed level of circulating catecholamine in trout blood: Ristori *et al.*, 1979) and modulate melatonin release during stress. Haloperidol was able to reduce melatonin secretion by 50% in the dark which suggests that some dopamine may be released at night. Since haloperidol does not totally depress secretion in the dark, there is clearly another mechanism enhancing melatonin secretion which may be potentiated by dopamine. In mammals, neuropeptide Y has been postulated as a likely candidate (Patel and Demaine, 1990), but such a neurotransmitter is not known in fish. It is not yet known, however, whether this dopamine release occurs in response to dark - it may be released generally under *in vitro* conditions. It is possible that the removal and subsequent treatment of pineals under *in vitro* conditions activates the dopaminergic neurones, perhaps by removing an inhibitory influence from centrally derived neurones.

Prolonged stimulation of trout pineals *in vitro* with high doses of norepinephrine (probably through activation of dopamine receptors)

resulted in a significant decline in pineal responsiveness. Since this decline in melatonin release was not observed in control pineals, it is not merely a product of limited precursor availability. It is therefore suggested that the decline in melatonin release from pineals treated with pharmacological doses of NE in light or dark is due to either receptor down regulation or dissociation of receptors from the second messenger system. Similar effects upon prolonged stimulation of adrenergic receptors have been reported in mammals. Firstly, the β-adrenoceptor density changes diurnally and can be changed by treatment with β-adrenergic agonists (Romero *et al.*, 1975). Secondly, there are biochemical changes which affect both β- and α_1-adrenoceptors following stimulation of pinealocytes with NE (cf. Sugden, 1989). Protein kinase C is initially activated by NE via α_1-adrenoceptors to increase intracellular cAMP levels (Sugden *et al.*, 1988). Almost immediately afterwards it begins to desensitise the α_1-adrenoceptor through phosphorylation (Leeb-Lundberg *et al.*, 1988). Similarly, β-adrenergic receptor kinase which is activated by β-adrenergic receptor stimulation, reduces the affinity of the β_1-adrenoceptors through phosphorylation (Lefkowitz *et al.*, 1986).

The administration of acetylcholine (ACh) to trout pineals *in vitro* did not alter melatonin secretion in either the light or the dark. Acetylcholinesterase-positive interneurones have been found in the teleostean pineal (Korf, 1974; Vigh-Teichmann *et al.*, 1982) together with considerable staining in the pineal tract, but this is not necessarily evidence that ACh is a neurotransmitter in these neurones (Eckenstein and Sofoniew, 1983). Ekstrom and Korf (1986), however,

also detected some interneurones which were immunoreactive against choline acetyltransferase. While it is possible that the situation *in vitro* differs from that *in vivo*, it seems likely, on the basis of the present work, that ACh is not an important neurotransmitter in melatonin release from the trout pineal.

Another neurotransmitter which influences melatonin secretion in higher vertebrates is GABA. It has been immunocytochemically localized in centrally projecting interneurones of the trout pineal stalk (Ekstrom *et al.*, 1987; Ekstrom and Meissl, 1989), and these authors suggested that, since GABA is known to be an inhibitor of pineal cell activity in frogs (Meissl and George, 1985), it may act as a neuromodulator of melatonin release from the trout pineal. Incubation of isolated trout pineals *in vitro* resulted in a significant decline in melatonin release but only in illuminated, rather than dark, conditions. This contrasts with the situation in frogs (Meissl and George, 1985) in which iontophoretic application of GABA *in vivo* inhibited the spontaneous activity of frog pinealocytes in the dark. Since GABA generally acts as a presynaptic neurotransmitter to inhibit the activity of depolarised neurones, one would normally expect GABAergic neurones to be active during the dark. The results of the present experiments, however, do not show any change in melatonin release from GABA treated pineals in the dark. The inhibition of melatonin release during the light suggests that some of the neurones within the dissected illuminated pineal were depolarised during the incubation experiment: the exogenous GABA may have inhibited the release of a stimulatory neurotransmitter (such as dopamine) from

these pineal cells. In the absence of light, the effect of GABA might have been masked by dark-induced stimulation of melatonin production.

In addition to aminergic neurotransmitters, several peptidergic neuromodulators have been implicated in the control of melatonin release from both mammalian and avian pineal glands. The present work examined the effects of some of these on trout pineals *in vitro*.

VIP-containing neurones have been localized in mammalian (Uddman *et al.*, 1980) and avian (Pratt and Takahashi, 1989) pineals. In both of these groups, VIP increases melatonin production when added to pineals *in vitro* (Pratt and Takahashi, 1989; Simmoneaux *et al.*, 1990; Zatz *et al.*, 1990) by increasing intracellular levels of cAMP (Chik *et al.*, 1988a, 1988b; Pratt and Takahashi, 1989). Although the response to VIP mimics that of NE in terms of its time course reaction kinetics (Chik *et al.*, 1988b), it acts through VIP (Kaku *et al.*, 1983) rather than adrenergic receptors (Simmoneaux *et al.*, 1990). Melatonin production from mammalian pineals can be stimulated by doses of 10^{-7}M VIP or more. The physiological role of this peptidergic neurotransmitter has not yet been determined in higher vertebrates but it is often associated with increasing blood flow. Although VIP modulates melatonin production in higher vertebrates, there was no detectable effect of high doses (10^{-4}M) of VIP on trout pineals *in vitro*. It would be interesting to see whether the trout pineal also contained immunoreactive VIP neurones and /or receptors.

An α-MSH-like material can be assayed in pineal extracts (Oliver and Porter, 1978), although its origin is uncertain. Such material can be demonstrated by immunocytochemistry, suggesting its possible production within the pineal (O'Donnohue *et al.*, 1980; Dupont *et al.*, 1975). A positive staining result with antisera against α-MSH has, on several occasions, been shown to be attributable to epitopes other than α-MSH itself: for example, neurofibrils, and an epitope within the MCH precursor (Powell and Baker, 1987; Bird *et al.*, 1989). Pevet and co-workers (1980) also showed that the α-MSH-like material in the pineal also bound to antisera against LHRH and AVT. The significance of any α-MSH-like immunoreactivity must therefore be interpreted with caution. On the other hand, some investigators have demonstrated that circulating radio-labelled α-MSH is concentrated in the pineal (Oaknin *et al.*, 1987; Dupont *et al.*, 1975; Swaab and Fisser, 1977). Despite its possible origin, α-MSH-like material within the pineal has been shown to fluctuate in concentration on a diurnal basis. O'Donnohue and co-workers (1979, 1980) therefore proposed that α-MSH might be a potential modulator of pineal melatonin release. In 1976, Sakai and co-workers demonstrated that iontophoretic application of exogenous α-MSH to mammalian pineals was able to inhibit the β-adrenergic response to norepinephrine, and depress the increase in intracellular levels of cAMP, although it had no effect when added to rat pineals alone. In trout, addition of 10^{-8}M MSH to pineals *in vitro* did not alter the rate of basal melatonin release rates in the light or dark but the effect of α-MSH in conjunction with NE or dopamine was not examined. If α-MSH acts to depress depolarization of other nerves (akin to GABA

effects) such an action would not be revealed by the present *in vitro* system.

Direct application of MCH to trout pineals in two *in vitro* experiments did not alter basal melatonin release in light or dark, but in initial experiments it appeared able to reduce forskolin-stimulated melatonin release from illuminated pineals, although not those in the dark. When a fresh preparation of MCH was used, however, this result could not be repeated. The initial inhibitory responses may have been caused by breakdown products or oxidation of the older preparation of MCH through storage and/or repeated freezing and thawing of the peptide. Oxidised MCH is equipotent in melanophore bioassays and therefore cannot be detected in this way. Pineals from trout reared on black and white backgrounds released similar amounts of melatonin in both light and dark incubation conditions which suggests that MCH may not modulate melatonin release *in vivo*. The presence of MCH fibres in the pretectal region of the brain nevertheless suggests that the peptide may modulate the actions of pinealofugal fibres but, although the existence of such fibres is certain, their physiological function is not known.

In conclusion, the regulation of diurnal pineal melatonin release from trout pineals seems to be under quite complex control. Trout melatonin production is similar to that of other vertebrates in that it appears to be modulated by cAMP. Unlike in higher vertebrates, however, neither the adrenergic nor VIP systems seem to be involved but dopamine may play a significant part. The present work shows that

melatonin release is unlikely to be affected by circulating titres of MCH but it is still theoretically possible that the dorsally projecting MCH fibres (Naito *et al.*, 1985) interact with pinealofugal (Hafeez and Zerihun, 1974) and retinal fibres (Pinganaud and Clairambault, 1979) in the pretectal area of the trout brain.

5.5 SECTION TWO: MELATONIN BINDING SITES IN TROUT BRAIN, RETINA AND MELANOPHORES.

INTRODUCTION

Immunocytochemical studies have revealed MCH-like neurones in the teleost brain (Naito *et al.*, 1985; Powell and Baker, 1987, 1988; Bird *et al.*, 1989; Bird and Baker, 1989). Further study showed that these areas also contain MCH bioactive material (Baker and Rance, 1983). MCH-like cell bodies are located in the NLT and the majority of axons extend into the pituitary (Naito *et al.*, 1985; Bird *et al.*, 1989). Some prominent fibres can also be traced caudo-dorsally, to terminate adjacent to the posterior commissure in the pretectal area of the brain (Naito *et al.*, 1985). The pretectal area of the brain also receives innervation from the retina (Pinganaud and Clairambault, 1979) and the pineal (Hafeez and Zerihun, 1974). It is therefore considered possible that some communication between these three types of fibres occurs (Naito *et al.*, 1985).

The aim of this work is to determine the distribution of melatonin binding sites in trout brain through autoradiographic techniques with a view to localizing possible sites of melatonin action, and to see whether the dorsally projecting MCH neurones might possess melatonin binding sites.

5.6 MATERIALS AND METHODS

Details of experimental procedure are given in Chapter Two (2.11-

2.12). Briefly, several white-adapted sexually immature trout (adapted to a 16L:8D photoperiod) were decapitated during mid- to late-photophase. Brains and retinal tissue were removed and snap-frozen in hexane (-30°C) before storing at -80°C. Within two weeks, frozen tissue was mounted in OTC compound (Gurr; BDH) and 16μm sections cut on a cryostat. The sections were thaw-mounted onto poly-L-lysine coated slides and left for at least 24 hours at 4°C. After rehydration in buffer, they were incubated with 70pM di-iodinated melatonin for an hour. Non-specific binding was determined by additionally incubating a few sections with 1μM melatonin. Trout scales were also removed, teased apart in MEM and mounted on slides. They were snap frozen and stored at -80°C, before being treated in the same way as the other tissue. The autoradiographic film was developed after one week.

5.7 RESULTS

Iodinated melatonin bound to discrete areas within the trout brain, to the retina and on the scale epidermis.

a. Brain

Localization of 2[^{125}I]-melatonin binding to trout brain is shown in Plates 5.10. All binding, apart from that in the saccus vasculosus, was displaced with 1μM unlabelled melatonin. The most intense specific melatonin binding was observed in the area thalamica lateralis (Pinganaud and Clairambault, 1979) also called the nucleus rotundus (Schnitzlein, 1962; Billard and Peter, 1982). The terminology of Pinganaud and Clairambault is used throughout this chapter as there is much confusion in the literature about which region is identified as

the 'nucleus rotundus' and the 'nucleus glomerulosum' in various teleost species. Moreover, this nucleus is not thought to be homologous to the nucleus rotundus of birds and reptiles (Schnitzlein, 1962). The area thalamica lateralis is characterized by its spherical or ovoid outline in cross-section. The cells within it are evenly dispersed, some with a glomerula organisation. This nucleus extends along the dorsal thalamus, caudal to the geniculate nucleus and dorsal to the optic tract throughout the greater part of its length, before fusing caudally with the area thalamica dorso-intermedius (Pinganaud and Clairambault, 1979), and together these areas have been named the corpus glomerulosum (Brickner, 1929). Melatonin binding was observed from the anterior-most level of the preoptic tectum for some 900μm posteriorly until just anterior to the saccus vasculosus.

Specific melatonin binding was also observed in the optic tectum (or mesencephalic tectum). The precise cellular localization of melatonin binding was difficult to discern, but appeared to be in layers 3-5: the fibrosum et griseum superficiale; griseum centrale; and album centrale, respectively (Pinganaud and Clairambault, 1979). These areas are the main targets of retinal neurones and they also give rise to efferent fibres from the tectum.

Melatonin binding was not observed lateral to the posterior commissure, where a large number of MCH fibres terminate, nor at the site of the MCH perikarya in the NLT of the ventral hypothalamus. Melatonin binding sites were also absent from the pineal, although they were observed on dermal melanophores which lie dorsal to the

Plates 5.10 (a-f)

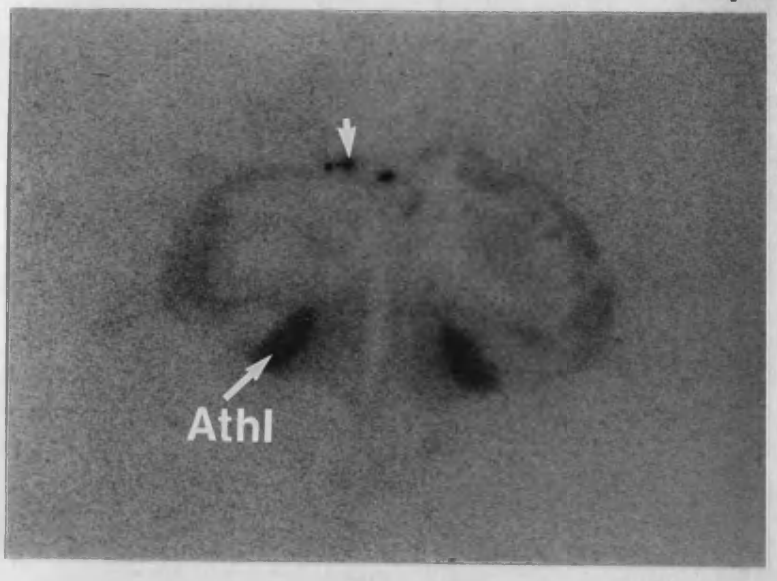
Distribution of ^{125}I-melatonin binding sites in trout brain.

Autoradiograms (a-d) show areas of specific ^{125}I-melatonin binding. Sections (16μm) were incubated with 70pM ^{125}I-melatonin for one hour, and the X-ray film developed after a week. Iodo-melatonin binding was most intense in the area thalamica lateralis (Athl) (or nucleus rotundus; Schnitzlein, 1962). Binding was also observed in the optic tectum (OT) and in melanophores in the meninges (arrows), but not in the pineal (p) or the pretectal region of the brain.

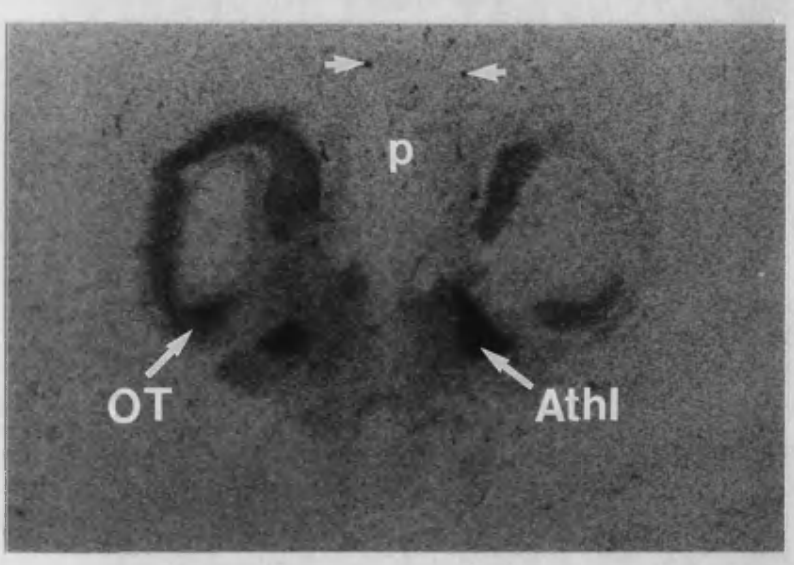
Autoradiograms (e, f) represent non-specific binding. Sections were incubated with 70pM ^{125}I-melatonin in the presence of 1μM melatonin. (f) shows non-specific ^{125}I-melatonin binding in the saccus vasiculosus (sv).

Magnification x10, i.e., 1cm represents 1mm.

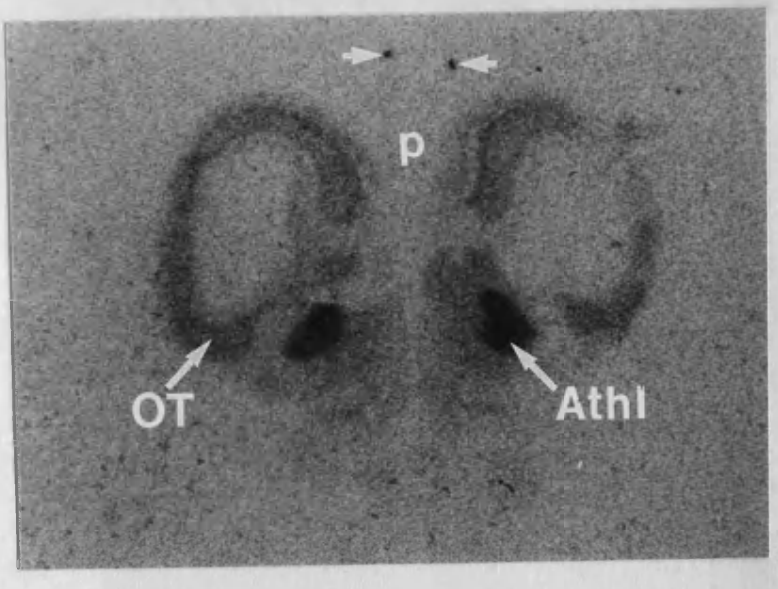
a



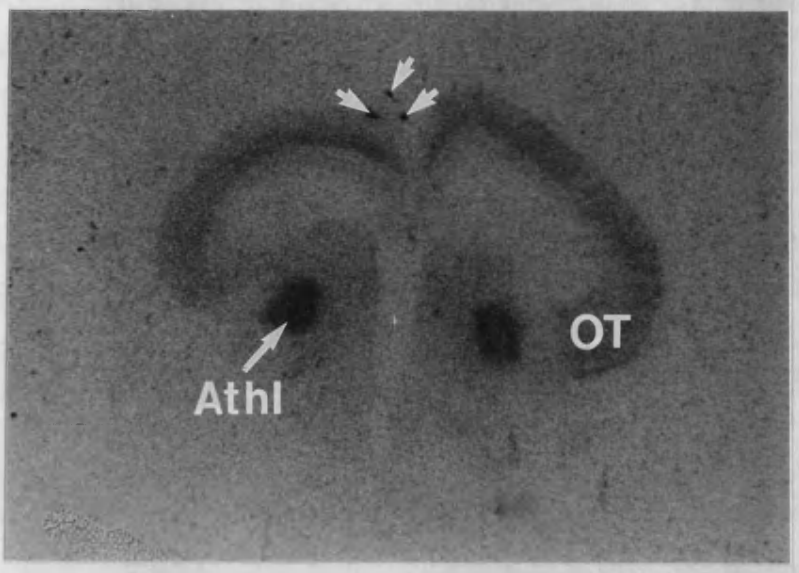
b



c

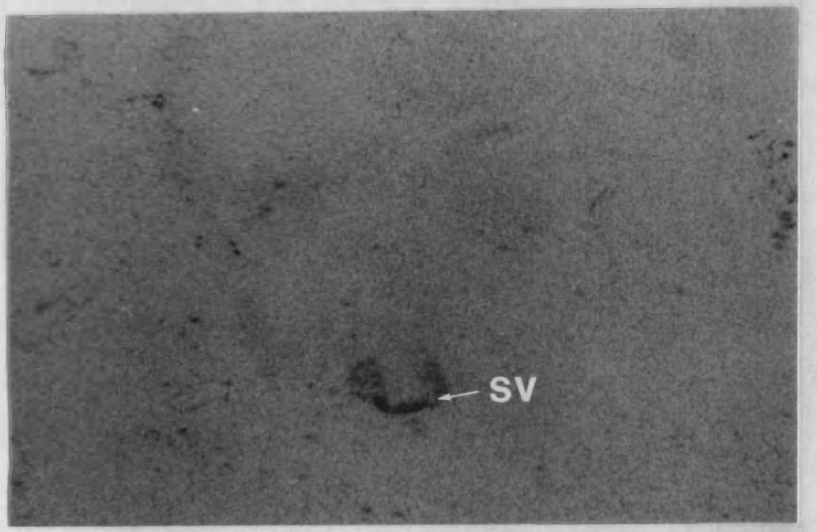


d



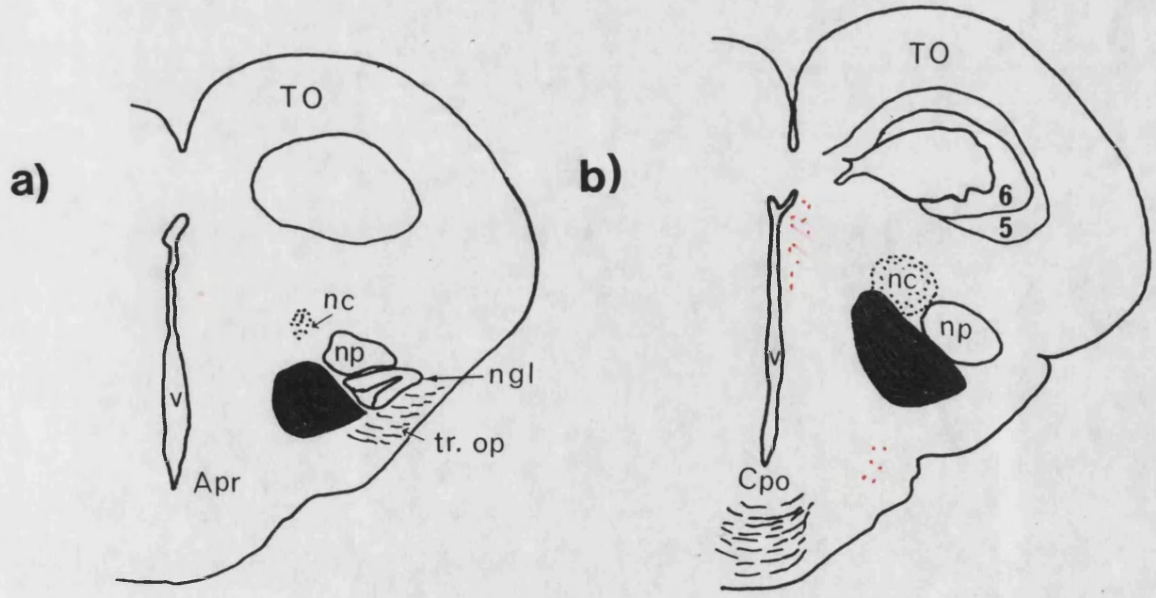
e

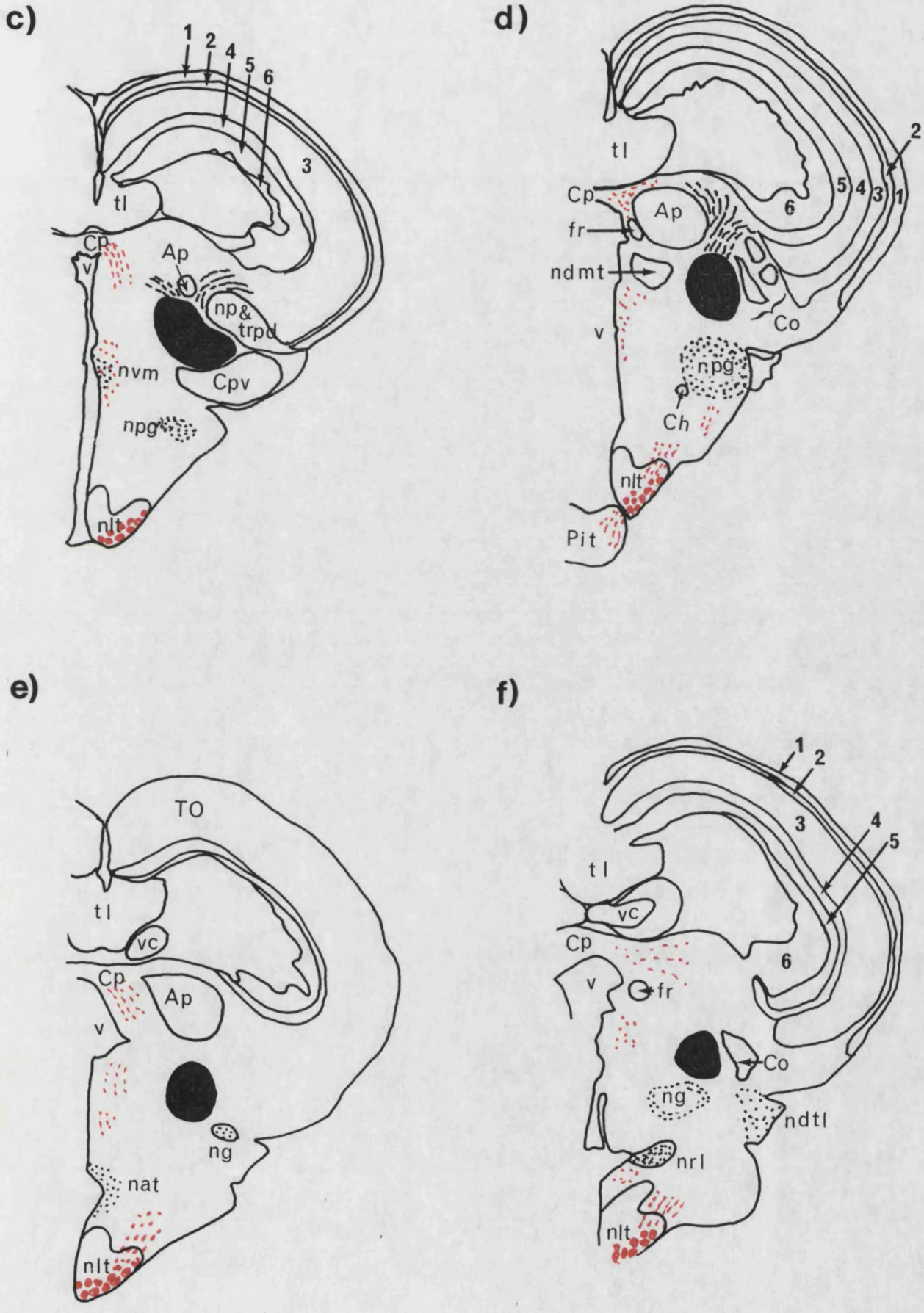
f



Camera lucida drawings of transferse 15µm sections through a trout brain, showing the area thalamica lateralis (dark region) and the distribution of MCH neurones (red). Cell bodies are shown as dots while axons are depicted by dotted lines. a) is the most anterior section.

Ap: area pretectalis; **Apr:** area preoptica; **Ch:** commissura horizontalis; **Co:** centrum opticum; **Cp:** commissura posterior; **Cpo:** commissura preoptica; **Cpv:** commissura postoptica ventralis pars ventralis; **fr:** fasciculus retroflexus; **nat:** nucleus anterior tuberis; **nc:** nucleus corticalis; **ndmt:** nucleus dorsolateralis thalami; **ndtl:** nucleus diffusus tori lateralis; **ng:** nucleus glomerulosum; **ngl:** nucleus geniculatus lateralis; **nlt:** nucleus lateralis tuberis; **np:** nucleus pretectalis; **npg:** nucleus preglomerulosa; **nrl:** nucleus recessus lateralis; **nvm:** nucleus ventromedialis; **Pit:** pituitary; **TO:** tectum opticum; **tl:** torus longitudinalis; **tr. op:** tractus opticus; **tr. pd:** tractus pretectalis descendens; **v:** third ventricle; **vc:** valvula cerebelli; **1:** stratum marginale; **2:** stratum opticum; **3:** stratum fibrosum and griseum superficiale; **4:** stratum griseum centrale; **5:** stratum album centrale; **6:** stratum periventriculare.




c)
1
2
4
5
6
3
tl
Cp
v
Ap
np&
trpd
nvm
Cpv
npg
nlt
d)
tl
2
Cp
Ap
6
5
4
3
1
fr
ndmt
Co
v
npg
Ch
nlt
Pit
e)
TO
tl
vc
Cp
Ap
v
ng
nat
nlt
f)
1
2
3
4
5
tl
vc
Cp
6
v
fr
Co
ng
ndtl
nrl
nlt

pineal and saccus dorsalis. Labelling was completely abolished when 1μM unlabelled melatonin was present in the incubation medium.

b. Retina

Radiolabelled melatonin bound avidly to retinal sections (Plates 5.11). In general, binding appeared to be uniformly distributed throughout the retina, that is, the pigmented and ganglion cell layer. Non-specific binding, however, appeared to be very high, as seen in sections which were incubated with excess unlabelled ligand.

c. Melanophores

Iodinated melatonin bound to trout melanophores *in vitro* both on scales and those over the pineal. The ligand bound avidly to trout scales and was not completely displaced in the presence of 1μM melatonin (Plates 5.12).

Plates 5.11

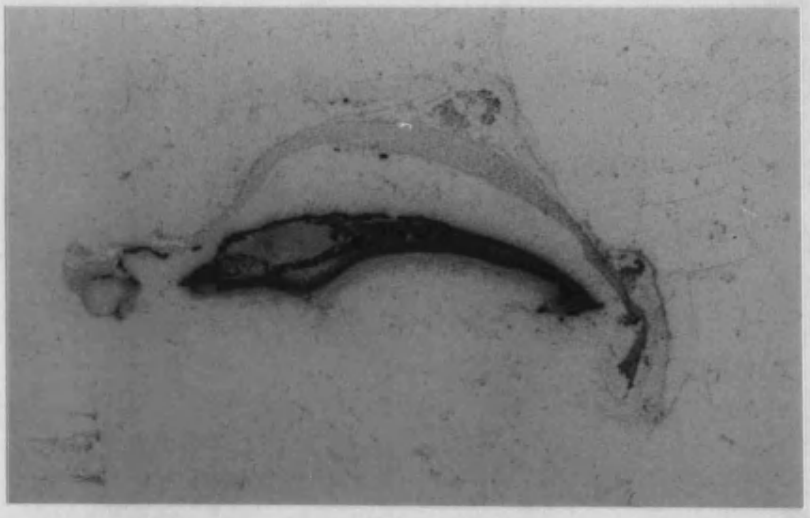
Distribution of ^{125}I-melatonin binding in trout retina.

a) Sections (16µm) were incubated with 70pM ^{125}I-melatonin for one hour.

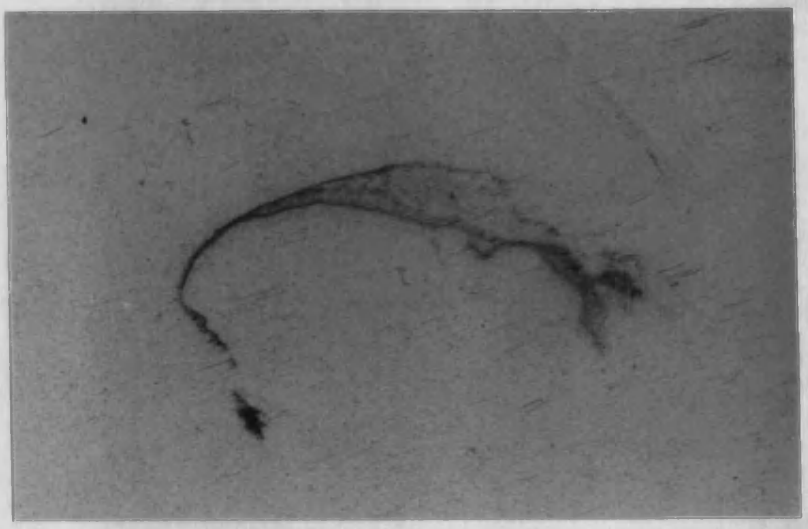
b) Labelled areas show complete displacement of binding in the presence of 1µM melatonin.

Autoradiographs were developed after one week.

a



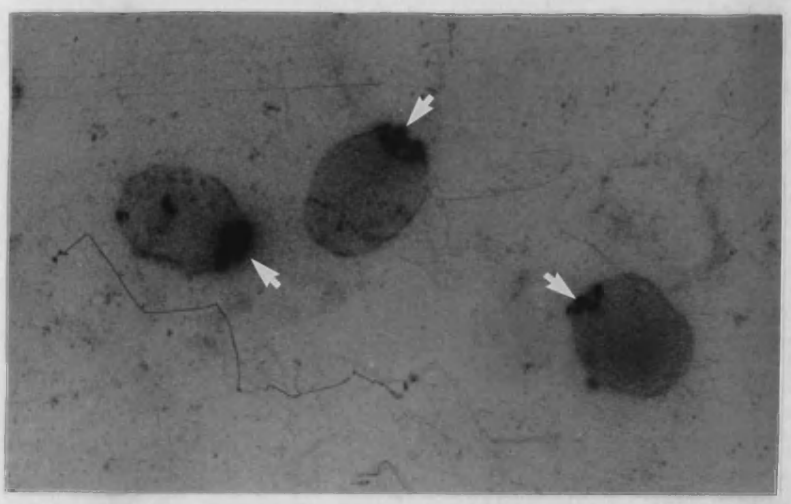
b



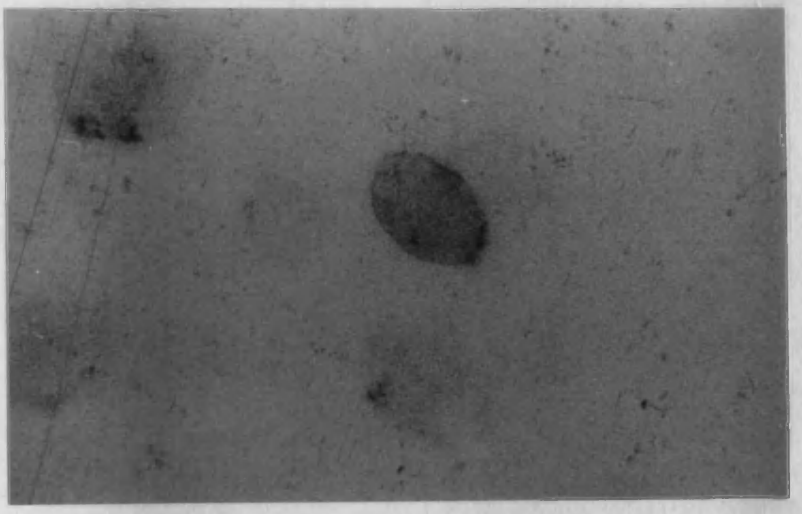
Plates 5. 12

Autoradiographs of 70pM ^{125}I-melatonin binding in trout scales in the absence (a) or presence (b) of 1μM unlabelled melatonin as a displacer. Dense labelling is seen over the melanophores (arrow).

a



b



5.8 DISCUSSION

In order to characterize the effects of melatonin, it is first necessary to discover its sites of action. Autoradiographic studies permit the location of melatonin binding sites in central or peripheral target tissues. The finding that melatonin binds to several different areas of trout brain and that this binding can be displaced by excess non-radiolabelled ligand, suggests the existence of specific binding sites. However, the physiological significance of these putative receptor binding sites is not known. In trout brain, iodinated melatonin bound in two main areas, the area thalamica lateralis (Athl) and the optic tectum. Since binding was most intense in the Athl, it is suggested that this may reflect a higher receptor density here than in any other area. A similar autoradiographic study in trout (Aggelopoulos and Demaine, 1990) also reported melatonin binding sites in the optic tectum. Unlike the present study, however, binding was reported more posteriorly, in the nucleus glomerulosum, torus semicircularis, lobus inferioris and the molecular layer of the cerebellum. No binding was observed in the saccus vasiculosus. It may be that these investigators did not examine the brain far enough anteriorly.

The Athl (Pinganaud and Clairamault, 1979) lies adjacent (ventral) to the pretectal nucleus (Schnitzlein, 1962; Niewenhuys and Pouwels, 1983) and receives direct innervation from the optic tract (Braford and Northcutt, 1983). Axons from the Athl extend to the optic tectum and the pretectal area (Ariens Kappers *et al.*, 1936; Schnitzlein, 1962; Ito and Kishida, 1977). The Athl nuclei on either side of the

brain are linked by the Athl tract which extends across the posterior commissure (Schnitzlein, 1962). As in many other vertebrates (with the exception of mammals), melatonin binding sites were found in the trout optic tectum. In chick, iodinated melatonin binds most intensely to the optic tectum (Stehle, 1990) and these putative receptors are even present two-days after hatching (Stehle, 1990). A small amount of non-specific binding was seen in the trout saccus vasculosus, a tissue which also non-specifically binds opioid peptides in the rainbow trout (Bird *et al.*, 1988).

Thus, like most other vertebrates, the majority of trout melatonin binding sites are located in areas of the brain concerned with processing visual information (Ehrlich and Mark, 1984, Moore, 1978). In rat and hamster brain melatonin binding sites are much more restricted, being found mostly in the SCN of the hypothalamus (Vanecek *et al.*, 1987; Duncan *et al.*, 1988; Reppert *et al.*, 1988; Weaver *et al.*, 1988, 1989; Laitenen *et al.*, 1989; Williams, 1989; Williams *et al.*, 1989), although some binding is also found in the median eminence/pars tuberalis (Duncan *et al.*, 1988; Williams and Morgan, 1988; Weaver *et al.*, 1989; Williams, 1989; Williams *et al.*, 1989; Williams, 1989), various thalamic nuclei (Duncan *et al.*, 1988; Weaver *et al.*, 1989) and the area postrema (Weaver *et al.*, 1989). In birds, melatonin binding sites have also been found in the visual suprachiasmatic nuclei (vSCN) (Stehle, 1990), an area of the brain thought to be homologous with the mammalian SCN (Cassone and Moore, 1987).

The SCN are considered the sites of a central circadian pacemaker which drives rhythmic events such as nocturnal melatonin release from the pineal (Review: Binkley, 1988). In mammals, this pacemaker is primarily responsible for phase-setting the melatonin rhythm (Cassone *et al.*, 1986b) via a neural network. Visual information about photoperiod is relayed to the SCN via the retino-hypothalamic pathway. Information is then relayed via the SCG to the pineal to alter melatonin release. Furthermore, experiments on rats suggest that melatonin may act in the SCN to alter circadian rhythmicity (Redman *et al.*, 1983). In birds, the vSCN alone do not drive the circadian rhythm of melatonin release. Dissociated chick pineal cells (Robertson and Takahashi, 1988) *in vitro*, will release melatonin with circadian periodicity in constant darkness, which suggests that the pinealocytes themselves may contain an intracellular endogenous oscillator. Extra-retinal photoreceptors have also been implicated in modulating avian pineal melatonin release (cf. Cassone and Menaker, 1984). Nevertheless, there is evidence that the vSCN are essential for maintaining circadian rhythmicity.

Although the secretion of melatonin in trout can be directly controlled by light (Gern and Greenhouse, 1988; present Chapter, Part I), it is conceivable that pineal activity is also modulated by a central pacemaker; analagous to the situation in birds and mammals. The SCN-equivalent in fish has not yet been located experimentally, nor is there any evidence for a pacemaker centre in the trout brain, although fish (like higher vertebrates) exhibit circadian activity rhythms (Kavaliers, 1980; Fingerman, 1976) and circannual reproductive

rhythms (Duston and Bromage, 1986b; 1987, 1988). On the basis of the present work it is proposed that should such a pacemaker exist in trout, the SCN-equivalent might be the Athl. As in higher vertebrates, the activity of such a pacemaker could be modulated by photoperiodic information (via the optic tectum) or by circulating melatonin release (high receptor density). Moreover, the Athl tract which connects nuclei on either side of the brain, traverses the pretectal area. It is therefore possible that the activity of the Athl might also be modulated by neurones innervating the pretectal area. These include MCH neurones. Thus, although the MCH fibres in this region are unlikely to be directly affected by circulating melatonin, through the absence of melatonin binding sites, they could modulate nervous connections from the Athl. In addition, it has recently been shown that the teleostean pineal (like that of higher vertebrates) is innervated by neurones of central origin (Ekstrom *et al.*, 1986, 1988). Some of these fibres have been traced from the habenular area of the brain but it is yet to be established whether any neural connection exists between these fibres and the Athl.

Iodinated melatonin also bound to trout retina (present work; Gern *et al.*, 1980, cited in Pautler and Hall, 1987). The eye and the pineal are closely related structures: embryologically, morphologically and functionally (Weichmann, 1986). Trout retinae have been shown to rhythmically release melatonin *in vitro* (Gern and Ralph, 1979), although, unlike the pineal, melatonin release from the trout retina occurs during the photophase rather than the scotophase. Retinal melatonin is thought to diffuse passively out of the eye (Pautler and

Hall, 1987) into the general circulation, as it is highly lipid soluble. The presence of iodomelatonin binding sites in trout retina suggests that the eyes act as target sites for melatonin. Indeed, melatonin has been implicated in a number of local retinal activities, including melanosome aggregation of pigment epithelium, rod outer-segment shedding, cone retino-motor movements and modulation of retinal neurotransmission (Weichmann, 1986). All of these activities would require the presence of retinal melatonin receptors. In addition, there is some evidence to suggest that the lowering of plasma melatonin concentrations following pinealectomy may result in a compensatory increase in retinal melatonin production (Reiter *et al.*, 1983; Osol *et al.*, 1985). This would explain why chick plasma melatonin levels are initially low following pinealectomy but are much higher several weeks later (Osol *et al.*, 1985), and suggests the existence of a direct feedback mechanism for the control of retinal melatonin production. In contrast, no iodo-melatonin binding was observed in the trout pineal which suggests that melatonin is not directly involved in regulating pineal activity. This further supports the hypothesis that the trout pineal does not contain an endogenous oscillator.

Melatonin was first noted for its potent melanosome-aggregating action on amphibian dermal melanophores (Lerner *et al.*, 1958). Administration of melatonin to trout, either orally via the ambient water (2g/l) or by intraperitoneal injection (6nmol/200g fish), results in intense body pallor (Hafeez, 1970). However, when 10^{-4}M melatonin was applied to isolated trout melanophores *in vitro* (Rance and Baker, 1979), no

melanosome aggregation was observed. This suggested that melatonin was not able to directly induce pallor at the melanophore level, but perhaps modulated the activity of the melanotropins or the local innervation. The results of the present work (Chapter Five) show that di-iodinated melatonin ($7x\ 10^{-8}M$) bound to both the melanophores on trout scales, and in the meninges which suggests the presence of specific melatonin binding sites on these cells. It therefore seems unlikely that melatonin is able to directly stimulate trout melanophores alone, although its action may be mainly synergistic in this species.

CHAPTER SIX

DISCUSSION AND GENERAL CONCLUSIONS

As with many areas of research, the work presented in this thesis has raised more questions than it has answered. Before discussing factors which may affect the control of MCH release or its possible roles in teleosts, the main problems investigated in this thesis will be briefly summarised.

In teleosts, MCH release occurs in response to perception of a pale background (Kishida *et al.*, 1989; present work, Chapter Three). Trout on a white background have significantly higher plasma MCH concentrations than those on black backgrounds, while fish maintained in the dark have circulating MCH titres which are lower still. Two factors were considered; the effect of different intensities of retinal illumination, and which region of the retina might be involved in mediating the paling response.

The results of the experiments in Chapter Three suggest that, in trout, the lateral or dorsolateral region must be illuminated to stimulate MCH release. It is not yet known whether retinal illumination of the ventral retina inhibits MCH release or whether it stimulates body darkening in some other way. Studies on other teleost species such as the three-spined stickleback, Gasterosteus aculeatus (Hogben and Landgrebe, 1940), the minnow, Phoxinus phoxinus, (Gentle,

1972) and the killifish, Fundulus heteroclitus, (Butcher, 1938), have shown that illumination of the ventral retina only results in body darkening, while illumination of either the dorsal retina (Butcher and Adelmann, 1937; Butcher, 1938; Gentle, 1972) or the lateral retinal area (Hogben and Landgrebe, 1940) elicits a paling response, depending on the species examined. The specific area of the retina concerned with paling responses may be dependent on differences in the shape of the eye. The simplest concept would be that the rate of MCH release is proportional to the intensity of light illuminating these dorsal/lateral regions of trout retina. However, this is unlikely to be the case, since a 20-fold difference in light intensity (75 lux or 1500 lux) did not significantly alter the plasma MCH concentrations in fish in either black or white tanks. Trout plasma MCH values were lower in brightly illuminated black tanks than in dimly illuminated white ones, even though the light intensity reflected from the sides of black tanks was about four-fold higher than that from those of the white tanks. The amount of MCH release seemed, instead, to be correlated to the ratio of incident to reflected light intensities. Hence, it is possible that its release is influenced by the contrast between illumination of the dorsal and lateral retinal regions (which receive mainly reflected light) and the ventral retina (which receives mostly incident light). It was, however, difficult to determine the retinal regions responsible for initiating the paling response (release of MCH)in unaneasthetised trout using this experimental approach for a variety of reasons. In an attempt to illuminate specific retinal regions, the fish were confined by placing six trout in a small tank which could be

illuminated either from above or below. Thus, fish were crowded and intra-specific competition could have been high. We now know that such stresses can influence both the synthesis and release of MCH. Secondly, the fish did not tolerate illumination of the dorsal retina alone: instead of remaining horizontal, they oriented themselves nose-downwards, facing the light source and making it impossible to illuminate the dorsal retina alone. Future experiments could be performed with individual fish housed in shallower tanks. This would eliminate crowding stress and make illumination of specific retinal areas more accurate. However, it is not possible to remove all stressful stimuli so that there is a chance that some other factors may interact to alter plasma MCH titres. To date very little is known about visual processing in trout. In mammals, electrical recordings are made from individual retinal cells of an anaesthetised animal in response to various illumination stimuli, and this provides information about visual fields and the most effective stimulus for each cell type, so that a wiring diagram can be constructed. Preliminary experiments have been carried out in the goldfish (Kaneko, 1970) where it seems the the bipolar cells behave in a similar way to mammalian ganglion cells, ie., they typically respond to contrast with on-centre and off-centre visual fields. Recording changes in electrical activity from the MCH cells of an anaesthetised trout in response to illumination of specific retinal areas would provide specific information about which retinal areas were responsible for initiating MCH release.

The next question raised in this thesis was whether the release of MCH was an immediate response to a pale background, or whether it was modulated by a central diurnal oscillator. The experiments of Chapter Four show that rather than merely responding to levels of illumination, there is a distinct diurnal rhythm of MCH release. Although probably also present in black-adapted trout (Chapter Four), the diurnal rhythm of plasma MCH concentrations can only be clearly seen in white-adapted fish, where plasma MCH concentrations are high (Kishida *et al.*, 1989; present work, Chapter Three). Plasma MCH concentrations rise with light onset to reach a peak at about the middle of the day, before declining again, prior to dark onset. When fish were maintained under continuous illumination for three days, a rise in plasma MCH titres was observed at the appropriate time on the second day, despite the total absence of a preceding dark period. This suggested that the morning rise in MCH concentrations may be regulated by a diurnal oscillator rather than by light onset itself. However, further experiments showed that darkness was able to override this diurnal oscillator to suppress the morning rise in plasma MCH concentrations. When the dark period was extended, delaying light onset for six hours, MCH values remained low but as soon as the lights came on, trout plasma MCH concentrations rose rapidly (about five times faster than before) to peak at about the same time as on the previous day. Thus, although an illuminated white background is a necessary condition for the release of MCH, the rhythm appears to be modulated also by a diurnal oscillator. When the photophase was advanced by six hours, MCH peaked after the normal interval, ie., 4.5h after light onset, then declined after the usual

period, before an apparent second peak at the normal time in the middle of the day. Unfortunately it was not confirmed that this noon-time increase in plasma MCH did represent a second peak as sampling was discontinued at this point. At first glance, this suggests that the rhythm had immediately phase-shifted by six hours, but the second increase in plasma MCH concentrations observed at noon may indicate that there is a diurnal oscillator modulating plasma MCH titres, which is aware of "real" time. Should such an oscillator exist, it is likely that there is a sensitive "window" during the second half of the night and early morning during which the rhythm can be manipulated. There was no evidence that trout plasma MCH concentrations are modulated by a circadian (as opposed to a diurnal) oscillator, since the diurnal pattern was lost within 36h of continuous lighting.

Trout plasma MCH concentrations also appeared to vary seasonally. Peak hormone titres in fish examined during long days (18L:6D) were about three times higher at their peak than those seen in fish on a short daylength regime (9L:15D). Yet measurements show that the total amount of MCH released throughout the day in summer is double that released in winter. Since the daylength in summer was twice that in winter, it seems likely that the increased daylight hours in summer were responsible for the elevated MCH concentrations.

Much more work needs to be done to clarify the sensory and/or neuronal influences on MCH secretion. The problems associated with this type of experimental approach are, however, numerous. Firstly,

the experiments are laborious and also very expensive, involving about a hundred fish over a 48h sampling period, since each fish yields only one plasma sample. The use of different fish for each plasma sample is likely to have increased the intra- and inter-group variation. Moreover, the different individuals may each have been affected by different levels of stress. Since stress is known to alter plasma MCH concentrations, and in some instances can more than double MCH release (Green and Baker, 1991), this may have altered MCH concentrations non-uniformly within each sampling group. For these reasons, this is perhaps not the best way to measure the diurnal MCH cycle in trout. Collecting blood samples periodically from the same fish (by cannulating the dorsal aorta) would reduce some of the inherent variation, but would not be possible without causing some stress to the individual, particularly over long periods of time. Given these technical difficulties, one could instead measure diurnal changes in MCH cell activity by monitoring changes in neuronal discharge in response to changes in the light/dark cycle with indwelling electrodes.

The aim of the experiments in Chapter Five was to determine whether MCH, or its antagonist, α-MSH, had a direct effect on pineal melatonin production. The evidence presented in this thesis suggests that neither MCH nor α-MSH alter melatonin release from trout pineals *in vitro*, but it was shown that pineal melatonin production was enhanced by exogenous dopamine administration. In mammals, in which the pineal is not directly photosensitive, pineal melatonin production is enhanced via sympathetic innervation. Information about

the light/dark cycle is conveyed from the eyes to the suprachiasmatic nuclei (SCN) (or pacemaker centres) and thence via the superior cervical ganglia (SCG) to the pineal, so that melatonin is produced and released into the circulation at night. The neurotransmitter, norepinephrine (NE) plays a major role in regulating mammalian pinealocyte activity, but other neurotransmitters, such as VIP, Substance P and α-MSH are also thought to be involved. The fish pineal, on the other hand is directly photosensitive: photoreceptive pinealocytes are hyperpolarised in the light and depolarised in the dark (Meissl and Ekstrom, 1988a, b; Ekstrom and Meissl, 1988). The pineals of some teleost species, like the pike (Falcon *et al.*, 1987; 1989) and goldfish (Kezuka *et al.*, 1988; 1989) will show a circadian pattern of pineal melatonin production. In other words, isolated pineals will continue to rhythmically release melatonin under conditions of continuous darkness. This is not true, however, for all teleost species. Isolated trout pineals *in vitro* show a continuous rather than cyclical release of melatonin in continuous darkness (Gern and Greenhouse, 1988). Hence, the trout pineal does not appear to contain an endogenous circadian oscillator but responds directly to light and dark. The present work shows that the stimulatory catecholamines in trout act via dopamine receptors, and suggest that dopamine may serve to control pineal melatonin production. It is not yet known whether the trout pineal contains dopaminergic neurones but an investigation into the presence of dopamine in trout pineals is planned. Dopamine has been detected in the retina in a variety of teleosts by Falck Hillarp immunofluorescence (Ehlinger *et al.*, 1969, cited in Graham, 1974). Thus melatonin production in the trout pineal

may be regulated, not only directly by light, but also by dopaminergic innervation, perhaps from a pacemaker centre in the brain. One might speculate that a possible candidate for such a pacemaker centre is the Anterior thalamica lateralis (Athl) - a region rich in specific melatonin binding sites. The advantage of neural modulation of melatonin production (in addition to direct photoreception) is that diurnal release of melatonin will continue during the dark phase despite, for example, bright moonlight, and will similarly be suppressed during the day, irrespective of whether, for example, the fish enters a dark place. Contrary to the situation in other vertebrates, there is no evidence that in trout NE, VIP, GABA, α-MSH or MCH altered melatonin production in isolated trout pineals in vitro. Although a stimulatory effect was observed with the addition of exogenous NE, this could not be repeated with specific adrenergic agonists but could be inhibited by haloperidol (a specific dopaminergic antagonist). It was therefore thought to be due to an action of NE on dopamine receptors. It must, however, be stressed that this *in vitro* method only tests the direct ability of the neuromodulator/neurotransmitter to alter melatonin production in cells of the pineal bulb. One cannot therefore comment on the putative role of any of these neuromodulators *in vivo*, since no account has been taken of any factors which might affect melatonin production by synapsing with pinealopetal neurones at the base of the pineal stalk. Hence, although it is unlikely from autoradiographical studies in trout (Chapter Five) that melatonin directly affects the activity of MCH neurones, it remains possible that MCH might modulate trout pineal activity *in vivo*.

In conclusion, although MCH has been immunocytochemically detected in the brains of all vertebrate classes so far examined, and in the optic lobes of some insects (Review: Baker, 1988b; Eberle, 1988) which suggests that MCH has been conserved over a long period of evolution, little is known of its role(s) in vertebrates. In higher vertebrates, it is presumed to act as a neuromodulator, neurotransmitter or hypophyseal regulatory peptide. The distribution of MCH-like neurones is often widespread (Skofitsch *et al.*, 1985; Naito *et al.*, 1986) which suggests that, like opiates, MCH may participate in a number of diverse functions. In teleosts, it appears to have two main functions. Firstly, the least validated role for MCH is in its involvement in the stress response. There is considerable circumstantial evidence for this, with white-adapted trout being less easily stressed than black-adapted fish. Generally there is a negative correlation between plasma MCH concentrations and the responsiveness of the hypothalamo-pituitary-adrenal axis (HPA) to stress (Baker and Rance, 1981; Gilham and Baker, 1985; Baker *et al.*, 1985b; Baker *et al.*, 1986). Direct evidence for the role of MCH in the stress response was provided by Green and co-workers (1991). Immunoneutralization of MCH in incubated hypothalami/ thalami from white-reared trout *in vitro* led to an increase in the release of bioactive CRF. MCH also appeared to have a direct inhibitory affect on pituitary corticotrophs, since it inhibited ACTH release in a dose-dependent manner *in vitro* (Baker *et al.*, 1985b), but this response has since been questioned for other vertebrates, eg. mammals (Navarra *et al.*, 1990). Secondly, MCH has a role in colour change responses, causing body pallor during adaptation to a pale background

colour, but this role is restricted to bony fishes. The effectiveness of MCH to cause melanosome aggregation *in vitro* has been well documented for a wide variety of teleost species (Wilkes *et al.*, 1984; Baker *et al.*, 1985a; Oshima *et al.*, 1985; Fujii and Oshima, 1986; Nagai *et al.*, 1986). In trout, MCH is released into the circulation in response to a pale background colour (Kishida *et al.*, 1989; present work, Chapter Three) but the circulating concentrations in this species are too low to achieve full melanosome aggregation. Hence, denervated melanophores of trout on a white background remain dark (Baker *et al.*, 1986). Green and Baker (1989) suggested that, *in vivo*, MCH synergises with norepinephrine, which is released from local nerve terminals, to stimulate melanosome aggregation. This may not be true for other teleost species, however, since denervated melanophores of the white- adapted molly, Poecilia latipinna remain pale. It may be that the plasma MCH concentrations in this teleost are high enough to elicit pallor alone, but this is not yet known.

It may be useful to consider how the role(s) for MCH might have evolved. Presumably there must have been some selective advantages for teleosts to adopt MCH as a colour-change hormone, and perhaps some function of MCH itself made it a suitable candidate for such a role. One might speculate that its suitabilty was determined by virtue of its antagonism to the darkening hormone, α-MSH. Interestingly, α-MSH is released by all vertebrates in response to stress, and therefore MCH might have originally been of some value to antagonise this release during the stress response in teleosts. Its role in stress response may have subsequently evolved. One might

further speculate that MCH neurones are responsive to light and this may have pre-adapted MCH for its role as a paling hormone. More may be understood of the ancient function of MCH once more is known of its role in tetrapods. In rats, the MCH neurones were first recognised as being αMSH-immunoreactive but ACTH-immunoreactive (Kohler *et al.*, 1984). Some neurones were traced to the hippocampus which is known to be the site of motivated, goal-oriented behaviour, such as food and water intake, aggression, reproduction and also memory (Canny, 1990). The hippocampus is also the source of corticosteroid-responsive inhibitory neurones, necessary for corticosteroid feedback. These negatively feedback onto the "CRF" component of the HPA axis in rats. If MCH neurones do innervate the hippocampus, it is possible that MCH might also have an indirect effect on goal-oriented behaviour and may indirectly affect the HPA via the "CRF" component. In this respect, the role of MCH in higher vertebrates may be similar to that in teleosts, since the dorsal hypothalamus/ thalamus (which also receives MCH innervation) is believed to be analagous to the mammalian hippocampus. It would be interesting to measure changes in plasma MCH titres in trout during some goal oriented behaviour such as during their migrations to and from the sea. Hence, a greater understanding of the role of MCH in teleosts and the factors which might influence its release, may shed some light on its role in higher vertebrates, including humans.

APPENDIX

A.1 IODINATION OF α-MSH AND MCH (Chloramine T Method)

Solutions required

Synthetic MCH (Peninsula Laboratories) or α-MSH (Sigma Chem. Co.)

2 μg/10 μl 0.01M HCl

10 μl $Na^{125}I$ (1 mCi)(Amersham)

0.25M Phosphate buffer pH 7.4

0.05M Phosphate buffer pH 7.4

1 % trifluoroacetic acid (TFA)

80 % methanol containing 1 % TFA

10 mg/ml polypep (Sigma) in 0.05M Phosphate buffer, pH 7.4

0.05M Phosphate buffer with 0.25 % BSA and 1 % mercaptoethanol, pH 7.4

A series of graded methanol solutions containing 1 % TFA ; 40 %, 42.5 %, 45 %, 46 %, 47 %, 48 %, 49 %, 50 %, 52.5 %, 55 %, 57.5 %, 60 %.

5 mg Chloramine T in 10 ml 0.25M Phosphate buffer (made up immediately before use).

Procedure

A reverse-phase RC-18 column (Sep-Pak; Waters Associates, Milford, MA) was primed by washing slowly with 2 ml of each solution in the following order: 1 % TFA, 80 % methanol plus 1% TFA, polypep, 80 % methanol plus 1% TFA, 1 % TFA.

The iodination reaction was carried out in an Eppendorf containing 10μl (1 mCi) ^{125}I and 20 μl 0.25M Phosphate buffer, pH 7.4. A 10 μl drop of

MCH solution and 15 µl Chloramine T (made up at the last moment) were pipetted onto one side of the Eppendorf tube wall. The Eppendorf lid was securely closed and its contents vortexed. The reaction was terminated after 30s by the addition of 600 µl 0.05M Phosphate buffer containing 0.25 % BSA and 1 % mercaptoethanol (stop solution).

The contents of the Eppendorf were applied to the primed Sep-pak and slowly forced through twice, followed by two washes with 600 µl 0.25M Phosphate buffer. The labelled peptide was eluted with 2 x 600 µl of each of the graded methanol solutions. All washing solutions were collected. The fractions containing iodinated MCH were detected by counting 10 µl volumes on a γ-counter. Both peptides eluted at approximately 48-50% methanol. Fractions with the highest cpm were diluted with 4 ml stop solution together with 4 ml of the appropriate methanol concentration and stored at -20 °C, at which temperature the solution did not freeze.

To ascertain which iodinated fraction was most suitable for use in the RIA, standard curves were run for each. The fraction with the steepest gradient or highest zero binding was used.

A.2 COUPLING OF MCH ANTISERUM TO IMMUNOBEADS

(As recommended by Bio-Rad).

Buffer A (0.003M Phosphate Buffer, pH 6.3)

A 0.3M stock solution KH_2PO_4 was prepared. This was diluted 1:80 with distilled water. The pH was adjusted to 6.3 with 0.1M KOH and the volume made up to one litre.

Buffer B

0.15M NaCl (4.39g) was dissolved in 250 ml distilled water and 33.3 ml 0.3M KH_2PO_4 stock solution was added. The pH was adjusted to 7.2 with 1M KOH and the volume made up to 500 ml with distilled water.

Buffer C

Sodium chloride (36.6g) was dissolved in 350 ml Buffer One stock solution. The pH was adjusted to 7.2 with 0.1M KOH and the volume made up to 500 ml with Buffer One.

Buffer D (0.005M Phosphate buffer pH 7.2

16.7 ml stock 0.3M phosphate buffer was diluted with 800 ml water and the pH adjusted to 7.2 with 1M KOH before being made up to a final volume of one litre with distilled water.

Buffer E (Solid Phase Triton X-100 Buffer)

0.05M Phosphate buffered saline (PBSG) pH 7.6, containing 0.1% Triton X-100, 0.9% NaCl, and 0.01% Thimerosal.

Procedure

Immunobeads (50 mg/5 ml) (Bio-Rad Laboratories, Cat No. 170-5910) were centrifuged at 2000g for 10 min. The supernatant was decanted and the beads were washed twice (by magnetic stirrer) with Coupling Buffer A.

They were resuspended in 1600 µl Buffer A and briefly centrifuged (800g, 1 min), before adding 200 µl of neat MCH antiserum (raised by Dr A N Eberle). The mixture was stirred and allowed to stand at 4°C for 1h. Next, 3mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC; Sigma) was added to the bead suspension. Again the mixture was stirred and left to stand at 4°C for an hour before the addition of a further 3mg EDAC. The immunobead suspension was stirred and left at 4°C overnight.

The next day the following steps were performed rapidly. The suspension was centrifuged at 2000g for 10min at 4°C. The pellet was washed twice with 20 ml Buffer B. The beads were then washed twice with 25 ml Buffer C, and then twice more with 25 ml Buffer B. After discarding the final supernatant, the beads were resuspended in 5 ml Buffer B and left at 4°C for at least four hours. The immunobeads were then centrifuged at 3000g for 10 min at 4°C, before being washed twice with 25 ml Buffer D and finally resuspended in 5 ml Buffer E. This stock bead preparation was stored at 4°C and remained usable for six months.

To test the optimum bead concentration for RIA, stock immunobeads were diluted (1:25, 1:50, 1:100, 1:200) with Solid Phase buffer (Buffer E). Radioimmunoassays were carried out for each bead dilution and the bead dilution falling on the steepest part of the curve was used.

A.3 DEXTRAN-COATED ACTIVATED CHARCOAL

In many radioimmunoassays, separation of free from radiolabelled tracer is achieved through the addition of 500 µl ice-cold dextran-coated activated charcoal suspension. Dextran T_{70} (Sigma; 1.25 g/l) and activated charcoal (5 g/l) were made up in the appropriate assay buffer at 4 °C and the suspension was left to stir on ice for at least 30 min before use. Using an Eppendorf multipette, 500 µl charcoal suspension was quickly added to each tube (except those containing labelled tracer alone). Fifteen minutes later tubes were centrifuged at 2000g for 15 min. Depending on the assay protocol, either the supernatant was discarded and the remaining pellet (containing unbound radiolabelled tracer) counted in a γ-counter; or the supernatant (containing the antibody-bound fraction) was tipped into 6ml Scintillant (Optiphase Safe, LKB Laboratories) and the vials were shaken well, left to stand for about 1h and then counted on a scintillation counter with the appropriate quench correction.

REFERENCES

Abrao MS, Castrucci AM de L, Hadley ME and Hruby VJ (1989)- Melanin concentrating hormone: mechanism of action. Abstract, XIth International Symposium on Comparative Endocrinology, Malaga, Spain.

Aggelopoulos N and Demaine C (1990)- Quantitative receptor autoradiography of melatonin receptors in three non-mammalian vertebrates: the green anole lizard (Anolis carolinensis), the edible frog (Rana esculenta) and the rainbow trout (Salmo gairdneri). Abs. 66; Vth Colloquium of the European Pineal Study Group, University of Surrey, UK.

Andersen AC, Pelletier G, Eberle AN, Leroux P, Jejou S and Vaudry H (1986)- Localization of melanin-concentrating hormone-like immunoreactivity in the brain and pituitary of the frog, Rana ridibunda. Peptides 7 (6) 941-952.

Andersen AC, Jegou S, Eberle AN, Tonon MC, Pelletier G and Vaudry H (1987)- Co-existence of Melanin-Concentrating Hormone (MCH) and Alpha-Melanophore-Stimulating Hormone (α-MSH) in the preoptic nucleus of the frog brain. Brain Res. Bull. 18257-259.

Ariens Kappers CU, Huber GC and Crosby CE (1936)- "The comparative anatomy of the nervous system of vertebrates, including man", Macmillan.

Armstrong SM (1989)- Melatonin and circadian control in mammals. Experientia 45 (10) 932-938.

Bagnara JT (1963)- The pineal and the body lightening reaction of larval amphibians. Gen. Comp. Endocrinol. 3 86-100.

Baker BI and Ball JN (1975)- Evidence for a dual pituitary control of teleost melanophores. Gen. Comp. Endocrinol. 25 145-152.

Baker BI and Rance TA (1981)- Differences in concentrations of plasma cortisol in the trout and eel following adaptation to black and white backgrounds. J. Endocrinol. 89 135-140.

Baker BI and Rance TA (1983)- Further observations on the distribution and properties of teleost melanin concentrating hormone. Gen. Comp. Endocrinol 53 423-431.

Baker BI, Wilson JF and Bowley TJ (1984)- Changes in pituitary and plasma levels of MSH in teleosts during physiological colour change. Gen. Comp. Endocrinol. 55 142-149.

Baker BI, Eberle AN, Baumann JB, Siegrist W and Girard J (1985a)- Effect of melanin-concentrating hormone on pigment and adrenal cells *in vitro*. Peptides 6 (6) 1125-1130.

Baker BI, Bird DJ and Buckingham JC (1985b)- Salmonid melanin-concentrating hormone inhibits corticotrophin release. J. Endocrinol. 106 R5-R8.

Baker BI, Bird DJ, and Buckingham JC (1986)- Effects of chronic administration of melanin-concentrating hormone on corticotrophin, melanotrophin, and pigmentation in the trout. Gen. Comp. Endocrinol, 63 62-69

Baker BI (1988a)- Relative importance of MCH and MSH in melanophore control. "Advances in Pigment Cell Research" Alan R. Liss Inc. pp 505-515.

Baker BI (1988b)- Melanin Concentrating Hormone. *In* "The melanotropic peptides, Vol. 2" (Ed. ME Hadley), CRC Press, Boca Raton FL.

Baker BI, Kinsman RG, Moss CA, White PD, Paul PKC, Brown DW, Campbell MM and Osguthorpe DJ (1990)- Structure-activity studies with fragments and analogues of salmonid melanin concentrating hormone. Peptides 11 1103-1108.

Barber LD, Baker BI, Penny JC and Eberle AN (1987)- Melanin concentrating hormone inhibits the release of αMSH from teleost pituitary glands. Gen. Comp. Endocrinol. 65 79-86.

Barton BA, Peter RE and Paulen CR (1980)- Plasma cortisol levels of fingerling rainbow trout (Salmo gairdneri) at rest and subjected to handling, confinement, transport and stocking. Can. J. Fish Aquat. Sci. 37 805-811.

Batten TFC and Baker BI (1988)- Melanin-concentrating hormone (MCH) immunoreactive hypophysial neurosecretory system in the teleost Poecilia latipinna: Light and electron microscopic study. Gen. Comp. Endocrinol. 70 193-205.

Billard R and Peter RE (1982)- A stereotaxic atlas and technique for nuclei of the diencephalon of rainbow trout (Salmo gairdneri). Reprod. Nutr. Develop. 22 (1A) 1-25.

Binkley SA, Reibman JB and Reilly (1978)- The pineal gland: A biological clock *in vitro*. Science. 202 1198-1201

Binkley S (1988)- "The pineal: Endocrine and neuroendocrine function". Endocrinology Series. Prentice Hall, New Jersey.

Bird DJ, Jackson M, Baker BI and Buckingham JC (1988)- Opioid binding sites in the fish brain: An autoradiographic study. Gen. Comp. Endocrinol. 70 49-62.

Bird DJ and Baker BI (1989)- An immunological study of the secretory activity of neurons producing melanin-concentrating hormone in a teleost. Neuroscience 28 (1) 245-251.

Bird DJ, Baker BI and Kawauchi H (1989)- Immunocytochemical demonstration of melanin-concentrating hormone and proopiomelanocortin-like production in the brain of trout and carp. Gen. Comp. Endocrinol. 74 442-450.

Birks EK and Ewing RD (1981)- Photoperiod effects on hydroxyindole-O-methyltransferase activity in the pineal gland of chinook salmon (Oncorhynchus tshawytscha). Gen. Comp. Endocrinol. 43 277-283.

Birks EK and Ewing RD (1986)- Seasonal changes in pineal melatonin content and hydroxyindole-O-methyltransferase activity in juvenile Chinook salmon, Oncorhynchus tshawytscha. Gen. Comp. Endocrinol. 64 91-98.

Bowley TJ, Rance TA and Baker BI (1983)- Measurement of immunoreactive α-MSH hormone in the blood of rainbow trout kept under various conditions. J. Endocrinol. 97 267-275.

Braford Jr. MR, and Northcutt RG (1983)- Organisation of the diencephalon and pretectum of the ray finned fishes. *In* "Fish Neuroendocrinology", Chapter Four, Vol. 2, (Eds. RG Northcutt and RE Davis), University of Michigan Press, Rexdale, Canada, USA.

Bresson JL, Clavequin MC, Fellmann D and Bugnon C (1989)- Human hypothalamic neuronal system revealed with a salmon melanin-concentrating hormone (MCH) antiserum. Neurosci. Letts. 102 39-43.

Brickner RM (1929)- A description and interpretation of certain parts of the teleostean mid-brain and thalamus. J. Comp. Neurol. 47 225-282.

Burton D (1987)- Differential effects of diurnal and continuous illumination regimes on melanophores and xanthophores of winter flounder, Pseudopleuronectes americanus Canad. J. Zool. 65 (5) 1140-1143.

Burton D (1991)- Acta Endocrinologica, in press.

Butcher EO and Adelmann HB (1937)- The effects of covering and rotating the eyes on the melanophoric responses in Fundulus heteroclitus. Bull. Mt. Desert Island Biol. 16-18.

Butcher EO (1938)- The structure of the retina of Fundulus heteroclitus and the regions of the retina associated with the different chromatic responses. J. Exp. Biol. 79 (2) 275-297.

Bylund DB, Rudeen PK, Petterbord LJ and Ray-Prenger C (1988)- Identification of α_2-adrenergic receptors in chicken pineal gland using [^{3}H] rauwolscine. J. Neurochem. 51 81-86.

Canny BJ (1990)- Hippocampal glucocorticoid receptors and the regulation of ACTH secretion. Mol. Cell. Endocrinol. 71 C35-C38.

Cassone VM and Menaker M (1984)- Is the avian circadian system a neuroendocrine loop?. J. Endocrinol. Zool. 232 539-549.

Cassone VM, Takahashi JS, Blaha CD, Lane RF and Menaker M (1986a)- Dynamics of noradrenergic circadian input to the chicken pineal gland. Brain Res. 384 334-341.

Cassone VM, Chesworth MJ and Armstrong SM (1986b)- Entrainment of rat circadian rhythms by daily injection of melatonin depends upon the hypothalamic suprachiasmatic nuclei. Physiol. Behav. 36 1111-1121.

Cassone VM and Moore RY (1987)- The retinohypothalamic projection of the suprachiasmatic nucleus of the house sparrow, Passer domesticus. J. Comp. Neurol. 266 171-182.

Castrucci AM de L, Hadley ME, Wilkes BC, Zechel C and Hruby VJ (1987)- The melanotropic peptides I. Melanin concentrating hormone exhibits both MSH and MCH activities on individual melanophores. Life Sci. 40 1845-1851.

Castrucci AM de L, Hadley ME, Lebl M, Zechel C and Hruby VJ (1989)- Melanocyte stimulating hormone and melanin concentrating hormone

may be structurally and evolutionarily related. Regulatory Peptides 24 27-36.

Chik CL, Ho AK and Klein DC (1988a)- α_1-adrenergic potentiation of VIP stimulation of rat cyclic AMP and cyclic GMP: evidence for a role of calcium and protein kinase C. Endocrinol. 122 702-708.

Chik CL, Ho AK and Klein DC (1988b)- Dual receptor regulation of cyclic nucleotides: α_1-adrenergic receptor potentiation of vasoactive intestinal peptide stimulation of pinealocyte adenosine 3',5'-monophosphate. Endocrinol. 122 1646-1651.

Collin JB, Meissl H, Voisin P, Brisson P and Falcon J (1986)- Rhythmic signals of pineal transducers: physiological, biochemical and cytochemical evidence. *In* "Advances in Pineal Research" (Eds. RJ Reiter and M Karasek), John Libbey and Co. Ltd., pp 41-50.

Deguchi T (1979a)- A circadian oscillator in cultured cells of the chicken pineal gland. Nature 282 94-96.

Deguchi T (1979b)- Role of adenosine 3',5'-monophosphate in the regulation of circadian oscillation of serotonin N-acetyl-transferase activity in cultured chick pineal gland. J. Neurochem. 33 45-51.

Deguchi T (1981)- Rhodopsin-like photosensitivity of isolated chicken pineal gland. Nature 290 706-707.

Dodt E (1963)- Photosensitivity of the pineal organ in the teleost Salmo irideus (Gibbons). Experientia 19 642-643.

Duncan MJ, Takahashi JS and Dubocovich ML (1988)- 2-[^{125}I]-iodomelatonin binding sites in hamster brain membranes: pharmacological characteristics and regional distribution. Endocrinol. 122 1825-1833.

Dupont A, Kastin AJ, Labrie F, Pelletier G, Puviani R and Schally AV (1975)- Distribution of radioactivity in the organs of the rat and mouse after injection of ^{125}I α-melanocyte stimulating hormone. J. Endocrinol. 64 237-241.

Duston J and Bromage N (1986a)- Serum melatonin profiles in rainbow trout maintained under long (16L-8D) and short (8L-16D) photoperiods. J. Endocrinol. 108 Abs. 77 "5th Joint Meeting of British Endocrine Societies". 1986, Sheffield.

Duston J and Bromage N (1986b)- Photoperiodic mechanisms and rhythms of reproduction in the female rainbow trout. Fish Physiol. and Biochem. 2 (1-4) 35-51.

Duston J and Bromage N (1987)- Constant photoperiod regimes and the entrainment of the annual cycle of reproduction in the female

rainbow trout (Salmo gairdneri). Gen. Comp. Endocrinol. 65 373-384.

Duston J and Bromage N (1988)- The entrainment and gating of the endogenous cirannual rhythm of reproduction in the female rainbow trout (Salmo gairdneri). J. Comp. Physiol. A. 164 259-268.

Ebadi M (1984)- Regulation of the synthesis of melatonin and its significance to neuroendocrinology. *In* "The pineal gland" (Ed. RJ Reiter), New York, Raven Press, pp 1-37.

Eberle AN, Atherton E, Dryland A and Sheppard RC (1986)- Peptide synthesis. Part 9. Solid synthesis of melanin concentrating hormone using a continuous-flow polyamide method. J. Chem. Soc. Pekin. Trans. I. (2) 361-368.

Eberle AN (1988)- "The melanotropins: chemistry, physiology, and mechanisms of action"., Karger: Basel, Switzerland; New York, USA.

Eberle AN, Baker BI, Kishida M, Baumann JB and Girard J (1989)- Current concepts: Melanin concentrating hormone. IV. Development of a sensitive solid-phase radioimmunoassay for melanin-concentrating hormone. Life Sci. 45 1149-1154.

Eckenstein F and Sofroniew M (1983)- Identification of central cholinergic neurones contaning both choline acetyltransferase and acetylcholinesterase and of central neurones containing only acetylcholinesterase. J. Neurosci. 5 2286-2291.

Eddy JM and Strahan R (1968)- The role of the pineal complex in the pigmentary effector system of the lampreys, Mordacia mordax (Richardson) and Geotria australis. Gen. Comp. Endocrinol. 11 528-534.

Ehinger B, Falck B and Laties AM (1969)- Adrenergic neurons in teleost retina. Z. Zellforsch Anat. Mikrosk. 97m 285-297.

Ehrlich D and Mark R (1984)- An atlas of the primary visual projections in the brain of the chick, Gallus gallus. J. Comp. Neurol. 223 592-610.

Ekstrom P and Korf HW (1986)- Putative cholinergic eliments in the photosensory pineal organ and retina of a teleost, Phoxinus phoxinus L. (Cyprinidae). Distribution of ChAT immunoreactivity, AChE-positive elements, and pinealofugally projecting neurons. Cell Tiss. Res. 246 321-329.

Ekstrom P, Reschke M, Steinbusch H and van Veen Th (1986)- Distribution of noradrenaline in the brain of the teleost, Gasterosteus aculeatus L.: an immunocytochemical analysis. J. Comp. Neurol. 254 297-313.

Ekstrom P, van Veen T, Bruun A and Ehinger B (1987)- GABA-immunoreactive neurones in the photosensory pineal organ of the rainbow trout: two distinct neuronal populations. Cell Tiss. Res. 250 87-92.

Ekstrom P and Meissl H (1988)- Intracellular staining of physiologically identified receptor cells and hyperpolerizing interneurons in the teleost pineal organ. Neurosci. 25 (3) 1061-1070.

Ekstrom P, Honkanen T and Ebbesson SOE (1988)- FMRFamide-like immunoreactive neurons in the nervus terminalis of teleosts innervate both retina and pineal organ. Brain Res. 460 68-75.

Ekstrom P and Meissl H (1989)- Signal processing in a simple vertebrate photoreceptor system: the teleost pineal organ. Phys. Bohemslovaca 38 (4) 311-326.

Enami M (1955)- Melanophore-concentrating hormone (MCH) of possible hypothalamic origin in the catfish, Parasilurus. Science 121 36-37.

Etkin W (1962)- Hypothalamic control of the pars intermedia activity in the frog. Gen. Comp. Endocrinol. 1 148-159.

Falcon J, Guerlotte JF, Voisin P and Collin JPH (1987)- Rhythmic melatonin biosynthesis in a photoreceptive pineal organ: A study in the pike. Neuroendocrinol. 45 479-486.

Falcon J and Collin J-P (1989)- Photoreceptors in the pineal of lower vertebrates: Functional aspects. Experientia 45 909-913.

Falcon J, Marmillon JB, Claustrtat B and Collin J-P (1989)- Regulation of melatonin secretion in a photoreceptive pineal organ: An *in vitro* study in the pike. J. Neurosci. 9 (6) 1943-1950.

Fingerman SW (1976)- Circadian rhythms of brain 5-hydroxytryptamine and swimming activity in the teleost, Fundulus grandis Comp. Bioch. Physiol. 54C 49-53.

Fraser S, Cowen P, Franklin M, Franey C and Arendt J (1983)- Direct radioimmunoassay for melatonin in plasma. Clin. Chem. 29 396-397.

Fujii R and Oshima N (1986)- Review: Control of chromatophore movements in teleost fishes. Zool. Sci. 3 13-47.

Gentle MJ (1972)- The eye and colour change in the minnow (Phoxinus phoxinus L). J. Exp. Biol. 57 701-707.

Gern WA and Ralph CL (1979)- Melatonin synthesis by the retina. Science 204 183-184.

Gern WA, Gorell TA and Owens DW (1980)- Melatonin and pigment cell rhythmicity. *In* "Melatonin: Current status and perspectives" (Eds. N Birau and W Schoolt), Permagon, Oxford, New York.

Gern WA and Greenhouse SS (1988)- Examination of in vitro melatonin secretion from perfused trout (Salmo gairdneri) pineal organs maintained under diel illumination or continuous darkness. Gen. Comp. Endocrinol. 71 163-174.

Gilham ID and Baker BI (1985)- A black background facilitates the response to stress in teleosts. J. Endocrinol 105 99-105.

Graham LT Jr (1974)- Comparative aspects of neurotransmitters in the retina. *In* "The eye: Comparative Physiology" Volume 6, (Eds. H Davson and LT Graham Jr), Academic Press, New York, London, Chapter Six, p 283.

Green JA and Baker BI (1989)- Influence of nerves and hormones on the control of trout melanophores. Life Sci. 45 1127-1132.

Green JA (1990)- The factors controlling the secretion of the melanin-concentrating hormone (MCH) in the rainbow trout. Thesis, University of Bath.

Green JA and Baker BI (1991)- The influence of repeated stress on the release of melanin-concentrating hormone in the rainbow trout. J.Endocrinol. 128 (2) 261-266.

Green JA, Baker BI and Kawauchi H (1991)- The effect of rearing trout on black or white backgrounds on their secretion of melanin-concentrating hormone and their sensitivity to stress. J. Endocrinol. 128 (2) 267-274.

Gwinner E (1974)- Testosterone induces 'splitting of circadian locomoter activity in birds. Science 185 72-74.

Hadley ME, Zechel C, Wilkes BC, Castrucci AM de L, Visconti MA, Pozo-Alonso M and Hruby VL (1987)- Differential structural requirements for the MSH activities of melanophore concentrating hormone. Life Sci. 40 1139-1145.

Hafeez MA (1970)- Effect of melatonin on body coloration and spontaneous swimming activity in rainbow trout, Salmo gairdneri. Comp. Bioch. Physiol. 36 639-656.

Hafeez MA and Quay WB (1970)- The role of the pineal organ in the control of phototaxis and body coloration in rainbow trout (Salmo gairdneri, Richardson). Z. Vergl. Physiol. 68 403-416.

Hafeez MA and Zerihun L (1974)- Studies on the central projections of the pineal tract in rainbow trout, Salmo gairdneri Richardson,

using cobalt chloride iontophoresis. Cell Tiss. Res. 154 485-510.

Hamm HE, Takahashi JS and Menaker M (1983)- Light-induced decrease of serotonin N-acetyltransferase activity and melatonin in the chicken pineal gland and retina. Brain Res. 266 287-293.

Healey EG (1948)- The colour change of the minnow (Phoxinus laevis AG). Bull. Anim. Behav. 6 5-15.

Hogben L and Slome D (1936)- The pigmentary effector system VIII. The dual receptive mechanism of the amphibian background response. Proc. Roy. Soc. Lond. B 120 138-150.

Hogben L and Landgrebe FW (1940)- The pigmentary effector system IX. Receptor fields of the teleostean visual response. Proc. Roy. Soc. B 128 317-342.

Ito H and Kishida R (1975)- Organisation of the teleostean nucleus rotundus. J. Morphology 147 89-107.

Joss JMP (1973)- The pineal complex, melatonin, and colour change in the lamprey, Lampetra. Gen. Comp. Endocrinol. 21 188-195.

Kaku K, Inoue Y, Matsutani A, Okubo M, Hatao K, Kaneki T and Yanaihara N (1983)- Receptors for vasoactive intestinal polypeptide on rat dispersed cells. Biomed. Res. 4 321-328.

Kaneko A (1970)- Physiological and morphological identification of horizontal, bipolar and amacrine cells in goldfish retina. J. Physiol. 207 623-633.

Kaneko T, Cheng P, Oka H, Oda T, Yanaihara N and Yanaihara C (1980)- Vasoactive intestinal polypeptide stimulates adenylate cyclase and serotonin N-acetyltransferase activities in rat pineal *in vitro*. Biomed. Res. 1 84-87.

Kavaliers M (1980)- Circadian locomotor activity rhythms of the Turbot, Lota lota: Seasonal differences in period length and the effect of pinealectomy. J. Comp. Physiol. 136 215-218.

Kawauchi H, Kawazoe I, Tsubokawa M, Kishida M, Baker B I (1983)- Characterization of melanin concentrating hormone in chum salmon pituitaries. Nature 305 321-323.

Kawauchi H, Kawazoe I, Hayashi Y, Minamitake Y, Tanaka S, Naito N, Nakai Y, Ide H and Hirano T (1986)- Melanin concentrating hormone: chemistry and biology. *In* "Natural products and biological activities." (Eds. M Imura, T Goto, T Murachi, T Nakajima) Nato Foundation Symposium. Univ. Tokyo Press, Tokyo.

Kawauchi H (1989)- Current concepts: Melanin concentrating hormone. II. Structure and biosynthesis of melanin concentrating hormone. Life Sci. 45 1133-1140.

Kawazoe I, Kawauchi H, Hirano T and Naito N (1987a)- Structure-activity relations of melanin-concentrating hormone. Int. J. Pep. and Prot. Res. 29 (6) 714-721.

Kawazoe I, Kawauchi H, Hirano T and Naito N (1987b)- Characterization of melanin-concentrating hormone in teleost hypothalamus. Gen. Comp. Endocrinol. 65 423-431.

Kezuka H, Furukawa K, Aida K and Hanyu I (1988)- Daily cycles of plasma melatonin levels under long or short photoperiod in the common carp, Cyprinus carpio. Gen. Comp. Endocrinol. 72 296-302.

Kezuka H, Aida K and Hanyu I (1989)- Melatonin secretion from goldfish pineal gland in organ culture. Gen. Comp. Endocrinol. 75 217-221.

Kishida M, Baker B I and Eberle AN (1989)- The measurement of melanin-concentrating hormone in trout blood. Gen. Comp. Endocrinol. 74 221-229.

Klein DC, Berg GR and Weller JL (1970)- Melatonin synthesis: Adenosine 3',5'-monophosphate and norepinephrine stimulate N-acetyltransferase. Science 168 979.

Klein DC and Moore RY (1979)- Pineal N-acetyltransferase and hydroxyindole-O-methyltransferase: control by retinohypothalamic tract and the suprachiasmatic nucleus. Brain Res. 174 245-262.

Klein DC, Sugden D and Weller JL (1983)- Postsynaptic α-adrenergic receptors potentiate the β-adrenergic stimulation of pineal serotonin N-acetyltransferase. Proc. Natl. Acad. Sci. 80 599-603.

Kohler C, Haglund L and Swanson LW (1984)- A diffuse α-MSH-immunoreactive projection to the hippocampus and spinal chord from individual neurons in the lateral hypothalamic area and zona incerta. J. Comp. Neurol. 233 501-514.

Korf H-W (1974)- Acetylcholinesterase-positive neurons in the pineal and parapineal organ of the rainbow trout, Salmo gairdneri (with special reference to the pineal tract). Cell Tiss. Res. 155 475-489.

Korf H-W, Zimmermam NH and Oksche A (1982)- Intrinsic neurones and neural connections of the pineal organ of the house sparrow, Passer domesticus, as revealed by anterograde and retrograde transport of horseradish peroxidase. Cell Tiss. Res. 222 243-260.

Kruk ZL and Pycock CJ (1983)- "Neurotransmitters and drugs" (Eds. ZL Kruk and CJ Pycock), Second Edition, Croom Helm, London and Canberra, pp 83-86.

Laitinen JT, Castren E, Vakkuri O and Saavedra JM (1989)- Diurnal rhythm of melatonin binding in the rat suprachiasmatic nucleus. Endocrinol. 124 (3) 1585-1587.

Lebl M, Hruby VJ, Castrucci AM de L and Hadley ME (1989)- Melanin concentrating hormone analogues: contraction of the cyclic structure. II. Antagonist activity. Life Sci. 44 451-457.

Leeb-Lundberg LMF, Cotecchia S, De Blasi A, Caron MG and Lefkowitz RJ (1986)- Regulation of adrenergic receptor function by phosphorylation. 1. Agonist-promoted desensitization and phosphorylation of α_1-adrenergic receptors coupled to inositol phospholipid metabolism in DDT, MF-2 smooth muscle cells. J. Biol. Chem. 262 3098-3105.

Lefkowitz RJ, Benovic JL, Kobilka B and Caron MG (1986)- β-Adrenergic receptors and rhodopsin: shedding new light on an old subject. TIPS 7 444-448.

Lerner AB, Case JD, Takahashi Y, Lee TH and Mori W (1958)- Isolation of melatonin, the pineal gland factor that lightens melanocytes. J. Am. Chem. Soc. 80 2587.

Matsunaga TO, Hruby VJ, Lebl M, Castrucci AM de L and Hadley ME (1989)- Melanin concentrating hormone(MCH): Structure-function aspects of its melanocyte stimulating hormone-like (MSH-like) activity. Peptides 10 773-778.

Meissl H and George SR (1985)- Effects of GABA and its antagonists, Bicuculline and Picrotoxin, on the nerve cell discharges of the photosensory pineal organ of the frog, Rana esculenta. Brain Res. 332 39-46.

Meissl H and Ekstrom P (1988a)- Photoreceptor responses to light in the isolated pineal organ of the trout, Salmo gairdneri. Neurosci. 25 (3) 1071-1076.

Meissl H and Ekstrom P (1988b)- Dark and light adaptation of pineal photoreceptors. Vision Res. 28 (1) 49-56.

Minth CD, Qui H, Akil H, Watson SJ and Dixon JE (1989)- Two precursors of melanin-concentrating hormone : DNA sequence analysis and *in situ* and immunochemical localization. Proc. Natl. Acad. Sci. USA. 86 4292-4296.

Moller M and van Veen Th (1982)- Fluorescence histochemistry. *In* "The Pineal Gland", Vol. 1: Anatomy and Biochemistry. (Ed. RJ Reiter), CRC Press, Boca Raton. pp 69-93.

Moller M and Korf K-W (1987)- Neural connections between the brain and the pineal gland of the golden hamster (Mesocricetus auratus). Cell Tiss. Res. 139 187-192.

Moller M, Reuss S, Olcese J, Stehle J and Vollrath L (1987)- Central neural control of pineal melatonin synthesis in the rat. Experientia 43 186-188.

Moore RY (1978)- Central nervous control of circadian rhythms. *In* "Frontiers of Neuroendocrinology" (Eds. WF Ganong and L Martin), Raven Press, New York. pp 185-206.

Mortia Y (1966)- Entladungsmuster pinealer neurone der regenbogenforelle (Salmo irideus) bei belichtung des zwischenhirns. Pfluger's Arch. Ges. Physiol. 289 155-167.

Morton DJ and Forbes HJ (1988)- Pineal gland N-acetyltransferase and hydroxyindole-O-methyltransferase activity in the rainbow trout (Salmo gairdneri): seasonal variation linked to photoperiod. Neurosci. Letts. 94 333-337.

Nagai M, Oshima N and Fujii R (1986)- A comparative study of melanin-concentrating hormone (MCH) action on teleost melanophores. Biol. Bull. 171 360-370.

Nagaishi H and Oshima N (1989)- Control of pigment migration in melanophores in the dorsal and ventral skin of the upside-down catfish. Comp. Bioch. Physiol. 93C 67-71.

Nahon J-L, Presse F, Bittencourt JC, Sawchencko PE and Vale W (1989a)- The rat melanin-concentrating hormone messenger

ribonucleic acid encodes multiple putative neuropeptides coexpressed in the dorsolateral hypothalamus. Endocrinol. 125 (4) 2056-2065.

Nahon J-L, Schoepfer R and Vale W (1989b)- cDNA sequence of salmon melanin-concentrating hormone exhibits similarities with 7SL RNA. Nucl. Acids Res. 17 (9) 3598.

Naito N, Nakai Y, Kawauchi H and Hayashi Y (1985)- Immunocytochemical identification of melanin concentrating hormone in the brain and pituitary gland of the teleost fishes Oncorhynchus keta and Salmo gairdneri. Cell Tiss. Res. 24241-48.

Naito N, Kawazoe I, Nakai Y, Kawauchi H and Hirano T (1986)- Coexistence of immunoreactivity for melanin-concentrating hormone and α-melanocyte-stimulating hormone in the hypothalamus of the rat. Neurosci. Letts. 70 75-80

Navarra P, Tsagarakis S, Coy DH, Rees LH, Besser GM and Grossman AB (1990)- Rat melanin concentrating hormone does not modify the release of CRF-41 from rat hypothalamus or ACTH from the anterior pituitary *in vitro*. J. Endocrinol. 127 R1- R4.

Nieuwenhuys R and Pouwels E (1983)- The brain stem of actinopterygian fishes, *In* "Fish Neurobiology", Vol. 1, Chapter Two, (Eds. RG Northcutt and RE Davis), University of Michigan Press, Rexdale, Canada, USA.

Oaknin S, Vaughan MK, Troiahi ME, Vaugham GM and Reiter RJ (1987)- Injections of α-melanocyte stimulating hormone affect pineal serotonin, melatonin, and N-acetyl-transferase activity. Comp. Bioch. Physiol. 86C 23-26.

O'Donohue TL, Miller RL and Jacobowitz DM (1979)- Identification, characterization and stereotaxic mapping of intraneuronal α-melanoctye hormone-like immunoreactive peptides in discrete regions of the rat brain. Brain Res. 176 101-123.

O'Donohue TL, Miller RL, Pendleton RC and Jacobowitz DM (1980)- Demonstration of an endogenous circadian rhythm of α-melanocyte stimulating hormone in the rat pineal gland. Brain Res. 186 145-155.

Ohno S, Wolf U and Atkin NB (1968)- Evolution from fish to mammal by gene duplication. Hereditas 59 169-187.

Okamoto K, Yasumura K, Fujitani K, Kiso Y, Kawauchi H, Kawazoe I and Yajima H (1984)- Synthesis of the heptadecapeptide corresponding to the entire aminoacid sequence of salmon melanin-concentrating hormone. Chem. Pharm. Bull. 32 (8) 2963-2970.

Oliver C and Porter J C (1978)- Distribution and charaterization of α-melanocyte-stimulating hormone in the rat brain. Endocrinol. 102 697-705.

Ono N, Wada C, Oikawa I, Kawazoe I and Kawauchi H (1988)- Structures of two kinds of mRNA encoding the chum salmon melanin-concentrating hormone. Gene. 71 433-438.

Oshima N, Kasukawa H, Fujii R, Wilkes BC, Hruby VJ, Castrucci AM de L and Hadley ME (1985)- Melanin concentrating hormone (MCH) effects on teleost (Chrysiptera cyanea) melanophores. J. Exp. Biol. 235 175-180.

Oshima N, Kasukawa H, Fujii R, Wilkes BC, Hruby VJ and Hadley ME (1986)- Action of melanin-concentrating hormone (MCH) on teleost chromatophores. Gen. Comp. Endocrinol. 64 381-388.

Osol G, Schwartz B and Foss DC (1985)- Effects of time, photoperiod and pinealectomy on ocular and plasma melatonin concentrations in the chick. Gen. Comp. Endocrinol. 58 514-520.

Owens DW , Gern WA, Ralph CL and Boardman TJ (1978)- Nonrelationship between background adaptation in the rainbow trout (Salmo gairdneri). Gen. Comp. Endocrinol. 34 459-467.

Pangerl B, Pangerl A and Reiter RJ (1990)- Circadian variations of adrenergic receptors in the mammalian pineal gland: A review. J. Neural Transm. 81 17-29.

Patel S and Demaine C (1990)- Effects of stimulation of the superior cervical ganglia and local application of noradrenaline on

electrical activity of the Syrian hamster pineal gland. J. Neuroendocrinol. 2 (4) 493-499.

Pautler EL and Hall FL (1987)- Movement of melatonin across the retinal pigment epithelium. Exp. Eye Res. 45 351-355.

Pelletier G, Guy J, Desy L, Li S, Eberle AN and Vaudry H (1987)- Melanin-concentrating hormone (MCH) is co-localized with alpha-melanocyte-stimulating hormone in the rat but not in the human hypothalamus. Brain Res. 423 247-253.

Pevet P, Ebels I, Swaab DF, Mud M and Arimura A (1980)- Presence of AVT-, α-MSH-, LHRH and somatostatin-like compounds in the rat pineal gland and their relationship with the UM05R fraction: An immunocytochemical study. Cell Tiss. Res. 200 341-353.

Pickering AD and Christie P (1981)- Changes in the concentrations of plasma cortisol and thyroxine during sexual maturation of the hatchery-reared brown trout, Salmo trutta, L. Gen. Comp. Endocrinol. 44 487-496.

Pickering AD and Pottinger AD (1983)- Seasonal and diel changes in plasma cortisol levels in the brown trout, Salmo trutta. Gen. Comp. Endocrinol. 49 232-239.

Pickering AD, Pottinger AD and Sumpter JP (1986)-Independence of the pituitary-interrenal axis and melanotroph activity in the brown

trout, Salmo trutta L., under conditions of environmental stress. Gen. Comp. Endocrinol. 64 (2) 206-211.

Pickford GE and Atz J (1957)- "The physiology of the pituitary gland of fishes". Zoological Society, New York.

Pinganaud G and Clairambault P (1979)- The visual system of the trout, Salmo irideus Gibb. A degeneration and radioautographic study. J. Hirnforsch. 20 413-431.

Pittendrigh CS (1960)- Circadian rhythms and the circadian organisation of living organism. Cold Spring Harb. Quant. Biol. 25 159-184.

Powell KA and Baker BI (1987)- Ultrastructural demonstration that melanin-concentrating-like and α-melanocyte-stimulating-like immunoreactive molecules co-exist in the same neurosecretory granules. Neurosci. Letts. 80 268-274.

Powell KA and Baker BI (1988)- Structural studies of nerve terminals containing melanin-concentrating hormone in the eel, Anguilla anguilla. Cell Tiss. Res. 251 433-439.

Pratt BL and Takahashi JS (1987)- Alpha-2 adrenergic regulation of melatonin release in chick pineal cell cultures. J. Neurosci. 7 3665-3674.

Pratt BL and Takahashi JS (1989)- Vasoactive intestinal polypeptide and α_2-adrenoceptor agonists regulate adenosine 3',5'-monophosphate accumulation and melatonin release in chick pineal cell cultures. Endocrinol. 125 (5) 2375-2384.

Presse F, Nahon J-L, Fischer WH and Vale W (1990)- Structure of the human melanin concentrating hormone mRNA. Mol. Endocrinol. 4 (4) 632-637.

Rance TA and Baker BI (1979)- The teleost melanin-concentrating hormone - a pituitary hormone of hypothalamic origin. Gen. Comp. Endocrinol 37 64-73.

Rance TA, Baker BI and Webley G (1982)- Variations in plasma cortisol concentrations over the 24-hour period in the rainbow trout Salmo gairdneri. Gen. Comp. Endocrinol. 48 269-274.

Redman, Armstrong S and Ng KT (1983)- Free-running activity rhythms in the rat: entrainment by melatonin. Science 219 1089-1091.

Reed BL (1968)- The control of circadian pigment changes in the pencil fish: a proposed role for melatonin. Life Sci. 7 961-973.

Reiter RJ, Richardson BA Mathews SA, Lane SJ and Ferguson BN (1983)- Rhythms in immunoreactive melatonin in the retina and harderian gland in rats: persistence after pinealectomy. Life Sci. 32 1229-1236.

Reiter RJ, Vaughan GM, Oaknin S, Troiani ME, Cozzi B and Li K (1987)- Norepinephrine or isoproterenol stimulation of pineal N-acetyltransferase activity and melatonin content in the Syrian hamster is restricted to the second half of the dark phase. Neuroendocrinol. 45 249-256.

Reiter RJ (1988)- *In* "Pineal research reviews. Volume 6". (Ed. RJ Reiter). Alan R. Liss Inc. New York .

Reppert SM, Weaver DR, Rivkees SA and Stopa EG (1988)- Putative melatonin receptors in a human biological clock. Science. 242 78-81.

Reuss S and Schroeder H (1987)- Neuropeptide Y effects on pineal melatonin synthesis in the rat. Neurosci. Lett. 74 158-162.

Ristori M-Th, Rehm J-Cl and Laurent P (1979)- Quantitative determination of plasma catecholamines in trout during controlled hypoxia. J. Physiol. Paris 75 67A.

Robertson LM and Takahashi JS (1988)- Circadian clock in cell culture. II. *In vitro* photic entrainment of melatonin oscillation from dissociated chick pineal cells. J. Neurosci. 8 (1) 22-30.

Rodrigues KT and Sumpter JP (1984)- Effects of background adaptation on the pituitary and plasma concentrations of some pro-

opiomelanocortin-related peptides in the rainbow trout (Salmo gairdneri). J Endocrinol 101 277-284

Romero JA, Zatz M, Kebabian JW and Axelrod J (1975)- Circadian cycles in the binding of ^{3}H-alprenolol to β-adrenergic receptor sites in rat pineal. Nature 258 435-436.

Ronnekliev OK, Kelly MJ and Wuttle W (1980)- Single unit recordings in the rat pineal gland: evidence for habenulo-pineal neural connections. Exp. Brain Res. 39 187-192.

Sakai KK, Schneider D, Felt B and Marks BH (1976)- The effect of α-MSH on β-adrenergic receptor mechanisms on the rat pineal. Life Sci 19 1145-1150.

Sawyer TK, Staples DJ, Castrucci AM de L and Hadley ME (1989)- Discovery and structure activity relationships of novel α-melanocyte stimulating hormone inhibitors. Peptide Res. 2 (1) 140-146.

Schnitzlein HN (1962)- The habenula and dorsal thalamus of some teleosts. J. Comp. Neurol. 118 225-266.

Sekiya K, Ghatei MA, Lacoumenta S, Burnet PWJ, Zamir N, Burrin JM, Polak JM and Bloom SR (1988)- The distribution of melanin-concentrating hormone-like immunoreactivity in the central

nervous system of rat, guinea-pig, pig, and man. Neurosci. 23 925-930.

Semm P, Demaine C and Vollrath L (1981)- Electrical responses of pineal cells to melatonin and putative transmitters. Exp. Brain Res. 43 361-370.

Shiotani Y, Yamano M, Shiosaka S, Emson PC, Hillyard CJ, Girie S and MacIntyre I (1986)- Distribution and origins of substance P (SP)- calcitonin gene-related peptide (CGRP)- vasoactive intestinal polypeptide (VIP)- and neuropeptide Y (NPY)- containing nerve fibres in the pineal gland of gerbils. Neurosci. Letts. 70 187-192.

Simonneaux V, Ouichou A and Pevet P (1990)- Vasoactive intestinal peptide stimulates melatonin release from perfused pineal glands of rats. J. Neural Transm. 79 69-79.

Skofitch G, Jacobowitz DM and Zamir N (1985)- Immunocytological localization of a melanin concentrating hormone-like peptide in the rat brain. Brain Res. Bull. 15 635-649.

Stehle J (1990)- Melatonin binding sites in the brain of the 2-day-old chicken: an autoradiographic localization. J. Neural Transm. 81 83-89.

Sugden D, Vanecek J, Klein DC, Thomaas TP and Anderson WB (1985)- Activation of protein kinase C potentiates isoprenaline-induced cyclic AMP accumulation in rat pinealocytes. Nature 314 359-361.

Sugden D, Ho AK, Sugden AL and Klein DC (1988)- Negative feedback mechanisms: Evidence that desensitization of pineal α_1-adrenergic responses involves protein kinase C. Endocrinol. 123 1425-1432.

Sugden D (1989)- Melatonin biosynthesis in the mammalian pineal gland Experientia 45 922-932.

Swaab DF and Fisser B (1977)- Immunocytochemical localization of α-melanocyte stimulating hormone (α-MSH)-like compounds in the rat nervous system. Neurosci. Lett. 7 313-317.

Takahashi JS, Hamm H and Menaker M (1980)- Circadian rhythms of melatonin release from individual superfused chicken pineal glands in vitro Proc. Natl. Acad. Sci. USA 77 2319-2322.

Takahashi JS and Menaker M (1984)- Multiple redundant circadian oscillators within the isolated avian pineal gland. J. Comp. Physiol. 154A 435-440.

Takayama Y, Wada C, Kawauchi H and Ono M (1989)- Structures of two genes coding for melanin concentrating hormone of chum salmon. Gene 80 65-73.

Tiligada E and Wilson JF (1990)- Minireview: Ionic, neuronal and endocrine influences on the proopiomelanocortin system of the hypothalamus. Life Sci. 46 (2) 81-90.

Uddman R, Malm L and Sundler F (1980)- Vasoactive intestinal peptide (VIP) occurs in nerves of the pineal gland. Experientia 36 1119-1120.

Underwood H (1977)- Circadian organization in lizards: the role of the pineal organ. Science 195 587-589.

Underwood H (1985)- Pineal melatonin rhythms in the lizard, Anolis carolinensis : effects of light and temperature. J. Comp. Physiol. A. 157 57-65.

Vacas MI, Keller Sarmiento MI, Pereyra EN, Etchegoyen GS and Cardinali DP (1979)- *In vitro* effect of neuropeptide-Y on melatonin and norepinephrine release in rat pineal gland. Cell Mol. Neurobiol. 7 (3) 309-315.

Vakkuri O, Leppaluoto J and Vuolteenaho O (1984)- Development and validation of a melatonin radioimmunoassay using radioiodinated melatonin as a tracer. Acta Endocrinologica 106 152-157.

Vallarino M, Andersen AC, Delbende C, Ottenello I, Eberle AN and Vaudry H (1989)- Melanin-concentrating hormone (MCH) immuno-reactivity in the brain and pituitary of the dogfish

Scyliorhinus canicula. Co-localization with alpha-melanocyte-stimulating hormone (α-MSH) in hypothalamic neurons. Peptides 10 375-382.

Vanecek J, Pavlik A and Illnerova H (1987)- Hypothalamic melatonin receptor sites revealed by autoradiography. Brain Res. 435 359-362

van Eys GJ and Peters PTW (1981)- Evidence for a direct role of α-MSH in morphological background adaptation of the skin in Sarotherodon mossambicus. Cell Tiss. Res. 217 361-372.

Vaughan JM, Fischer WH, Hoeger C, Rivier J and Vale W (1989)- Characterization of melanin-concentrating hormone from rat hypothalamus. Endocrinol. 125 (3) 1660-1665.

Vermes I, Tilders FJH and Stoof JC (1985)- Dopamine inhibits the release of immunoreactive beta-endorphin from rat hypothalamus *in vitro*. Brain Res. 326 41-46.

Vigh-Teichmann I, Korf HW, Oksche A, and Vigh B (1982)- Opsin-immunoreactive outer segments and acetylcholine-positive neurones in the pineal complex of Phoxinus phoxinus (Teleostei, Cyprinidae). Cell Tiss. Res. 227 351-369.

Voisin P and Collin JP (1986)- Regulation of chicken pineal arylalkylamine-N-acetyltransferase by postsynaptic α_2-adrenergic receptors. Life Sci. 39 2025-2032.

Voisin P, Martin C and Collin JP (1987)- α_2-adrenergic regulation of arylalkylamine N-acetyltransferase in organ-cultured chick pineal gland. J. Neurochem. 49 1421-1426.

Voisin P, van Camp G and Collin JP (1990)- N-acetylation of serotonin is correlated with α_2- but not with β-adrenergic regulation of cyclic AMP levels in cultured chick pineal cells. J. Neurochem. 54 (6) 1953-1960.

Wainwright SD (1980)- Diurnal cycles in serotonin and acetyl-transferase activity and cyclic GMP content of cultured chick pineal glands. Nature 285 478-480.

Wainwright SD and Wainwright LK (1980)- Regulation of cycle in chick pineal serotonin N-acetyltransferase activity *in vitro* by light. J. Neurochem. 35 451-457.

Weaver DR, Namboodiri MAA and Reppert SM (1988)- Iodinated melatonin mimics melatonin action and reveals discrete binding sites in fetal brain. FEBS Lett. 228 123-127.

Weaver DR, Rivkees SA and Reppert SM (1989)- Localization and characterization of melatonin receptors in rodent brain by *in vitro* autoradiography. J. Neurosci. 9 2581-2590.

Weichmann AF (1986)- Melatonin: parallels in pineal gland and retina. Exp. Eye Res. 42 507-527.

Whitaker (1987-89)- Whitaker's Almanack, William Clowes Ltd., Suffolk.

Wight P (1971)- "Physiology and Biochemistry of the domestic fowl" (Eds. D Bell and T Freeman), Academic Press, London., Vol. 1, pp 549.

Wilkes BC, Hruby VJ, Castrucci AM de L, Sherbrooke WC and Hadley ME (1984)- Synthesis and biological actions of melanin concentrating hormone. Bioch. Biophys. Res. Comm. 122 613-619.

Williams LM and Morgan PJ (1988)- Demonstration of melatonin-binding sites on the pars tuberalis of the rat. J. Endocrinol. 119 R1-R3.

Williams LM (1989)- Melatonin-binding sites in the rat brain and pituitary mapped by *in vitro* autoradiography. J. Mol. Endocrinol. 3 71-75.

Williams LM, Morgan PJ, Hastings MH, Lawson W, Davidson G and Howell HE (1989)- Melatonin receptor sites in the Syrian hamster brain and pituitary. Localization and characterization using [^{125}I]iodomelatonin. J. Neuroendocrinol 1 (5) 315-320.

Wilson JF and Dodd JM (1973)- The role of melanophore-stimulating hormone in melanogenisis in the dogfish, Scyliorhinus canicula L. J. Endocrinol. 58 685-686.

Wilson JF and Morgan MA (1979)- A radioimmunoassay for alpha-melanotropin in rat plasma. J. Pharmacol. Meths. 2 97-116.

Young JZ (1935)- The photoreceptors of lampreys. II. The functions of the pineal complex. J. Exp. Biol. 12 254-270.

Yuwiler A (1987)- Synergistic action of post-synaptic alpha-receptor stimulation on VIP-induced increases in pineal N-acetyl-transferase activity. J. Neurochem. 49 806-811.

Zamir N, Skofitsch G, Bannon MJ and Jacobowitz DM (1986)- Melanin-concentrating hormone: unique peptide neuronal system in rat brain and pituitary gland. Proc. Natl. Acad. Sci. USA 83 1528-1531.

Zatz M and Mullen DA (1988)- Norepinephrine, acting via adenylate cyclase, inhibits melatonin output but does not phase-shift the

pacemaker in cultured chick pineal cells. Brain Res. 450 137-143.

Zatz M, Mullen DA and Moskal JR (1988)- Photoendocrine transduction in cultured chick pineal cells: effect of light, dark, and potassium on the melatonin rhythm. Brain Res. 438 199-215.

Zatz M, Kasper G and Marque Z (1990)- Vasoactive intestinal peptide stimulated chick pineal melatonin production and interacts with other stimulatory and inhibitory agents but does not show α_1-adrenergic potentiation. J. Neurochem. 55 (4) 1149-1153.