LOCALIZATION OF MELATONIN RECEPTORS IN CHICK RETINA

by

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A thesis submitted in conformity with the requirements for the degree of M.Sc.
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ABSTRACT

Binding of melatonin occurs in a large number of tissues in avian species, reflecting the potent effects of this pineal hormone; implicated in many biological processes under rhythmic control. In vertebrates, melatonin is also secreted from the retina where it is thought to act locally, affecting such processes as dopamine synthesis and retinal adaptation to darkness. The existence of multiple melatonin receptor subtypes suggests different functional roles for melatonin at the cellular level.

Previously, autoradiography studies using 2-[\textsuperscript{125}I]iodomelatonin have revealed high affinity binding in the inner plexiform layer of the retina in chick. \textit{In situ} hybridization using chick cRNA probes for the Mel\textsubscript{1a} and Mel\textsubscript{1e} receptor subtypes displayed mRNA expression in the retinal inner nuclear layer in this species. Evidence of Mel\textsubscript{1a} mRNA expression was also reported in the ganglion cell layer.

It was the purpose of these studies to specifically localize the Mel\textsubscript{1a} receptors within the chick retina, and to investigate the presence of the chick Mel\textsubscript{1b} subtype in this tissue. Here, we directly identify melatonin receptors within the chick retina by immunocytochemistry, employing a peptide-specific polyclonal antibody to the human Mel\textsubscript{1a} receptor. Specific, blockable binding was seen as two distinct bands in the inner plexiform layer. Furthermore, immunoblot studies revealed a 37 kilodalton peptide blockable band in the retina. In order to determine whether melatonin receptors colocalize with retinal dopaminergic amacrine cells, we used an anti-tyrosine hydroxylase antibody and immunocytochemistry to stain the dopaminergic cells. We report that the melatonin Mel\textsubscript{1a} receptor staining is distinct from that of the dopaminergic cell staining. Finally, we also report the first evidence of chick Mel\textsubscript{1b} expression in retina as revealed by \textit{in situ} hybridization, using a DIG labeled cRNA probe, transcribed from a chick Mel\textsubscript{1b} cDNA clone. We show Mel\textsubscript{1b} mRNA expression in distinct amacrine cells of the inner nuclear layer and throughout the ganglion cell layer.

This work reports the first specific localization of the melatonin Mel\textsubscript{1a} receptor protein in chick retina. Additionally, we report the first evidence of both chick Mel\textsubscript{1b} expression and localization in retina, establishing the presence of all three subtypes in this tissue. Further work is required to determine whether or not there is cellular colocalization between the three melatonin receptor subtypes in retina, as well as possible colocalizations between dopaminergic cells and the Mel\textsubscript{1b} and Mel\textsubscript{1e} melatonin receptor subtypes. In addition, specific localization of chick Mel\textsubscript{1b} melatonin receptor subtype in other tissues remains to be investigated, but this work advances our knowledge of melatonin receptor subtype distribution in chick retina.
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TERMS AND ABBREVIATIONS

Ab - antibody
BCIP - X-phosphate/ 5-bromo-4-chloro-3-indolyl-phosphate
BSA - bovine serum albumin
Cb - cerebellum
ddH₂O - double deionized water, Milli-Q
dH₂O - deionized water
DAB - diaminobenzidine
DEPC - diethyl pyrocarbonate
DIG - digoxigenin
DTT - dithiothreitol
EDC - 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDTA - ethylenediaminetetraacetic acid
G3PDH - glyceraldehyde-3-phosphate dehydrogenase
GABA - y-aminobutyric acid
GCL - ganglion cell layer
IgG - immunoglobulin G
kDa - kilodalton
INL - inner nuclear layer
IPL - inner plexiform layer
Mel₁₈ - melatonin receptor subtype ‘a’; if mammalian then currently known as MT₁
Mel₁₉ - melatonin receptor subtype ‘b’; if mammalian then currently known as MT₂
Mel₁₆ - melatonin receptor subtype ‘c’; in chick only
NBT - 4-nitro blue tetrazolium chloride
N-P40 - Nonidet-P40
PB - phosphate buffer
PBS - phosphate buffered saline
PFA - paraformaldehyde
PMSF - phenylmethylsulfonyl fluoride
PNGase F - peptide-N-glycosidase F
RT - room temperature
SDS - sodium dodecyl sulfate
SSC - standard sodium citrate
TBS - Tris buffered saline
TEA - triethanolamine
TEMED - N,N,N',N''-tetramethylethylenediamine
TCA - trichloroacetic acid
T.H.-tyrosine hydroxylase
TIL₃ - third intracellular loop
VIP - vasoactive intestinal peptide
INTRODUCTION

1.1. The Pineal Gland and the Discovery of Melatonin

The role of the pineal gland has been, up until this century, very poorly understood. In 1917, McCord and Allen discovered that bovine pineal extracts could cause blanching in the skin of tadpoles. Later, in 1958, dermatologist Dr. Aaron Lerner, who was interested in the causes of vitiligo, furthered these studies. He was successful in isolating and characterizing the pineal factor responsible; the hormone, melatonin (N-acetyl-5-methoxytryptamine) (Lerner et al, 1958 & 1959). Since then, the role of both the pineal and melatonin itself have attracted considerable attention. The capacity for skin blanching in some animals is now only one of many roles for the gland and its hormone.

The pineal gland is regulated by the external photoperiod. That is, it behaves in a rhythmic fashion in response to day-night cues which it receives, in part, from the eyes. In fact, in birds and other lower vertebrates, the pineal is itself also directly photosensitive. This allows the gland to provide a measurement of day length to the other organs, by releasing its hormone, melatonin, in a pulsatile manner (Ralph et al, 1975). Indeed, the highest levels of melatonin secretion occur at night (Axelrod, 1974), earning for itself the title "hormone of darkness". By transducing photoperiod information into a rhythmic hormone signal, the animal can adapt to seasonal challenges and exploit prime breeding times. Both physiological and behavioral seasonal adaptations have been shown to be affected by pineal gland activity. Experiments from the 1960's onward have specifically identified melatonin as the carrier of this pineal time message (for reviews see Pang, 1985, and Arendt, 1985).
1.2. Roles of Melatonin

The effect of melatonin on reproductive activity in seasonally breeding animals is potent and tightly regulated. This is facilitated by the fact that the synthesis of melatonin occurs quickly once activated, and the half-life of the hormone in the circulation is short (about 20 minutes, Gibbs & Vriend, 1981). Long winter nights, and therefore extended melatonin secretion, cause a decrease in the gonadal activity of long-day breeders such as hamsters (Reiter, 1978), while, conversely, short-day breeders such as sheep become sexually active (Bittman, 1983). It follows that melatonin behaves as a neuromodulator on the hypothalamic-pituitary axis and both autoradiographic and binding studies have revealed melatonin ligand binding in nuclei of both regions (Vanecëk, 1988, and Stankov et al, 1991). Melatonin appears to be able to either stimulate or inhibit GnRH (gonadotropin-releasing hormone) secretion from the hypothalamus in mice by modifying the mechanisms in GnRH neurons that regulate its release (review by Tamarkin et al, 1985). As well, melatonin may have direct effects on the reproductive organs themselves (Ayre et al, 1991, and Carneiro et al, 1993).

In addition to regulating reproductive function in seasonally breeders, melatonin also influences coat colour (Kuderling et al, 1984), hibernation (Stanton et al, 1987), migration (Schneider et al, 1994), and thermoregulation (Cagnacci et al, 1992). As well, in lower vertebrates such as birds and reptiles, melatonin maintains a strict circadian rhythm in the physiological activity of the target tissues. In mammals, its role is less potent, but still serves to synchronize biological rhythms as well as acting as a sleep promoter (Anton-Tay, 1974). It has recently been used to treat some circadian rhythm disorders, seasonal depression and even jet lag (for a review see Brown, 1994).
Melatonin has also been found in the retinas of several vertebrate species, acting as a modulator. Both retinal physiology and morphology are affected by daily melatonin rhythms, regulating the tissue's adaptation to darkness (for review see Cahill & Besharse, 1995). The presence of melatonin causes; aggregation of melanin pigment granules in the retinal pigment epithelium (Pang and Yew, 1979), activation of disc shedding in rod photoreceptors (Besharse and Dunis, 1983), elongation of cone photoreceptors (Pierce and Besharse, 1985), and the inhibition of retinal dopamine synthesis and release (Dubocovich, 1983). Retinal pigment granules aggregate at night to best expose the rod photoreceptors for dim light. The retinomotor movements of cone photoreceptors enhance this action by making way for the encroaching rods. The elongation of the cones allows for the maximal positioning of the rods in dark (scotopic) vision. Disc shedding allows for a continuous renewal of photoreceptor discs at their tips, and in rods this disc shedding begins in the morning with first light. However, it requires a "dark-priming" period, regulated by melatonin (for a review see Besarshe et al, 1988).

Melatonin also acts, as stated above, to inhibit dopamine synthesis in vertebrate retina. Dopamine is synthesized in amacrine cells in the retina and acts as a light signal as it is activated by photic stimulation, with opposing effects to those of melatonin (Dowling & Ehinger, 1978, Ehinger, 1977, and Pierce and Besharse, 1985). The inhibition of dopamine synthesis by melatonin occurs at night, when melatonin levels are at their highest. Conversely, during the day, dopamine acts to inhibit melatonin synthesis through D2 dopamine receptors (see review by Iuvone, 1986). The role of melatonin in dopamine inhibition was investigated by Dubocovich in 1983, who used picomolar concentrations of melatonin administered to dissected, superfused rabbit retinas and showed a selective inhibition of calcium-dependent dopamine release. In 1992, Nowak et al showed similar findings in chick, using intraocular injections of melatonin to cause a dose-dependent
decrease in dopamine release. The exact mechanism of dopamine inhibition by melatonin in the retina is not known. It may occur directly through melatonin receptors on dopaminergic cells, or through an intermediary route using one or more other neurotransmitters. For example, GABA and enkephalin have both been shown to have inhibitory effects on dopaminergic cells (Morgan & Kamp 1980, and Dubocovich & Weiner, 1983). Therefore, whether melatonin exerts its effects on these cells directly or through one of these neurotransmitters remains to be seen.

Although research into the actions of melatonin has become extensive, there may be, in addition to the actions of melatonin cited above, other possible roles and potentials for this hormone, yet unknown. Research continues today to further elucidate the roles of this indole.

1.3. A Role in Birds

There are many diverse roles for melatonin in animals. It influences reproduction, circadian rhythms, metabolism, as well as retinal dark adaptations. Many tissues are able to bind melatonin, in a wide variety of species (Arendt, 1985, and Morgan et al, 1994). The more an animal depends on light cycles to entrain its physiology and behaviour, the more widespread is melatonin binding, and with higher density. Lower vertebrates in general show much higher melatonin binding than higher vertebrates. For example, birds and reptiles show overall much higher melatonin binding in binding assays than mammals (Stankov et al, 1991). It occurs both with higher density and with a far wider distribution in brain and other tissues. Indeed, birds show a 125-fold higher concentration of melatonin binding sites in brain than mammals (Rivkees et al, 1989b). This makes the bird an ideal
animal to use as a model in melatonin research. The high level of melatonin in the plasma and high density binding as seen in avian tissues (primarily reported in chicken and quail), together with this observed wide-spread distribution of binding in various avian tissues facilitates investigative studies on this hormone's actions. As mammals produce so little melatonin in comparison to birds, detection of signals with molecular techniques becomes more difficult. The stronger binding signals in birds facilitates cellular and molecular investigations. Consequently, the domestic chicken (*Gallus domesticus*) was the species of choice in these investigations.

1.4. **Melatonin Secretion**

Melatonin in the plasma is secreted almost exclusively from the pineal gland; most, if not all circulating levels of melatonin in the blood are derived from this source. In fact, pinealectomy eliminates virtually all serum and brain melatonin (Pelham et al, 1972, and Pang et al, 1974). Although other sites of melatonin synthesis have been reported (the gut, retina, harderian gland, and lacrimal gland (Reiter, 1991b and Pang et al, 1992)), these seem to involve paracrine roles only, so that melatonin acts locally within these tissues.

For example, the actions of melatonin on retinal physiology and cell morphology as described in the preceding section are a result of this local synthesis of melatonin in the retina. Pinealectomy does not eliminate melatonin levels in the retina of chicken (Osol et al, 1985), and isolated retinas from rats maintain their capacity to synthesize melatonin from radiolabeled precursors (Cardinali & Rosner, 1971). The source of retinal melatonin has been localized to photoreceptors by immunohistochemistry which revealed the presence of one of the melatonin synthesizing enzymes, HIOMT (hydroxyindole-O-methyltransferase),
in the photoreceptors of rat and chicken (Wiechmann et al, 1985, and Voisin et al, 1988). Furthermore, melatonin itself has been localized to photoreceptors by immunohistochemical studies (Bubenik et al, 1974). The melatonin that is synthesized within the retina acts as a paracrine substance only, and does not enter the general circulation. It is ultimately metabolized within the retina itself (for a review on retinal melatonin see Cahill and Besharse, 1995).

The effects of the hormone as seen on gonadal activity, circadian rhythms, behaviour and metabolism all result from melatonin released into the general circulation from the pineal gland (Pelham et al, 1972, and Pang, 1985). It is the tight regulation of the hormone's synthesis in the pineal, as well as in the retina and other extrapineal sources, that allows for appropriate adaptations to external photic cues.

1.5. Melatonin Synthesis

1.5(a) The pineal rhythm

Light acts to suppress the synthesis of melatonin in the vertebrate pineal (Lewy et al, 1980, and Illnerova, 1991). In birds, light detected by the retina is conveyed as an excitatory signal to the SCN (suprachiasmatic nuclei) of the hypothalamus via the retinal-hypothalamic tract. The SCN then acts as the chick's biological clock, using visual information to entrain endogenous oscillators within these nuclei. The excitatory effect of the light stimulus travels from the SCN through the sympathetic SCG (superior cervical ganglia), and results in the release of NE (norepinephrine) from SCG postganglionic neurons onto pineal β-adrenergic receptors. In birds, NE acts to inhibit cAMP production
in pinealocytes, which in turn results in decreased NAT (N-acetyltransferase) production. This is the rate-limiting enzyme that controls the amount of melatonin that will be made from pineal serotonin (figure 1.1 and figure 1.2) (for review see Cassone and Menaker, 1984). As melatonin is thought to leave the pineal passively, the origin of melatonin's rhythmic release can be found in the rhythmic regulation of NAT. In contrast to the above pathway, mammalian melatonin is controlled in the opposite manner: NE has a stimulatory effect on the pineal. The SCN is in fact inhibited by light and stimulatory NE release is thereby restricted to night. In birds, the pineal is itself directly photosensitive and also contains endogenous pacemakers (Takahashi & Zatz, 1982) which, in combination with the SCN message, all contribute to a highly sensitive and finely-tuned biological rhythm.

1.5.(b) The retinal rhythm

As described above, melatonin is thought to be produced in photoreceptor cells, released only as a paracrine secretion at night to act upon retinal tissue specifically. Its synthesis follows the same precursor and enzyme route as described in pinealocytes. One difference is that it is dopamine, and not norepinephrine, that serves as the inhibitory signal during the day. As with the avian pineal, the retina is of course photosensitive, and also contains endogenous oscillators in the photoreceptor cells (Pierce et al., 1993). Neither optic nerve section nor removal of sympathetic nerve input affects the retinal melatonin rhythm (for review see Cahill & Besharse, 1995). These oscillators act to establish a daily melatonin rhythm, which is entrained by photic cues and dopamine inhibitory actions. Dark adaptations within the eye are dependent on this cyclic melatonin synthesis, just as brain and peripheral tissues require pineal melatonin rhythmicity.
Figure 1.1 Light Inhibition of the Chick Pineal
SCN: suprachiasmatic nuclei, SCG: superior cervical ganglia, NE: norepinephrine, Mel: melatonin
Figure 1.2 Synthetic Pathway of Melatonin

1.6. **Tissue Target Sites**

Brain and retina constitute two of the main sites of action for melatonin. The potency of melatonin in chick brain, for example, is understood better when one considers the fact that chick brain concentrates melatonin from the serum to a ratio of 7:1 (Pang & Ralph, 1975). Autoradiography and binding studies in chicken show target sites in hypothalamus, thalamus, pons-midbrain region, tectum opticum, and auditory system, among others (Rivkees et al, 1989b, Stankov et al, 1991, and Stehle, 1990). Pang et al (1974) showed highest binding in the hypothalamus; the site of the SCN, and putatively the biological clock. Dubocovich et al (1989) added the pituitary to binding target sites in chicken, implying a direct action by melatonin on the hypothalamo-pituitary axis to regulate reproductive functions. (Since then Ayre et al, 1991, have shown melatonin binding directly in chick ovaries and testes as well). As noted above, large regions of brain involved in visual, auditory and even olfactory processing have also been labeled as binding sites for melatonin in chicken, either through binding studies or autoradiography, using 2-[125I]iodomelatonin. Binding in mammalian brain is less extensive than in avian species, although the binding pattern in hypothalamus, pituitary and thalamus is similar in most species (for a review on mammalian melatonin binding, see Morgan & Williams, 1989 and Krause & Dubocovich, 1990).

In 1989, Dubocovich et al showed melatonin binding in chick retinal membranes, and in 1990 Laitinen and Saavedra used autoradiography to localize the melatonin binding to the inner plexiform layer of the chick retina (for a figure of the retinal structure, see figure 3.5 in Results, page 132). The inner plexiform layer (IPL) contains only processes (dendrites) extending from amacrine cells in the proximal portion of the inner nuclear layer
and from ganglion cells in the ganglion cell layer. Binding in the IPL implies that processes from one or both cell sources contain functional melatonin receptors.

Amacrine cells are classified into a number of subsets, depending mostly on their neurotransmitters. They synapse with many other retinal cells, including other amacrine cells, and are thought to relay and modify neural information as it is transmitted through the retina. Dopamine, as previously mentioned, is synthesized by a subset of amacrine cells.

Ganglion cells are located in the innermost region of the retina, the ganglion cell layer. Their processes also extend into the IPL, as stated, and their axons form the optic nerve which projects into the brain (including the retinal-hypothalamic tract which projects to the SCN). Little is known about ganglion cells except for their role in transmitting information from the retina to the brain.

Although binding assays and autoradiographic studies were certainly helpful in elucidating the precise role of melatonin by revealing target sites, the techniques are limited in that they can only imply the presence of a functional receptor. To better understand the function of melatonin at the cellular level, the receptors themselves needed to be identified. The precise localization of specific melatonin receptors to target cells would provide important insight into melatonin function. Functional studies could then be performed to determine exactly how melatonin exerts its effects in various tissues, and what means of regulation control the melatonin signal. Recent cloning of the melatonin receptor has contributed to our understanding of the hormone by revealing cell-specific expression of the receptor.

It was the goal of these investigations to contribute to the current information about these receptors through their localization in chick retina.
1.7. Melatonin Receptors

In 1994, the first clone of the melatonin receptor was successfully obtained from *Xenopus laevis*, and then shortly thereafter, from mammals (Ebisawa et al., 1994, and Reppert et al., 1994 and 1995a). Pharmacological characterization of the recombinant receptors revealed similarities to the characteristics of the binding with 2-[125I]iodomelatonin. That is, the receptors bind melatonin with high affinity (in the picomolar range), the binding is reversible and saturable. Additionally, the receptors were characterized as members of the superfamily of seven transmembrane, G-protein coupled receptors (figure 1.3); each clone (from *Xenopus* and mammals) inhibits cAMP accumulation within the target cell.

As with many hormones and neuromodulators, melatonin does not act through a single receptor. Two structurally distinct subtypes of the high affinity melatonin receptor have now been cloned in mammals; denoted Mel₁a and Mel₁b (Reppert et al., 1994 and 1995a) (now known as mt₁ and MT₂, respectively, based on the extent of pharmacological characterization, Dubocovich et al., 1998). Interestingly, these two clones show only 60% homology to the clone from *Xenopus* at the amino acid level. In these studies, the Mel₁a mammalian receptor subtype was shown to be expressed in the mammalian SCN and pars tuberalis of the pituitary. In contrast, the mammalian receptor subtype Mel₁b is expressed in retina, raising the possibility of distinct functional roles for these subtypes.

Currently, only two high affinity melatonin receptor subtypes have been found in mammalian species. However, since then, three melatonin receptor subtypes have been found in birds; again, high affinity, G-protein coupled receptors. The third clone found in birds turns out to be a structural homologue to the *Xenopus* clone (Reppert et al., 1995b). The presence of an additional receptor subtype in birds is not surprising considering the
Figure 1.3 Basic Structure of the Melatonin Receptor
potent effects of melatonin in lower vertebrates. It is quite possible that with the observed evolutionary trend toward a decrease in melatonin binding density, as previously mentioned, a loss of one receptor subtype could have occurred. In 1995 (year reference b), Reppert et al cloned two of the chick melatonin receptor subtypes; Mel_1a (77% homologous to mammalian Mel_1) and Mel_1c (unlike the mammalian receptors, but 80% homologous to *Xenopus* at the amino acid level). Reppert et al found the chick Mel_1a subtype to be expressed in optic tectum, hypothalamus, thalamus and retina. Mel_1c was expressed in the same tissues (though less in retina), as well as in pineal gland and cerebellum. The chick melatonin receptor subtype Mel_1b was, in fact, the last to be found and cloned. Previous work in this lab succeeded in cloning this subtype (Liu et al, 1995), based on homology probing and using a chicken brain cDNA library. RT-PCR studies showed Mel_1b expression in the chick brain regions of optic tectum and cerebellum (chick retina had not been probed with Mel_1b). Finally, melatonin may act as a ligand, not just through membrane bound receptors, but also through nuclear hormone receptors (Dubocovich, 1995, and Park et al, 1996). A subfamily of nuclear receptors, RZRα/β orphan receptors are putative melatonin receptors; RZRβ mRNA expression is found in rat retina, pineal gland and SCN (Park et al, 1996), while RZRα is expressed in peripheral tissues (Steinhilber et al, 1994). These nuclear receptors control gene expression by binding to specific response elements on the target DNA. However, they are low affinity receptors, binding melatonin in the nM range (Dubocovich, 1995, and Carlberg & Wiesenber, 1995). Some controversy remains, therefore, as to whether or not melatonin is the true physiological ligand for these receptors as other indole, such as N-acetyl-5-hydroxytryptamine, show similar affinities for the receptors (Becker-Andre et al, 1994, and Reppert et al, 1996).
With the availability of these new clones, comes the possibility to localize receptors at both the tissue and cellular level. Identifying the actual 'target' cells within the tissues brings us closer to understanding the specific functions of the hormone. If specific cells can be identified, we can use current knowledge of the cell's function to speculate as to the pathways used by melatonin. Additionally, interesting information can be obtained regarding melatonin's role through its putatively selective interaction with the different receptor subtypes. Therefore, as stated in the previous section, it was the goal of these studies to further our knowledge of melatonin through the localization of the melatonin receptors.

1.8. Objectives and Rationale

The objectives of this research were: 1) to localize Mel₁a receptors at the cellular level in chick retina, 2) to determine the presence of chick melatonin receptor subtype Mel₁b in chick retina, and 3) to determine whether melatonin receptors colocalize with retinal dopaminergic cells. Retina was the primary tissue investigated in these studies as it is one of the main sites of action for melatonin. Due to the complex structure of brain tissue, some investigations were furthered in this tissue only after techniques had been successfully established in retina.

1.8.(a) Melatonin Mel₁a receptor localization and the first hypothesis

Autoradiography had shown melatonin binding in the inner plexiform layer of the chick retina (Laitinen & Saavedra, 1990, and Dubocovich et al, 1989). Therefore, we first
hypothesized that actual melatonin receptors, specifically Mel_{1a}, would be localized to the IPL of chick retina using immunocytochemistry. To investigate this, we used a polyclonal, human peptide-specific antibody to the human melatonin receptor subtype Mel_{1a} (Song et al, 1997) and immunocytochemistry to localize the Mel_{1a} receptor at the cellular level. The human peptide region used as an antigen was the third intracellular loop (TIL_{3}) of human melatonin receptor (subtype Mel_{1a}). This human peptide region shows 77% homology in its sequence to the chick receptor subtypes Mel_{1a} and Mel_{1b}. This degree of homology between these regions of the mammalian and chick receptors allowed for a reasonable expectation that the antibody would cross react with chick tissue. However, because of this antibody's ability to recognize either chick subtype Mel_{1a} or Mel_{1b}, blocking of the Ab with the corresponding chick-specific peptides was necessary. This would help to determine any selective binding of the Ab to one of the chick subtypes. Additionally, and for the first time, chick-specific antibodies were raised for these investigations to further discriminate between the subtypes in the chick retina.

The use of these antibodies as probes for melatonin receptors in immunocytochemical studies would allow us to specifically localize the receptor protein. This would be the first direct localization of the melatonin receptor, in contrast to indirect ligand binding studies. To support our immunocytochemical evidence of melatonin receptor presence in chick retina, we used the above-mentioned antibodies in Western Blot investigations. This latter technique is highly sensitive and can detect proteins of even low expression levels (Constantine et al, 1994, and Nesbitt & Horton, 1992). The technique is, however, limited by its inability to separate retinal cell types. The first hypothesis, that melatonin Mel_{1a} receptors would localize to the chick retinal IPL, would be initially supported by evidence of the receptor's presence in retinal tissue through both the Western
Blots and the immunocytochemistry. The putative, specific IPL localization would be investigated by the immunocytochemical studies.

1.8.(b) Subtype Mel₁₆ and the second hypothesis

The second objective of these investigations was to determine the presence of the melatonin receptor subtype Mel₁₆ in chick retina by in situ hybridization. This chick receptor subtype has recently been cloned by this lab (Liu et al, 1995) and little is known about its localization. Reppert et al (1995b) reported from their cloning work that chick subtypes Mel₁₆ and Mel₁₄ both localized to the retina. Using radiolabeled cRNA probes for the two subtypes, they showed by in situ hybridization that the localization was specific to cells at the INL/IPL boundary, as well as Mel₁₆ in the GCL. It is possible that, if Mel₁₆ localizes to chick retina, it may be expressed in the same cells. Reppert et al (1995a) had shown mammalian Mel₁₆ to localize to the retina by RT-PCR. Our second hypothesis was that the chick Mel₁₆ subtype would also be localized to retinal tissue.

We investigated this hypothesis through the use of the chick-specific antibodies and immunocytochemistry, which were subtype-specific. To further confirm the presence and localization of the Mel₁₆ subtype in chick retina, we used a chick cRNA Mel₁₆ clone and in situ hybridization to determine chick Mel₁₆ mRNA expression. This technique would also reveal the actual cell types that express this receptor. This was the first investigation of chick retina for the subtype Mel₁₆ and the first use of in situ hybridization with this probe. Results can then be compared to those obtained by Reppert et al (1995b) to determine possible differential distribution of the subtypes.
1.8(c) Colocalization with dopaminergic cells and the third hypothesis

Finally, the third goal of these investigations was to determine whether melatonin receptors colocalize with chick retinal dopaminergic cells. Melatonin inhibits the synthesis of dopamine in chick retina (Dubocovich, 1983). Interestingly, dopamine is synthesized in amacrine cells at the INL/IPL boundary (Dowling & Ehinger, 1978, and Ehinger, 1977); the same retinal region in which the melatonin receptors Mel1a and Mel1e were localized. The inhibition may involve a direct interaction between melatonin and dopaminergic cells via one or more of the melatonin receptor subtypes. Colocalization of the melatonin receptors Mel1a and Mel1e to dopaminergic cells has not yet been investigated, and, as the presence of Mel1a in the retina is not yet known, a possible colocalization for this subtype remains to be seen.

Our third, and final, hypothesis was that chick melatonin receptors, of one or more subtypes, would colocalize to dopaminergic cells in retina. Preliminary colocalization studies were performed to investigate the possible direct effect of melatonin on these cells. A commercially available antibody to the enzyme tyrosine hydroxylase, involved in dopamine synthesis, was used to identify dopaminergic cells in chick retina. Using immunocytochemistry and the antibodies to both tyrosine hydroxylase and melatonin receptors, a colocalization between the two was investigated. However, the possibility of melatonin exerting its inhibitory influence on dopamine synthesis through an intermediary neurotransmitter also cannot be ruled out. Colocalization of melatonin receptors with other inhibitory cells were investigated. As mentioned in section 1.2 (page 4), both GABA and enkephalin have also been reported to have inhibitory effects on dopaminergic cells. In fact, Morgan & Kamp (1980) reported a tonic inhibition of dopaminergic amacrine cells by GABAergic amacrine cells in darkness only; not in light. Melatonin may modulate GABA's
inhibition, using this indirect route to affect dopamine synthesis. We used commercially available antibodies to GABA (γ-aminobutyric acid) and other known retinal neurotransmitters or neuromodulators (enkephalin, substance P, VIP (vasoactive intestinal peptide), neurotensin, and serotonin) or their enzymes (choline acetyltransferase for acetyl choline) and immunocytochemistry to colocalize these neurotransmitters in chick retina with melatonin receptors. Should the third hypothesis of a direct colocalization between melatonin receptors and dopaminergic cells be refuted, these preliminary colocalization studies with the above neurotransmitters and melatonin receptors may offer information on other factors in the melatonin inhibition of dopamine. (See Table 1, next page, for antisera information.)

1.9 Summation of Research Goals and Choices of Techniques

Detecting the specific localization of the receptors at the cellular level, as well as discriminating between the receptor subtypes has become possible with the isolation of these melatonin receptor clones and antibodies. The specific, cellular localization of the receptor subtypes can be achieved through the use of immunocytochemistry and in situ hybridization. The presence of Mel₁β in chick retina can be established through Western blots, using the subtype-specific probes, as well as by immunostaining and in situ hybridization. Finally, the possible colocalization of melatonin receptors and retinal dopaminergic cells can be investigated through immunocytochemistry and the use of the antibodies listed above. To test the second hypothesis that the chick Mel₁β subtype would localize to retinal tissue in chick would provide insight into the role of this subtype at the
Table 1. Antisera Against Retinal Neurotransmitters and Their Enzymes

<table>
<thead>
<tr>
<th>Antisera</th>
<th>Against which species</th>
<th>Raised in what animal</th>
<th>Monoclonal</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-choline acetyltransferase (enzyme for ACh)</td>
<td>porcine brain extract</td>
<td>mice</td>
<td>yes</td>
</tr>
<tr>
<td>anti-enkephalin</td>
<td>ubiquitous neurotransmitter</td>
<td>mice</td>
<td>yes</td>
</tr>
<tr>
<td>anti-GABA</td>
<td>ubiquitous neurotransmitter</td>
<td>mice</td>
<td>yes</td>
</tr>
<tr>
<td>anti-neurotensin</td>
<td>ubiquitous neurotransmitter</td>
<td>rabbit</td>
<td>polyclonal</td>
</tr>
<tr>
<td>anti-serotonin</td>
<td>ubiquitous neurotransmitter</td>
<td>mice</td>
<td>yes</td>
</tr>
<tr>
<td>anti-substance P</td>
<td>ubiquitous neurotransmitter</td>
<td>rat</td>
<td>yes</td>
</tr>
<tr>
<td>anti-tyrosine hydroxylase (enzyme for dopamine)</td>
<td>rat</td>
<td>mice</td>
<td>yes</td>
</tr>
<tr>
<td>anti-VIP</td>
<td>porcine intestine extract</td>
<td>rabbit</td>
<td>polyclonal</td>
</tr>
</tbody>
</table>
cellular level and either confirm or deny the presence of all three subtypes in chick retina; with interesting implications as to possible subtype-specific functions.

However, the techniques mentioned above require specific optimizations in terms of both species and probes. Although they are sensitive techniques, they are also very susceptible to providing either negative or nonspecific data if they are not properly optimized. For example, the human Mel₁₄ antibody has never been used in chick tissue, and although the homology suggests a possible cross reaction in this species, optimization of both Western Blotting and immunostaining protocols were required in order to obtain a specific signal. In addition, in situ hybridization with the probe for subtype Mel₁₅ has also never been performed, and needed to be optimized. The use of radiolabeled probes in the in situ technique was decided against in favour of the substrate-based chromogen detection using digoxigenin (DIG) labeled probes. The probe choice was made for reasons of technical practicality; the use of DIG labeled probes is a safer alternative to radiolabeled probes, and most radiolabeled in situ investigations involving chick tissue (including Reppert et al's work in 1995b) required weeks to develop the films as opposed to a few minutes with DIG. This decision was made despite the limitation that data produced from DIG probes are not as easily quantifiable.

A large part of the research itself involved the optimization of the protocols. Reported here is the process of optimization and the results of the localization studies with chick subtype Mel₁₅ as well as the colocalization studies. For clarity, the Methods section will present the final protocol used to obtain results followed by the process of optimization for each technique. There will also be a discussion of this optimization process at the end of the Methods section. The Discussion section will therefore involve strictly an analysis of the final results and their relation to the research goals.
METHODS

Each protocol is followed by a section entitled "Specific Optimizations for this Protocol". In that section, the optimization process is described, with figures to illustrate various stages of this process. Therefore, figures shown in this section are not final results. Final data are presented only in the Results section. The following section here includes some description of the major stages of adjustments. Analysis of these optimizing processes are presented at the end of the Methods section, entitled "Discussion of Methods".

Cellular Localization of Melatonin Mel_{1a} Receptors (1st Hypothesis)

In order to investigate the presence of melatonin Mel_{1a} receptors in chick retina, as well as to reveal the specific localization of these receptors in this tissue, we used Western Blots and immunocytochemistry, respectively. As a probe for the Mel_{1a} receptor subtype in these techniques, we employed a polyclonal, peptide-specific antibody against the TIL_{3} region (third intracellular loop) of the human Mel_{1a} receptor peptide. To discriminate between the subtypes in these investigations and to specifically investigate the presence of Mel_{1b} in this tissue, we used chick-specific antibodies against the TIL_{2} peptide region of each chick receptor subtype.
2.1 Antibodies used in cellular localization of melatonin Mel subtype receptors
(for Western Blots and Immunocytochemistry)

Previous work in this lab had used synthetic peptides corresponding to the sequence in the third intracellular loop of the human Mel subtype receptor (TIL, residues 226-238; KPKLKPQDFRNFV), to generate a polyclonal, human anti-Mel antibody (Ab), by coupling the peptide to Keyhole Limpet hemocyanin (KLH) and then immunizing rabbits (Song et al, 1997). For the current studies, IgG fractions of the antisera were isolated using protein A-conjugated Sepharose (SIGMA). Specific antibodies were further purified by affinity chromatography using EAH Sepharose 4B (Pharmacia Biotech) coupled to the protein. For this, 30 mg of peptide was added to 1 ml of EAH Sepharose 4B in a column. 60 mg of EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) was added to the suspension (pH 4.5) and left to mix overnight. The product was then washed 5 times successively with 10 ml each of 0.1 M acetate buffer (pH 4.0) / 0.5 M NaCl and 0.1 M Tris-HCl (pH 8.0) / 0.5 M NaCl. A 10 ml dH₂O wash was followed by a 10 ml wash of 0.1 M Glycine / HCl (pH 3.0) and three washes with TBS buffer (20 mM Tris, 0.15 M NaCl, pH 7.5). 300 µl of crude antisera (diluted to 1 ml in autoclaved ddH₂O) was added to the column and incubated for 1 hr at RT (room temperature). The column was then washed once with 10 ml each of; TBS, 3 X with TBS-Triton, and then 3 X again with TBS. Finally, the column was washed twice with 0.15 M NaCl. To collect the affinity purified product, the column was incubated with 50 µl of 0.1 M Glycine-HCl (pH 3.0) for 5 min. at 0°C. The eluate was collected and the latter step was repeated again. The final 100 µl of purified Ab was neutralized with 5 µl of 1 M Tris-HCl and 1 µl of 500 mM sodium azide. The Ab was then aliquotted and stored at -70°C. The concentration of the affinity purified antisera is based on the column’s recovery
efficiency and, therefore, the resultant eluate concentration after purification (recovery of Ab in the eluate is estimated at 70%, and is approximate only).

The TIL2 region of the human peptide used to generate the antibody shows 77% homology to both the chick Melα and Melβ subtypes. To determine subtype specificity of the antisera in chick tissues, chick-specific peptides for the corresponding TIL2 region were synthesized for the chick melatonin receptor subtypes Melα and Melβ (residues NPRLKPHDFRNVF and KPRLKPSDFRNFL, respectively (Biotechnology Service Center, Department of Clinical Biochemistry, University of Toronto)).

Chick-specific antibodies for the three chick melatonin receptor subtypes (Melα, Melβ, and Melγ) were raised in rabbits (SynPep) in order to discriminate between the subtypes in chick tissue. The peptide sequence from the second intracellular loop (TIL2) was chosen as the immunogen, as it showed the highest sequence variation among the three subtypes (chick Melα: YSDKNSLCYVG, chick Melβ: YSCWNTMLYVS, chick Melγ: FNKLKTCCYIC). We then both IgG and affinity purified the antisera as described above.
2.2 Detection of the Melatonin Mel$_{1a}$ Receptor Protein in Chick Tissue:

WESTERN BLOTS

2.2.1. Western Blot PRELIMINARY Protocol:

Two protocols were used for the Western Blot investigations. The first protocol used, presented as "preliminary protocol", was unsuccessful in detecting a signal despite optimization of the technique. However, due to the extensive optimizations, both of the Western Blot protocols are presented here.

2.2.1. Preliminary Protocol: PMSF/EDTA (phenylmethylsulfonyl fluoride / ethylenediaminetetraacetic acid) and N-P40 (Nonidet-P40) tissue preparation method

Previous work in this lab had performed Western Blots successfully on human and rat retinal tissue using this tissue preparation method (Song et al, 1997 and Fujieda et al, 1999). These investigations to detect melatonin receptors in chick retinal tissue were therefore first performed using this method. Additionally, chick brain sections (cerebellum (Cb), tectum and thalamus) were also investigated.

2.2.1.(a) Tissue collection and preparation (preliminary protocol):

Day-old chicks were kept under LD (light:dark) 14hrs : 10hrs photoperiod (lights on at 07:00) and decapitated under anesthesia with carbon dioxide between 10:00-14:00.
Tissues were removed, frozen on dry ice and stored at -70° C until use. 1 g of each brain section (Cb, tectum and thalamus) were immersed in 5 ml of 0.01 M TBS. To this, 10 µl of EDTA (0.001 M), 25 µl of PMSF (phenylmethylsulfonyl fluoride, 100 µg/ml (SIGMA)), and 30 µl of Protease Inhibitor Cocktail (SIGMA) were added. The retinas from 6 eyeballs were dissected out and immersed in the same (0.6 X volume). The samples were homogenized (retina was sonicated) and then centrifuged at 31,000 x g for 20 min at 4° C. To the pellets 5 ml of TBS was added, along with; 25 µl of the cocktail, 10 µl of EDTA, and 12 µl of PMSF (1 ml and corresponding 1/5 volumes for retina). These were again homogenized and centrifuged and the final pellets were dissolved in 1 ml TBS (brain tissues) or 160 µl TBS (retina), 5 µl PMSF (brain) or 1 µl PMSF (retina), and 40 µl cocktail (brain) or 30 µl cocktail (retina). N-P40 (Nonidet-P40, Tergitol) (10%) was used to solubolize the membrane fraction of tissue proteins. It was added to the samples in a 9:1 ratio (9 parts sample, 1 part N-P40 (10%)). This mixture was shaken for 20 min on ice. Finally, the samples were spun at 12,000 x g for 15 min at 4° C. The supernatant was kept in 3 X Sample Buffer (Biolabs) (0.5 X volume) and 30 X Reducing Agent (1.25 M DTT, Biolabs) (0.3 X volume) at -70° C until use in the immunoblot studies -- 2-3 weeks.

2.2.1.(b) Gel electrophoresis and detection (preliminary protocol):

Prior to gel electrophoresis, samples were pretreated with sodium dodecyl sulfate (SDS) buffer and a reducing agent (1.25 M DTT). For this, tissue load amounts were 34 µl of each brain sample (cerebellum Cb, tectum, and thalamus) and 28 µl of retina sample from the tissue preparation stage, and each sample was mixed with 17 µl of 3X SDS Sample Buffer (Biolabs) (brain) or 14 µl (retina) and 1.7 µl of 30 X Reducing Agent (1.25
M DTT, Biolabs) (brain) or 1.4 μl (retina). Samples were then denatured at 90° C for 5-10 min and subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then finally transferred to a nitrocellulose membrane. Gel electrophoresis was performed using a SDS-polyacrylamide gel (10%) (acylamide (10%), 0.37 M Tris (pH 8.8), SDS (0.1%), ammonium persulfate (0.1%), TEMED (0.04%) (ICN Biomedicals)) in a Tris-glycine electrophoresis buffer for 1.5 hrs at 100 V (Volts) at RT (buffer: 25 mM Tris, 250 mM glycine (pH 8.3), SDS (0.1%)). A prestained SDS-PAGE standard, low-range (Biorad), was run along with the tissues in order to verify protein size. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Xymotech Biosystems) in transfer buffer (39 mM glycine, 48 mM Tris, SDS (0.037%), methanol (20%)) at 30 V overnight at RT. The membrane was washed in Tris-buffered saline -Tween 20 (0.2%) (TBS: 20 mM Tris, 137 mM NaCl, 1 M HCl, pH 7.6) and then incubated for 1 hr in blocking solution (defatted dry milk (5%) (Biorad) in TBS-Tween buffer, in order to reduce nonspecific binding with the nitrocellulose membrane. The membrane was subsequently washed 1 X 10 min and then incubated for 1 hr at RT with IgG-pure anti-Mel Ab, diluted 1:1000 - 1:5000 (stock Ab: 1.36 μg/μl) with 10 ml blocking solution. In the case of the chick-specific Abs, dilutions of 1:500 - 1:2000 were used for the crude chick-specific antisera, and 1:500 -1:1500 with IgG purified chick-specific antisera. In the case of peptide blocking with the human Mel peptide or the chick peptides, each peptide was preincubated with its corresponding Ab (2:1 ratio, Ab:peptide) in blocking solution for 0.5 hrs prior to incubation of the membrane. After washing 1 x 15 min and 2 X 5 min, the blots were incubated with 1:10,000 diluted horseradish peroxidase (HRP)-conjugated donkey antirabbit IgG secondary Ab (Amersham), under the same conditions. A final wash of 1 X 15 min and 4 X 5 min was followed by a 1 min incubation in equal portions of ECL detection
reagents 1 & 2 (Amersham) and then the immunoreactive proteins were detected by exposure to X-ray film (Amersham).

Adapting this protocol to chick tissue required extensive optimization, which is outlined below.

2.2.1.(c) Specific Optimizations for PRELIMINARY Protocol:

2.2.1.(c). i. Detection of melatonin Mel₁₆ receptor protein with human Mel₁₆ Ab:

This protocol was the first to be tried. However, due to high background and poor specificity of signals, the protocol was not long used. The tissue amounts required to obtain a signal were at maximal recommended loading amounts (Bollag et al, 1996) and excessive washing could not yield a cleaner blot. Figure 2.1 shows a Western Blot as described above, using the Ab for human Mel₁₆ (IgG pure). Retinal tissue is representative of results obtained with all tissues (i.e. brain tissue blots were similar). This blot used human Mel₁₆ Ab, diluted to 1:4000 (IgG pure) in an attempt to decrease background. Blots with higher Ab concentrations resulted in complete 'blackout' smearing of the blots. No other blots with this tissue preparation method (and this Ab) were successful, regardless of the fact that Ab dilutions were varied as mentioned above and multiple washes were tried. Doubling the Tween to 0.2% in the wash buffers did not reduce background and higher Tween was not recommended due to possible Ab-antigen interference.
2.2.1.(c).ii. Discrimination of melatonin receptor subtypes with chick specific antisera:

Attempts at Western Blotting with the chick specific antibodies and this tissue preparation protocol were unsuccessful. All of the chick-specific antisera produced for these investigations (multiple batches) resulted in a very low titer of antibody, as determined by SynPep using ELISA. Figure 2.2 shows an immunoblot with chick specific antisera against chick subtype Melβ in chick cerebellum. High background and multiple non-specific bands cover the blot (peptide blocking was unsuccessful - not shown). Additional washing did not clear the blot of background smears. The Ab used in this blot was a chick Ab, specific for subtype Melβ (IgG pure) (Ab dilution 1:1000). The lack of specific signal was likely due to the low antibody titer. Antisera for the other two subtypes yielded similar blots, that is, no specific signal could be detected in retina with these antisera or other brain sections in subsequent blots. Different dilutions of the chick specific Abs were tried as described in the preceding subsection; all without positive results.

Because of this low chick specific antisera titer (for all three receptor subtypes), and because of a possible loss of specific Ab during IgG purification, crude antisera was used in an attempt to yield a signal. However, no specific signal was obtained (data not shown). Due to this lack of specific results with this protocol, despite extensive optimization, the final protocol listed below was adopted.

A discussion of this protocol is provided at the end of Methods
2.2.2. **Western Blot FINAL Protocol:**

**2.2.2. FINAL Protocol: TCA and urea-Triton tissue preparation method**

Despite previous success in this lab with the above protocol (Song et al, 1997, and Fujieda et al, 1999) in human and rat tissue, this protocol did not yield specific signals in chick tissue. The following protocol utilizes TCA (trichloroacetic acid) to directly precipitate the membrane proteins and then solubilize them using urea - Triton. This protocol was successful in these Western Blot studies and was based on the protocol of Bollag et al, 1996, Hames, 1981, and van Renswoude & Kempf, 1984. A description of this final protocol used to obtain final results is presented below.

**2.2.2.(a) Tissue collection and preparation (FINAL protocol):**

Tissues were collected as described for the preliminary protocol (page 25). Retinas were dissected out from 4 eyeballs and immersed in 600 μl of the TCA (10%) (trichloroacetic acid) solution, while 0.067 g of each brain section (cerebellum (Cb), tectum, and thalamus) was separated and immersed in the same amount of the above solution. All samples were first sonicated in the TCA (10%) to ensure the latter's penetration, then incubated for 15 min at 0° C. TCA (10%) was used in order to inhibit endogenous enzymes (acts as protease inhibitor) and to precipitate the proteins. Following the incubation, the tissues were centrifuged at 12,000 x g for 10 min at 4° C and the TCA poured off the pellets. The pellets were then resuspended in 0.1 M Tris-HCl (pH 8.0),
sonicated and then centrifuged at 12,000 x g for 10 min at 4° C. The supernatant was discarded, and the pellet was resuspended in a 9 M urea -Triton (2%) X-100 mixture, sonicated, and then incubated on ice for 20 min. Approximately 700 µl of the urea-triton mixture was used for each tissue preparation, except retina, for which 300 µl was used. Following the incubation, the samples were centrifuged at 12,000 x g for 20 min at 4° C and the supernatant was collected for the immunoblot studies (kept at -70° C until use -- 2-3 weeks). (Protein concentrations in each sample were determined by a standard protein assay as per Lowry et al, 1951 (retina protein concentration: 40.9 µg/ µl, Cb: 125.6 µg/ µl, tectum: 278.9 µg/ µl, thalamus: 247.1 µg/ µl.)

2.2.2.(b) Gel electrophoresis and detection (final protocol):

Prior to gel electrophoresis, samples were first heated for 2.5 hrs at 37° C, and then denatured with sodium dodecyl sulfate (SDS) (0.5 X volume). Briefly, after heating, each tissue preparation sample was further diluted in urea-Triton (9 M urea - Triton (2%)) as determined by optimization ( 1:4 Cb, 1:2 all other tissues except thalamus - no further dilution, so that final protein loads were: 30.9 µg retina, 201 µg Cb, 892.5 µg tectum, and 15814 µg of thalamus ). Then 6.4 µl of each was mixed with 3.2 µl of 3X SDS Sample Buffer (Biolabs) and 0.3 µl of 30 X Reducing Agent (1.25 M DTT, Biolabs), for a total 10 µl load of each sample. In some cases, samples were incubated with 1-100 µl of the deglycosylation enzyme PNGase F (peptide-N-glycosidase F, Boehringer Mannheim) during the heating stage. This was done to test for possible presence of glycosylated proteins (see end of protocol and discussion). The samples were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (10%), and then finally transferred to a
nitrocellulose membrane. Gel electrophoresis and protein transfer to the membrane were performed as described for the preliminary protocol (page 26-27). Detection of the signal was also performed as per the preliminary protocol, except that the human Mel_{1a} Ab (IgG pure) was diluted 1:4000 (stock Ab: 1.36 μg/μl) with 10 ml blocking solution. For peptide blocking of the human Mel_{1a} Ab with; the human Mel_{1a} peptide, the chick-specific Mel_{1a} peptide, or the chick-specific Mel_{1b} peptide, each peptide was preincubated with the anti-Mel_{1a} Ab (2:1 ratio, Ab:peptide) in blocking solution for 0.5 hrs prior to incubation of the membrane. In the case of the chick-specific Abs, a range of dilutions 1:500 - 1:2000 of crude chick-specific antisera, and 1:500 -1:1500 of IgG purified chick-specific antisera were used. All other stages of detection were as described above (page 26).

2.2.2.(c) Specific Optimizations for FINAL Protocol:

2.2.2.(c).i. Detection of melatonin Mel_{1a}-receptor protein with human Mel_{1b} Ab (final protocol):

Antisera against human Mel_{1a} receptor (IgG pure) produced specific, blockable bands in Cb, retina, tectum and thalamus at approximately 37 kDa (expected molecular size of the receptor), as well as a blockable band at 50 kDa (figure 2.3 (a - d) chick tectum). The use of this protocol was immediately successful, as a signal could easily be detected with this Ab. Note the clarity of the signal and the relatively low background. Ab dilution for this blot was 1:4000 and initial load amounts had been decreased to 10μl.
2.2.2.(c).ii. Deglycosylation of the receptor protein (final protocol):

The second, unexpected band at 50 kDa remained despite these variations in conditions. It is possible that a second band, of larger molecular size, could be the result of the melatonin receptor being glycosylated during processing stages in the cell. Dubocovich (1995) identified glycosylation sites on the Mel₁₁ melatonin receptor during characterization of the protein from mammals and *Xenopus laevis*. To test for the possibility of a heavier protein due to glycosylation, the samples were subjected to a deglycosylating enzyme, PNGase F (peptide-N-glycosidase F, Boehringer Mannheim), to determine if the second band would then disappear from the blots after enzyme treatment. As the enzyme required an incubation of 37°C for activation (though, according to manufacturer, 25°C is sometimes sufficient), samples were incubated prior to gel loading for varying amounts of time (10 min - 2 hrs) and temperatures (25°C - 90°C). As a control, samples were also heated without exposure to the enzyme to ensure the persistence of the signal after long heating. As seen in figure 2.3 (c), the enzyme did not eliminate the 50 kDa band, even after 2 hrs of 37°C incubation. In this particular figure, a 25°C incubation for 0.5 hrs is also shown as one of many enzyme incubation times and temperatures (figure 2.3 (d)); also unsuccessful. However, the control sample subjected to heating for 2 hrs without enzyme treatment resulted in a fading of the 50 kDa band (figure 2.3 (b)).

As urea-Triton acts to denature the sample, no heating had been performed on these samples prior to gel loading before enzyme treatments were attempted. The heating of the sample prior to gel loading, and in the absence of the enzyme, was sufficient to remove the 50 kDa band. Further heating to a final incubation of 2.5 hrs at 37°C resulted in a complete disappearance of this band, while the expected 37 kDa band was unaffected (see final results, and discussion for data interpretation).
2.2.2.(c).iii. Specificity of the human Mel_{14} Ab (final protocol):

Blocking of the immunoblot signal by preincubation with human Mel_{14} peptide determined the specificity of the human Mel_{14} Ab for the melatonin receptor, and blocking with corresponding chick specific peptides yielded results as described in the Results section.

2.2.2.(c).iv. Discrimination of melatonin receptor subtypes with chick specific antisera (final protocol):

Attempts at Western Blotting with the chick specific antibodies and this tissue preparation protocol were unsuccessful. As mentioned in the preliminary protocol, all of the chick-specific antisera produced for these investigations resulted in a very low titer of antibody, as determined by SynPep using ELISA. Figure 2.4 shows the blot performed with crude antisera for chick subtype Mel_{14}. (All subtypes resulted in similar blots). Peptide blocking with chick Mel_{14} peptide was unable to block any of the signals, implying a lack of specific signal in the blot. Further attempts with an IgG purified antisera were no more successful (data not shown).

*A discussion of the optimization process for this protocol is provided at the end of Methods*
**Cellular Localization of Melatonin Mel₁₅ Receptors (continued)**

To further investigate the presence of melatonin Mel₁₅ receptors in chick retinal tissue, as well as to specifically localize the receptor within the tissue, we used immunocytochemistry and the human Mel₁₅ and chick specific antisera described above for the immunoblots. Previous success in this lab with immunostaining, using the human Mel₁₅ Ab in rat and human tissues (Fujieda et al., 1999 and Song et al., 1997) was the basis for the choice of the protocol. This was this first use of any of the above-mentioned antisera in chick tissue immunocytochemistry.

**2.3 Specific Localization in Chick Retina:**

**IMMUNOCYTOCHEMISTRY**

**2.3. Immunocytochemistry (final protocol)**

**2.3.(a) Tissue collection and slide preparation (final protocol)**

Tissues were obtained from day-old chicks after decapitation between 11:00 a.m. and 12:30 p.m. Whole eyeballs and brain were removed and immediately immersed in O.C.T. compound (embedding medium for cryostat (Sakura)), and then frozen using acetone on dry ice. Immunostaining with chick specific Abs was performed with both non-fixed (fresh - as per human Mel₁₅ staining) as well as perfused tissue. This decision was based on the fact that perfusion protects protein (i.e. receptor) degradation after death,
which may strengthen the signal. For perfused tissue, eyeballs were perfused with PFA (4% paraformaldehyde in PBS) during the tissue collection stage. For this, chicks were anesthetized with halothane gas (0.7%, mixed with O₂ and N₂O). Chicks were then immediately (and briefly) perfused through the left ventricle with saline (1 X PBS in DEPC- H₂O) to drain the blood, followed by a 5 min perfusion with PFA (4%). Whole eyeballs were removed and immersed in sucrose (15%, in 0.1 M PB) until saturated (approximately 3 hrs), followed by a saturation-immersion in sucrose (30%) and, finally, immersed in a 50-50 mixture of sucrose (30%) -OCT for 0.5 hrs. These eyeballs were then frozen, as above, after immersing the tissue in pure O.C.T. compound.

All tissues were stored at -70° C. Cryostat sections of 10 μm thickness were mounted onto gelatin-coated slides (gelatin (0.5%), CrK(SO₄)₂·H₂O (0.05%)) at -20° C. Slides were kept at -70° C until use (6 weeks or less).

2.3.(b) Detection of signal (final protocol)

Slides were dried for 30 min and then the non-fixed retina slides were fixed in PFA (4%) / 0.1 M PB as above. All the slides were then washed 3 X 5 min in 0.1 M PBS with Triton X-100 (0.03%) (PBS-Triton) and endogenous peroxidase was blocked by a 20 min incubation in H₂O₂ (0.3%) / methanol. After a 3 X 5 min wash, the slides were incubated in non-immunized (normal) swine serum (DAKO), diluted 1:20 with PBS-Triton for 30 min. Another wash routine was then followed by an overnight incubation with the anti-human Mel₁₈ Ab (affinity purified), diluted 1:50 - 1:100 in PBS-Triton / BSA (1%). For chick specific antibodies, both crude and affinity purified antisera were used; dilutions from 1:100 - 1:1000 and 1:10 - 1:100, respectively. (The affinity purified chick antisera, like the
affinity purified human Mel₁₈ Ab, is the eluate from the purification column and final
concentrations depend on recovery efficiency, as mentioned in section 2.1.) For peptide
blocking with the human Mel₁₈ peptide, the chick-specific Mel₁₈ peptide, or the chick-
specific Mel₁₉ peptide, the peptide was preincubated for 0.5 hrs in the Ab solution prior to
overnight slide incubation, as per Fujieda et al, 1999.

Due to the possible cross reaction of the human Mel₁₈ Ab with either chick subtype
Mel₁₈ or Mel₁₉, immunostaining with a human Mel₁₉ polyclonal antisera was performed.
Chick Mel₁₉ shows 46% sequence variation from the human Mel₁₉ (chick Mel₁₈ variation
is 62%). The Ab against human Mel₁₉ was affinity purified, and dilutions from 1:100 -
1:500 were tried.

Slides were then washed as above and incubated for 30 min at RT in secondary
antibody; biotinylated swine anti-rabbit immunoglobins (DAKO), which was diluted 1:800
in PBS-Triton / BSA (1%). Following the standard wash routine, the slides were then
subjected to a 30 min incubation with peroxidase-conjugated streptavidin (DAKO), diluted
1:500 in PBS-Triton / BSA (1%). After 2 X 5 min washes, and 1 X 5 min wash in 0.05 M
Tris-HCl buffer (pH 7.4), the immunoreaction was detected by a chromogen substrate
reaction using DAB (diaminobenzidine) (0.03%) as chromogen and H₂O₂ (0.01%) as
substrate for the above peroxidase. The immunodetection took place in Tris-HCl buffer for
approximately 6 min. at RT in the dark. The reaction was stopped by washes in PBS-
Triton and deionized water. (Reference staining of the retina was performed on separate
slides with hematoxylin and eosin.)
2.3.(c) **Initial Trials with Immunostaining and Specific Optimizations**

2.3.(c) i. **Receptor localization with antisera against human Mel\(_{1a}\) and Mel\(_{1b}\):**

The Ab against human Mel\(_{1a}\) was successful in staining after minor adjustments of the technique (see final Results), but the success was limited to retinal tissue. Fresh retina (no paraformaldehyde fixation during tissue collection) offered stronger staining than retina that had been fixed for 1 hr in PFA (4% paraformaldehyde in PBS) directly after tissue collection, using affinity purified antisera. IgG pure human Mel\(_{1a}\) antisera was also capable of revealing bands in the IPL, as shown in Figure 2.5 (a & b), even in fixed tissue, background was much higher and the bands themselves were weak.

As no cell bodies could be labeled with the immunostaining, and as the IPL is composed of processes from many amacrine cell types as well as from ganglion cells, we used human Mel\(_{1a}\) Ab and perfused tissue to stain cell bodies, despite the lack of success with fixed tissue (see page 35 for justification of perfusion). Figure 2.6 shows that perfusion of the tissue did enhance the IPL staining, but cell bodies did not stain specifically and, therefore, final results included only dendritic staining with human Mel\(_{1a}\) (see Results).

Immunostaining was also performed on chick brain tissue, using the human Mel\(_{1a}\) antisera. High background with no specific staining was the only result after many attempts at varying the amount of fixation of the tissue. Neither crude, nor IgG pure, nor even affinity purified antisera could elicit a signal. Dilutions of Ab as low as 1:10 were used (data not shown).

Immunostaining with affinity purified human Mel\(_{1b}\) antisera was performed on both chick retina and brain, using fresh tissue. Figure 2.7 shows the lack of results in
retina tissue (brain was similar). No signal could be detected, and the antibody was considered nonreactive in chick tissue. For this reason, it was not employed with Western Blots.

2.3.3 ii. Chick specific Abs:

In an attempt to discriminate between receptor subtypes in the chick retina, chick specific antibodies were used, despite the lack of success with Western Blots. Figure 2.8 shows fresh retina with affinity purified chick Mel₁₆ Ab. No staining could be obtained with this antisera, and slides were similar for Abs against the other two subtypes. Here, the Ab was diluted 1:50, and further staining with the Ab as high as 1:10 were also unsuccessful. Perfused retina was also used; as was perfused brain. Figure 2.9 shows perfused chick retina with Ab for chick subtype Mel₁₆. Only background (nonspecific) staining increased, without any blockable signal.

Finally, crude antisera of various dilutions (up to 1:100) was used on both retina and brain to elicit a signal with these antibodies, but figure 2.10 (retina) shows that even the highest titer antisera, chick Mel₁₆, did not yield a specific reaction. Attempts to discriminate between the subtypes were therefore limited to the peptide blocking of the human Mel₁₆ antibody, as shown in Results.

A discussion of the optimization process for this protocol is provided at the end of Methods.
Melatonin Receptor Subtype Mel_{1b} Expression (2nd Hypothesis)

In order to determine the presence of the melatonin receptor subtype Mel_{1b} in chick retina, as well as to identify the specific cell types expressing Mel_{1b}, we used *in situ* hybridization. For these investigations, a DIG labeled RNA probe for chick subtype Mel_{1b} was used.

### 2.4. Localization of Mel_{1b} Expression:

*In situ* Hybridization

The protocol for the *in situ* hybridization investigations was originally as described by Boehringer Mannheim (1996). Previous success in this lab with this protocol for rat tissue (Fujieda et al, 1999) was the basis for its use in these investigations. The final protocol described here is the final, optimized version used to obtain data from chick tissue.

### 2.4. *in situ* Hybridization (final protocol)

2.4.(a) cDNA for *in situ* hybridization

cDNA clones for chick melatonin receptor subtypes Mel_{1a} and Mel_{1c} were given to this lab as a generous gift from the laboratory of Dr. Steven Reppert (Harvard Medical School, Boston), who had previously cloned them from a chick brain cDNA library, using the plasmid vector pcDNA3 (InVitroGen) (Reppert et al, 1995b). Previous work in this lab
had also generated a cDNA clone for the melatonin Mel₁β receptor subtype from a chicken brain cDNA library (Clontech), using the plasmid vector pSP73 (Promega) (Liu et al., 1995). Each cDNA clone was introduced into competent XL-1 cells, and then the plasmid vectors were isolated using a plasmid vector purification kit (Qiagen), according to the manufacturer's instructions. cDNA was kept at -20° C until use.

2.4.(b) Chick RNA probes for in situ hybridization (final protocol)

Linearization of the plasmid DNA was performed using the appropriate restriction enzyme (Xba I for Mel₁α antisense, Sac I for Mel₁α sense, EcoRV for Mel₁β antisense, BamH I for Mel₁β sense, Hind III for Mel₁c antisense, Xho I for Mel₁c sense—all from Pharmacia Biotech, except BamH I from Stratagene). Linearization was complete after a 3 hr incubation in a 37° C bath. To confirm proper linearization, 1% agarose gel electrophoresis was performed with small amounts of both circular and linearized DNA. The differences in migration patterns were visualized with a transilluminator. Proteinase K was added to the linearized product (30 min incubation at 37° C) in order to inactivate the restriction enzyme, and then the DNA was isolated by column purification using the commercially available, GFX PCR DNA and Gel Band Purification Kit (Pharmacia Biotech), according to the manufacturer's instructions. After elution from the column, the DNA was resuspended in DEPC-H₂O and kept at -20° C until transcription.

Transcription of the chick cDNA inserts to DIG (digoxigenin)-labeled RNA probes was performed using the appropriate RNA polymerase (T7 for Sac, Eco, Xho, and SP6 for Xba, Bam, and Hind) and NTP labeling mixture (DIG-UTP labeling mix, Boehringer Mannheim) in transcription buffer (Boehringer Mannheim) in the presence of RNase
inhibitor (Boehringer Mannheim) and 0.2 M DTT. Incubation was 2 hrs at 37° C. Briefly, the transcribed RNA probe was subjected to DNase I (Boehringer Mannheim) to digest the cDNA (15 min at 37° C). This reaction was stopped by addition of 0.5 M EDTA and TE buffer (Tris-EDTA), while the RNA was precipitated by addition of 10 mg/ml tRNA, 3 M sodium acetate and ethanol, the solution was centrifuged, after a 10 min incubation, at 12,000 x g for 30 min at RT. The final pellet was resuspended in DEPC-H₂O and a 1% agarose gel electrophoresis was performed to confirm transcription.

Finally, the chick RNA probe was cut to a 300 bp size by alkaline hydrolysis. For this, the RNA was spun and dried, then dissolved in RNA sizing solution (60 mM Na₂CO₃, 40 mM NaHCO₃, pH 10.2) and incubated in the above for 16 - 23 min at 60° C. The RNA solution was cooled on ice and then precipitated as described above. The final, spun pellet was dissolved in DEPC-H₂O and 0.5 µl of RNase inhibitor. A final 2% agarose gel electrophoresis was performed to ensure proper hydrolysis.

RNA probes were purified to remove small fragments (<70 bp) of RNA, as well as unincorporated DIG-UTP nucleotides. Purification was done using G-50 Sephadex Quick Spin columns (Boehringer Mannheim), according to the manufacturer's instructions, and then the final RNA probe concentration was determined by spectrophotometry. All probes were kept at -70° C until use.

2.4.(c) Tissue collection and slide preparation (final protocol)

Day-old chicks were kept under the same conditions (see Western Blot) and, during the hours 10:00 -14:00, anesthetized with 0.7% halothane gas (mixed with O₂ and N₂O). Chicks were then immediately (and briefly) perfused through the left ventricle with saline.
(1 X PBS in DEPC- $H_2O$) to drain the blood, followed by a 5 min perfusion with PFA (4% paraformaldehyde in PBS). Whole eyeballs were removed and immersed in PFA (4%) for an additional 5 hrs to extra fix the tissue. (Some eyeballs were also collected as above, but not extra-fixed after perfusion (i.e. no additional 5 hrs in PFA) for comparative experiments. The tissues were compared during optimization of the protocol.) To preserve the tissue and cells during the freezing process, eyeballs were placed in sucrose (15%) (in PBS diluted with DEPC-$H_2O$) for 15 min, then again for approximately 4 hrs until the tissue was saturated. The eyeballs were then immersed in sucrose (30%) solution overnight (4° C). Prior to freezing, the eyeballs were placed in a 50-50 mix of sucrose (30%) and OCT (tissue mounting medium for cryostat) for approximately 1 hr. Then eyeballs were individually dipped into OCT mounting medium and frozen by acetone-dry ice. Sections were obtained on a cryostat as per immunostaining, except that slides were coated with RNase free BSA (1%) and glutaraldehyde (25%). Slides were kept at -70° C until use (6 weeks or less).

2.4.(d) Hybridization and detection (final protocol)

Because the chick Mel$_{18}$ and Mel$_{18}$ in situ hybridization are already reported by Reppert et al (1995), this protocol was used for chick Mel$_{18}$ RNA probes only. Therefore, 'slides' refers to in situ slides with chick Mel$_{18}$ RNA probes, either antisense or sense (or no probe).

All slides were treated with DEPC-treated solutions prior to and during hybridization to prevent RNA degradation. Pretreatment of slides involved the following: slides were dried for 1/2 hr and then washed 2 X 5 min in PBS to remove residual OCT.
Slides were then subjected to a 10 min incubation with 0.5 μg/ml proteinase K at 37°C. Slides were briefly rinsed with a 0.1 M TEA buffer (0.1 M triethanolamine pH 8.0) and then acetylated for 10 min RT with acetic acid (0.25%) in 0.1 M TEA. This was followed by 3 X 5 min of washes with 1 X PBS, then the tissue was fixed onto the slides with PFA (4%) (10 min incubation at RT). Following 3 X 5 min washes with 1 X PBS, the slides were dehydrated with, first H2O, then ethanol-H2O mixes 3 min each, each wash with a successively higher ethanol content, from 70 - 100%. Finally the slides were air dried for 20 min.

All slides were prehybridized with a lower stringency hybridization solution (formamide (40%), 4 X SSC (0.6 M NaCl), 2.5 mM EDTA, 1 X Denhardt's solution, 1 mg/ml E. coli tRNA, 1 mg/ml fish sperm DNA, SDS (0.25%), dextran sulfate (10%)) at RT for approximately 20 hrs (120 μl/slide), kept in a moist chamber (formamide (50%), 0.3 M NaCl, and ddH2O in the chamber bottom). Slides were then hybridized with probes (1 μg probe / 50 μl hybridization solution per slide --2 retinas per slide) in a high stringency hybridization solution (as above except formamide (50%) and 2 X SSC (0.3 M NaCl)) at 48°C overnight in the same moist chamber.

Washing of the slides involved: 1 X 5 min 2 X SSC (different probes kept separate), 3 X 5 min 0.1 X SSC, all at 42°C (2 X SSC: 0.3 M NaCl, 1.3 M sodium citrate, pH 7.0). No formamide was used in these washes.

After 2 X 5 min washes in DIG 1 buffer (0.1 M Tris-HCl, 0.15 M NaCl, Triton X-100 (0.3%), pH 7.5), the slides were blocked with normal goat serum (DAKO) diluted 1:5 in DIG buffer 1 for 30 min. Ab incubation was performed for 3 1/2 hrs with anti-DIG Ab alkaline phosphatase - fab fragments (Boehringer Mannheim) diluted 1:500 in DIG buffer 1. The slides were then washed 2 X 7 min in DIG 1 and 1 X 7 min DIG 2 (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM MgCl2, pH 9.5).
Detection of the signal was then achieved by incubating the slides overnight in 0.34 mg/ml NBT (4-nitro blue tetrazolium chloride) and 0.18 mg/ml BCIP (X-phosphate/5-bromo-4-chloro-3-indolyl-phosphate) (both Boehringer Mannheim) in DIG buffer 2 in the dark.

2.4. (e) Optimization of the In Situ Hybridization Protocol:

Silan coated slides (silan (2%) in acetone) were replaced with the above described BSA (1%) / glutaraldehyde (25%) coating. The strong adhesion of tissue to slides with the latter coating method was required to prevent tissue dissociating from the slides.

Figure 2.11 (a) shows an example of an early attempt at in situ hybridization with the Mel sub antisense probe (Eco) in chick retina. Next to this, figure 2.11 (b) shows the chick retina hybridized with Mel sub sense probe (Bam), as a control. As these slides were from very early attempts, the unstable substrate reaction has degraded and left the entire retina a dark purple colour (as seen in the photograph as gray). Also, spots of substrate precipitate are scattered across the slide, seen especially in the INL--not to be confused with cell staining. In the original slides these are obvious crystals from substrate precipitation. However, it is still possible to take note of the failure to produce any signal, which would appear as dark purple cell bodies. A brief description of the protocol differences as used in these early attempts is as follows:

- retinal tissue was perfused, but not extra-fixed (5 hrs in PFA after perfusion)
- proteinase K concentration was 1.0 μg/ml (not 0.5 as described in final protocol)
- no prehybridization step was used
- Probe concentration was 3.0 μg of probe in 50 μl hybridization solution (not 1.0 μg) and left to hybridize overnight at 54°C.
- Probes were not further purified (by Sephadex column) after the ethanol precipitation purification.
- Wash solutions contained 50% formamide and all washes were at 54°C, a 30 min incubation with RNase A (20 μg/ml, Boehringer Mannheim) at 37°C preceded the 0.1 X SSC step (also, wash times were 2 X 20 min for all the SSC washes).
- Ab incubation was only 1 hr.

The stringencies of both the probe incubation and the washes were repeatedly adjusted. Ultimately lower and lower levels of stringencies were used in order to secure a signal. With reductions in the temperature of probe incubation, as well as decreasing the temperatures of the washes, a signal in the ganglion cell layer (GCL) was obtained. However, this signal appeared in both antisense and sense (control) slides. Addition of a ‘prehybridization’ step (incubating slides in hybridization solution without probes prior to the actual probe hybridization) for 40 min at 42°C did little to reduce the sense signal, but did clear up some of the background. The hybridization solution used for these prehybridizations was the same stringency solution as used for the probes; i.e. with 50% formamide and 0.3 M NaCl. Figure 2.12 (a & b) shows these results with Eco (antisense) and Bam (sense), respectively for the probe chick Mel. Note the cell bodies now stained in the GCL. It had become necessary by this stage to lower the temperature of the probe hybridization to 42°C and to eliminate the use of formamide in the washes (decrease stringency), in order to secure this signal. Wash temperatures had been lowered to 37°C (from 54°C) and Ab incubation had been extended to overnight to elicit the signal.
To protect the tissue, proteinase K treatment was reduced to 0.5 μg/ml of enzyme, without any noticeable differences in signal intensity. (Previous trials with no enzyme treatment resulted in a poor signal.)

Note, however, that background is still high in figure 2.12 and that the sense probe still yields a strong signal in the GCL. A stronger signal was required in order to allow for further attempts to decrease background and reduce nonspecific sense staining without a loss of the specific signal. Extra-fixed retina (5 hrs in 4% PFA) resulted in a stronger signal, as seen in figure 2.13 (a & b). Also note that now some signal is detectable in cells along the innermost region of the inner nuclear layer (INL) with the use of extra-fixed tissue. Multiple trials with this tissue began to yield a difference between the Melβ antisense and sense slides, as can be seen in this figure, although sense GCL staining remains (figure 2.13 (b)). Among the changes to the protocol: the RNase A incubation step had been eliminated which also helped to strengthen the overall signal, a reduction in probe concentrations to 1 μg of probe/50 μl of hybridization solution assisted in the reduction of sense staining, and an increase in prehybridization incubation time from 40 min to overnight (approximately 16 hrs) at 42° C also reduced this nonspecific signal. Washes at this stage (no formamide) were all at 37° C and as follows: 1 X 5 min with 2 X SSC, 3 X 5 min with 0.2 X SSC and then 1 X 5 min with 2 X SSC; a much lower stringency wash routine than had been originally used. Ab incubation was reduced to 4 hrs (from overnight) with a slight reduction in some background without affecting the signal intensity.

Figure 2.14 (a & b) shows some additional success in reducing the sense signal after probes were further purified by column purification (page 42). GCL staining in the sense slide is weaker, while the specific signal in the antisense slide remains strong. INL staining is also strong by this stage.
Final stages of optimization focused on the elimination of nonspecific sense signals to ensure the specificity of the antisense INL and GCL staining. Prehybridization was extended to approximately 20 hrs at RT and a lower stringency hybridization solution was for the prehybridization (40% formamide instead of 50% as used for probe hybridization, and 4 X SSC (the equivalent of 0.6 M NaCl, instead of 0.3 M NaCl (2 X SSC), again as for probes). Long incubation in this low stringency buffer reduced the sense signal as seen in figure 2.15 (a & b). Also, this figure shows the difference that a slightly higher stringency wash routine effected. The wash routine at this stage became the final protocol wash method; i.e. “washing of the slides involved: 1 X 5 min 2 X SSC (different probes kept separate), 3 X 5 min 0.1 X SSC , all at 42° C. No formamide was used in these washes”. The higher temperature and lower salt (0.1 X SSC as high stringency wash rather than 0.2 X SSC) improved the antisense - sense difference.

Finally, not shown in the figure 2.15, but rather shown in the final Results, was the success obtained from final adjustments. These included; increasing the probe incubation temperature to 48° C (from 42° C), Ab incubation of 3.5 hrs, and an important addition of an acetylation step prior to the proteinase K treatment (see section ‘Hybridization and detection”). This acetylation step, as well as the fine tuning of the probe incubation stringency, finally eliminated the last of the nonspecific, sense signal. The protocol therefore, is as described under ‘Hybridization and detection’ , and the slides are shown as final Results.

A discussion of the optimization process for this protocol is provided at the end of Methods
Colocalization with Dopaminergic Cells (3rd Hypothesis)

In order to determine whether melatonin receptors colocalize with chick retinal dopaminergic cells, we used immunocytochemistry to specifically identify dopamine positive amacrine cells. In order to determine whether melatonin receptors colocalize with amacrine cells positive for other neurotransmitters / neuromodulators, we used immunocytochemistry and commercially available antisera against the neurotransmitters / neuromodulators listed below. Immunostaining from these studies was then compared to the melatonin receptor immunostaining.

2.6. Colocalization Studies:

immunocytochemistry

2.6. Immunocytochemistry (final protocol)

2.6.(a) Antibodies used in colocalization immunocytochemistry

In order to specifically localize dopamine positive retinal amacrine cells, we used a commercially available, monoclonal antibody against the enzyme tyrosine hydroxylase (SIGMA); involved in dopamine synthesis. Colocalization of melatonin receptors with other neurotransmitters / neuromodulators was also investigated by immunocytochemistry, using commercially available antisera against: enkephalin, substance P, and choline acetyltransferase (for acetyl choline) (all from Chemicon), GABA and VIP (vasoactive
intestinal peptide) (SIGMA), neurotensin (Serotec), and serotonin (DAKO). (see Table 1 for further antisera information; page 19 of Introduction).

2.6.(b) Tissue collection and preparation (final protocol)

Tissues were obtained from day-old chicks after decapitation between 11:00 a.m. and 12:30 p.m. Whole eyeballs were removed and those used to localize dopaminergic cells by tyrosine hydroxylase (T.H.) staining, as well as those used for GABAergic cell colocalization, were prefixed in paraformaldehyde (4% PFA) in 0.1 M PB (pH 7.4) for 1 hr. This procedure was modified from Mariani & Hokoc, 1988, Reiss et al, 1997, and Dos Santos & Gardino, 1998, whose fixation times varied from 10 minutes to 3 hrs. These eyeballs were then immersed in sucrose (15%) (in 0.1 M PB) until saturated (approximately 3 hrs), followed by a saturation-immersion in sucrose (30%) and, finally, immersed in a 50-50 mixture of sucrose (30%) - O.C.T. for 0.5 hrs. These prefixed eyeballs were then frozen using acetone on dry ice after immersing the tissue in pure O.C.T. compound.

For colocalization studies with the neurotransmitters (or neuropeptides) serotonin, enkephalin and substance P, eyeballs were first perfused with PFA (4%) during the tissue collection stage as described for 'Cellular localization of melatonin receptors' (section 2.3.(a)). This fixation procedure was as per Cuello et al, 1979, Millar et al, 1988, and Karten & Brecha, 1980. After perfusion, whole eyeballs were removed and immersed in PFA (4%) for an additional 5 hrs to extra fix the tissue. Some eyeballs were subjected to this extra fixation after perfusion (5 hrs), while others were simply perfused and then immersed in sucrose as described above. For example, for colocalization with VIP, and
neurotensin, all eyeballs were PFA-perfused without extra fixation. This was modified from Yang et al., 1997, and Watt & Florack, 1994, who fixed tissue in PFA (4%) / glutaraldehyde (0.1%) in PB for 1 hr instead of PFA (4%) perfusion, followed by extra fixation in PFA.

All tissues were stored at -70° C. Cryostat sections of 10 μm thickness were mounted onto gelatin-coated slides (gelatin (0.5%), CrK(SO₄)₂·12H₂O (0.05%)) at -20° C. Slides were kept at -70° C until use (6 weeks or less).

2.6.6 (c) Detection of signal (final protocol)

Slides were subjected to the same detection protocol as described for 'Cellular Localization of Melatonin Receptors' (section 2.3.(b), page 36). The following dilutions were used for the primary Abs in the colocalization studies, according to the manufacturer's recommendations: GABA 1:50, choline acetyltransferase 1:5, serotonin 1:5, enkephalin 1:100, substance P 1:200, VIP 1:8000, and neurotensin 1:500; all diluted in PBS-Triton / BSA (1%) or, in the case of anti-T.H. Ab (SIGMA), a dilution of 1:5000 was used. As a secondary antisera, we used commercially available, biotinylated swine anti-rabbit immunoglobins (DAKO), diluted 1:800 in PBS-Triton / BSA (1%), for the VIP and neurotensin studies. Secondary antisera used against the primary anti-substance P antibody was a commercially available, biotinylated rabbit anti-rat immunoglobin (DAKO), diluted 1:200. Finally, for the primary Abs against choline acetyltransferase, GABA, tyrosine hydroxylase, serotonin, and enkephalin; biotinylated goat anti-mouse immunoglobins (DAKO), diluted 1:500 was used as secondary antisera.
Specific Optimizations for Colocalization Immunocytochemistry:

Immunostaining with the Ab against tyrosine hydroxylase (for dopaminergic cell localization) was successful, using the procedure described above and results are shown in the Results section.

Anti-GABA staining was, however, unsuccessful. The Ab did not appear to show a specific reaction in chick tissue, with a resultant all-over stain as seen in figure 2.16; impossible to determine if specific or not. Immunostaining with anti-serotonin Ab, in order to localize this melatonin precursor, were also unsuccessful (figure 2.17). Both perfused retina and extra-fixed (5 hrs in PFA (4%) after perfusion) were used without success. The figure illustrates the latter form of tissue preparation with an Ab dilution of 1:5. Still no specific signal is visible, and like the GABA staining, results in a dark, all-over nonspecific reaction.

Anti-substance P antisera appeared nonreactive in chick tissue (figure 2.18), as was anti-enkephalin (figure 2.19), anti-choline acetyltransferase, anti-VIP, and anti-neurotensin. See Table 1 in Introduction (page 19) for antisera information. Results from these immunostaining studies are not shown as they were all identical to figures 2.18 & 2.19. No positive controls were used in these colocalization studies.

A discussion of the optimization process for this protocol is provided at the end of Methods
2.7. Discussion of Methods

2.7.(a) Western Blots:

2.7.(a) i. A comparison of two protocols

The fundamental difference between the PMSF / EDTA - N-P40 and the TCA / urea-Triton tissue preparation methods for Western blotting is the manner of protein isolation. In the former method (preliminary immunoblot protocol), membrane fractions are separated out from the rest of the cell by the homogenizing and spinning process. This leaves a precipitate of cell membranes as well as the proteins bound to them. The proteins are then solubilized by the Nonidet-P40 (the PMSF and EDTA act as the protease inhibitors in this protocol). The TCA / urea-Triton protocol, the final protocol used, is based on the direct precipitation of proteins from the tissue (both the soluble and membrane-bound proteins) by the use of TCA, which also acts as a protease inhibitor. Homogenization allows for the TCA to penetrate the tissue and 'free' the membrane-bound proteins. The urea-Triton then solubilizes the proteins from the precipitate.

As the preliminary protocol requires membrane fractions to be first separated from the cells, and then the proteins to be solubilized, the only proteins that can be captured in this solubilizing step are those proteins that remained with the membrane fraction precipitate. In this case, where the melatonin receptor protein is indeed a membrane-bound protein, this would suffice to capture our desired protein from tissue. But any membrane fractions that are lost during the stages of homogenization and precipitation would carry away their bound proteins as well. A lower protein yield in the loading samples would
affect the strength of the signal in the blots, and in the case of proteins with a low level of expression, may hinder any appearance of a signal. The melatonin receptor, although expressed highest in birds, is still a relatively low expression level receptor. It is possible that lack of signal with the preliminary protocol with the use of the human Mel 

Ab was a result of low receptor amounts in the preparation; not due to a lack of receptor in the original tissue, but rather due to a poor yield after sample preparation. Only high background smearing is observable. Smaller sample loads in the gel eliminated the smear, without any discernible blotting at all, and higher loads used to elicit even a faint signal resulted in all-over blackout smears. The idea that this protocol gave poor melatonin receptor yield is also suggested by the fact that far less sample was required to obtain a signal with the final protocol (TCA / urea- Triton), showing it to be not simply due to a lack of receptor protein within those particular tissues.

As chick-specific Abs could reveal at least nonspecific bands, the preliminary protocol was not 'ineffective' for Western Blot tissue preparation. It does in fact produce detectable protein signals on a film; albeit nonspecific in these studies. This lack of specific, blockable bands in any of the blots with this PMSF / EDTA - NP-40 protocol, which were detectable only with the final protocol, further suggests a low melatonin receptor protein yield after this preparation. Additionally, the fact that the human Ab could not even detect nonspecific signals in these investigations suggests that either the Ab-antigen interaction was highly specific and not detectable only due to low protein levels, or conversely, a poor interspecies cross reaction with this Ab and the low overall protein content of the sample, combined together, inhibited even nonspecific binding. However, the fact that the final protocol produced films free of nonspecific reactions suggests that the human Mel 

IgG antisera was quite specific in its reaction with the melatonin receptor and, therefore, protein levels are most likely to blame.
It is possible that a specific signal might have been obtained with the chick-specific antisera, had the Ab titer been higher. This can be speculated from the success in achieving at least nonspecific signals, even with the preliminary protocol, as seen in figure 2.2. The Ab within this antisera, specific for the melatonin receptor, must have been too low to detect the receptor protein, and further blots with the chick Abs were abandoned for this protocol.

2.7.(a) ii. Optimization of the final, TCA/urea-Triton tissue preparation method

Using chick Abs in combination with the TCA/urea-Triton protocol to prepare tissue was also unsuccessful in eliciting a specific signal. Cb is shown in the Methods as representative of the tissues tried; Cb, retina, tectum, and thalamus. All resulted in multiple nonspecific bands with each of the three chick specific Abs. As the figure illustrates, peptide blocking (preincubation of the Ab with its corresponding peptide) revealed a similar blot; no blockable bands.

However, this final protocol was successful with the human Mel$_{1a}$ Ab. Figure 2.3 shows the presence of blockable bands in both chick tectum. The success with this protocol (and the human Mel$_{1a}$ Ab) was immediate, and optimization of this protocol did not, therefore, focus on obtaining a signal per se, but on strengthening the signal, as well as investigating the second band which appears at around 50 kDa. First of all, load amounts could be dramatically reduced from 34 µl down to 10 µl sample loads, without losing the signal. In fact, the signal is much stronger with the reduced protein load. This enhancement of the signal may be due to the fact that higher loads, and consequently excessive protein amounts, may have interfered with the Ab - antigen interaction. Too much protein may
have masked the target antigen and therefore hindered maximal Ab binding. The final 10 μl loads still contained > 240 μg of protein (depending on the tissue), which is already in excess of what is often required for this technique. It is also known that blots become clearer with less loading, often reducing the background clouding of the film.

The second band which appeared at about 50 kDa was originally thought to be nonspecific, even though the band itself was blocked with Ab-peptide preincubation. It was thought that the excessive load amounts which had been used to ensure a preliminary signal might have been forcing a nonspecific reaction between the human Mel Ab and other tissue proteins. A simple reduction in load amounts was therefore thought to be the means of its elimination from further blots. However, despite these variations in the loads, the second band persisted. The Ab was further diluted in order to increase the specificity of the Ab binding, with dilutions as low as 1:4000 (from an original 1:1000), which was also unable to eliminate the second band.

As mentioned in the Methods section, a second, larger band could have been the result of the melatonin receptor being glycosylated during processing stages in the cell. In fact, as mentioned, Dubocovich (1995) had identified glycosylation sites on the receptor during the characterization of the protein. This seemed to be a possible explanation for the presence of the 50 kDa signal, and the trials with deglycosylation enzyme, PNGase F, are summarized in Figure 2.3. The samples were preincubated with the enzyme for 10 minutes to 2 hours (the figure shows incubation of 2 hrs) at 37° C, prior to gel loading. The tissue shown is chick tectum, and lane C marks the sample with the enzyme. (The break in the second band in lane C is actually due to an air bubble in the gel). The 50 kDa band is virtually unaffected by enzyme treatment. Lane C, in fact, resembles the control sample (lane A), which is simply the sample loaded without previous heating. Lane D also has sample with enzyme treatment, incubated for half an hour at 25° C, as one of many
variations in the immunoblots with the enzyme; also with no change in the blot. However, there was a striking reduction in the 50 kDa signal in lane B of figure 2.3. This lane marks sample that was heated to 37°C for 2 hours, without enzyme treatment, used as a control. It is interesting to note that other attempts to eliminate the 50 kDa band by the conventional denaturing process of heating to 90°C for 10 minutes did not work. In fact, 90°C heating eliminated both bands with this protocol; possibly a result of excessive denaturation and, therefore, damaged proteins. Only the 37°C, 2 - 2.5 hour incubation was successful and reproducible.

Denaturing of the samples by heating had not, originally, been performed with the TCA / urea-Triton protocol, as it had been for the preliminary protocol, because it was known that urea-Triton itself acts to denature proteins (Bolag et al., 1996), and therefore heating was thought to be unnecessary. Heating the samples to 90°C for 10 minutes had eliminated the signals, while the 37°C incubation only eliminated the 50 kDa band. The reason for this may be due to the fact that 37°C is the biochemically optimal temperature for cell function. It may be that urea-Triton denatures proteins best at this temperature, and the tissue protein, therefore, may not have been properly denatured. This may have been a problem during tissue preparation, which kept the tissue at 0°C at all stages (to prevent protein degradation), or else the freezing at -70°C which may have resulted in a crystallization of some urea with receptor protein. The 37°C incubation may have been needed to redissolve the urea crystals or otherwise allow the denaturation process to be completed. Ultimately, the blot is left with the one specific signal at 37 kDa in each tissue.

The idea that the 50 kDa band was actually a dimerization of melatonin receptors is unlikely, due to the fact that the size is not really double that of the expected 37 kDa receptor size. Finally, the Ab dilution in figure 2.2 was 1:3000, and a final dilution to
1:4000, combined with the 10 μl sample load, resulted in the sharp signal and clear blots as seen in Results.

2.7.(b) Optimization of the Immunocytochemistry Protocol for the Human Mel很棒 Ab:

As the Western blots had ultimately led to a specific signal with the human Mel很棒 Ab, it was reasonable to expect a specific signal after immunocytochemistry as well. However, with chick brain tissue, the immunostaining technique was unresponsive. No signal could be obtained despite the variations to tissue fixations and Ab dilutions mentioned in. This lack of Ab reactivity may have been due to large amounts of lipids in brain tissue which could have inhibited the Ab - antigen interaction. High levels of lipids in the tissue might have restricted the Ab's access to target cell proteins. Previous trials in this lab had attempted to solubolize brain lipids by treating the tissue with acetone (which would also help to fix the tissue proteins), in an attempt to facilitate the Ab - antigen interaction and thereby elicit a signal. These trials had used the same human Mel很棒 Ab with brain tissue from rat (which shows 100% antigen sequence homology with human), however, these lipid solubolizing trials were unsuccessful and no signal was ever detected by this Ab in either rat or chick brain tissue. Therefore, it is also a possibility that the receptors themselves, when expressed in brain tissue, are somehow altered in their final structure by post-translational modifications which render them unrecognizable to the human Ab, even in the presence of excess Ab (dilutions as low as 1:10 had been tried). Whatever the reason, only chick retina was responsive to the human Mel很棒 Ab in these investigations.
Experiments with this Ab and chick retina were, in fact, quite successful. However, no cell bodies are visible in either fresh or fixed tissue, and in the fixed tissue, the bands themselves were barely visible (data not shown). Fresh chick retina and affinity purified Ab seemed to be the best combination for immunostaining, but a last set of trials were attempted in the hope of revealing cell body staining. As perfusion of tissue with fixative (4% PFA) serves to protect proteins from degradation and therefore often enhances the staining signal, as mentioned above, perfused chick tissue was subjected to immunostaining in the hope that this enhancement of signal strength would elicit cell body staining. The result of this trial was that the signal was indeed enhanced, as was background, but no cell bodies were specifically stained. Fresh retinal tissue still maintained the clearest immunostaining as well as the sharpest bands.

2.7.(c) Optimization of the In situ Hybridization Protocol:

In order to secure a specific in situ hybridization signal, extensive optimization was required for the protocol. Retina was ultimately the focus of the experiments due to its simple structure, and time constraints precluded optimization of the protocol in brain. However, the delicate nature of the retinal tissue posed problems at the outset of the trials. A switch from silan coated slides to BSA/glutaraldehyde coated slides was necessary to ensure tissue adhesion. The proteinase K treatment was determined to be a necessity, however, as a subsequent removal of this step resulted in a greatly decreased signal.

The lack of any detectable signal at the outset of these trials was most likely attributable to the excessively high stringencies used in the original protocol. For example, the probe hybridization was originally carried out at 54° C, which may have prevented even
specific annealing between probe and target mRNA. The problem may have been confounded by the 54° C washes to which the slides were originally subjected. Any hybrid formed between probe and mRNA may have been severed with the high temperature wash. The presence of 50% formamide in the wash solutions would have further increased the stringency by acting to decrease the thermal stability of the probe - mRNA hybrid. As it reduces the probe - mRNA duplex melting temperature by 0.72° C for each % of formamide, the effect of the 54° C wash temperature would have been closer to that of an 85 - 90° C wash in the presence of the 50 % formamide. This may have made it impossible to maintain a hybridization. Any signal that would have survived these conditions would probably have been too small to detect after only a 1 hour antibody incubation.

Initial adjustments involved a decrease in the temperature of both the hybridization incubation and the posthybridization washes. GCL staining was then observed, although a similar signal with the sense probe cast doubt on the specificity of the signal. The wash solutions no longer contained formamide and wash temperatures had been reduced to 37° C, while the probe hybridization was performed at 42° C instead of 48° C. The lower stringency obviously allowed for the GCL staining, but fine tuning was still required for specificity in the signal. An initial prehybridization step did serve to reduce background, but the relatively short incubation (40 minutes) did little to decrease the sense signal.

Extra fixed tissue (5 hrs in 4% PFA after perfusion) was instrumental in eliciting the INL staining, as well as strengthening the overall signal intensity. This was probably due to the fixative’s ability to prevent tissue degradation and therefore preserve the mRNA. A reduction in the amount of probe used to 1/3 the original amount (now 1 μg probe/ 50 μl hybridization solution) reduced the level of nonspecific binding during hybridization, as seen with the sense signal. An increased prehybridization time to 16 hrs increased the blocking of nonspecific sites in the tissue.
The fact that the Ab incubation, which had been lengthened to overnight, could be reduced to 4 hrs without affecting signal intensity confirmed the strength of the signal. The staining pattern observed for Mel10 and Mel15 at this stage was similar to the data from Reppert et al (1995b), and final alterations to the protocol centered on the specificity of the Mel16 signal alone. Wash solution stringencies were quite low (0.2 X SSC and 37° C routine), which allowed for adjustments to higher temperatures and lower salt without loss of signal. The final adjustment to a 42° C and 0.1 X SSC wash routine increased the stringency enough to improve the antisense - sense signal difference. The lower stringency hybridization solution, used here for the prehybridization step, also contributed to the observed reduction in sense staining. This was probably due to an increase in blocking of nonspecific sites by the tRNA and fish sperm DNA present in the solution.

Purification of the probe reduced the stubborn GCL sense staining. Small RNA fragments and unincorporated DIG-UTP nucleotides must have been reacting nonspecifically in this region to produce the strong sense signal. Although some sense signal remained, final adjustments were successful in eliminating the last of the sense staining in the GCL. An increase in probe hybridization temperature from 42° C to 48° C increased the specificity of the hybrid, and the last of the nonspecific reaction in the GCL was successfully blocked by the added acetylation step. This latter step reduces positive charges on molecules in the tissue which may nonspecifically attract the probe nucleotides.

Ultimately, the final adjustments of the protocol involved establishing a careful balance between the level of stringency used (and blocking steps) with the efforts to secure a strong signal. The protocol finally elicited strong, reproducible staining in the INL and GCL, with a virtual lack of sense staining as seen in Results.
FIGURES FOR METHODS
**Figure 2.1**

Preliminary protocol. Western Blot using anti-human Mel_{1a} Ab (IgG purified). Chick retina is shown for all tissues. Ab dilution is 1:4000 and sample loads are 43 µl. Lane B shows Ab preincubated with human Mel_{1a} peptide.
Retina

Figure 2.1
Figure 2.2

Preliminary protocol. Western Blot with chick-specific antisera against chick Mel1
(IgG purified). Dilution of Ab was 1: 1000. Cb= cerebellum. Peptide blocking failed to
block signals (data not shown).
Figure 2.2
**Figure 2.3**

Final Protocol. Western Blots with Ab against human Mel1a (IgG purified). Chick tectum is shown. Ab dilution is 1: 4000 and sample loads are 10 µl. Lane A shows sample without heating or PNGase F enzyme. Lane B shows sample heated 2 hrs at 37°C without enzyme. Lanes C & D show sample after PNGase F enzyme incubations of 2 hrs at 37°C and 0.5 hrs at 25°C, respectively. (Lane C shows an air bubble artifact in the 50 kDa band.)
Tectum

51 kDa - 

34 kDa - 

A  B  C  D

Figure 2.3
Figure 2.4

Final protocol. Western Blots with crude, chick-specific antisera against chick Mel₁₆. Chick cerebellum is shown. Lane B shows the results of peptide blocking.
Figure 2.4

34 kDa - Cb

A B
Figure 2.5

Immunostaining in chick retina with anti-human Mel1a antisera (IgG pure, diluted 1:100) using a) fresh and b) fixed tissue. Fixation was 1 hr in 4% PFA. Staining with IgG purified Ab also yielded bands in IPL, even in fixed tissue, but staining was weaker and background was higher.
Figure 2.6
Immunostaining in chick retina with anti-human Mel$_{la}$ Ab (affinity purified, diluted 1: 100) in perfused tissue (4% PFA). Staining was stronger in IPL, but background was high and no cell bodies are stained.
Figure 2.7

Immunostaining in chick retina with anti-human Mel19 Ab (affinity purified, diluted 1:100) using fresh tissue. Note the lack of signal with this antisera.
**Figure 2.8**

Immunostaining in chick retina with chick-specific Ab against chick Mel₁₆ (affinity purified, diluted 1: 50) using fresh tissue. Note the lack of staining.

**Figure 2.9**

Immunostaining in chick retina with chick-specific Ab against chick Mel₁₆ (affinity purified) using perfused (4% PFA) tissue. Only nonspecific staining increased.
Immunostaining in chick retina with crude, chick-specific antisera against chick Melₐ (diluted 1:100) using fresh tissue. Note the lack of specific signal. Staining was similar for the antisera against the other subtypes (not shown).
Figure 2.11

*In situ* hybridization in chick retina with DIG-labeled RNA a) antisense and b) sense probes for chick Mel14. Degradation of the reaction has darkened the appearance of the slides and precipitates from substrates speckle the slides. No specific reaction could be detected at this stage of optimization. INL= inner nuclear layer, IPL= inner plexiform layer, GCL= ganglion cell layer.
Figure 2.12

*In situ* hybridization in chick retina with a) antisense and b) sense DIG RNA probes for chick Mel1b. GCL staining is visible with both probes (though not clear in reproductions of figure 2.12 (b)).
Figure 2.12
**Figure 2.13**

*In situ* hybridization in chick retina with a) antisense and b) sense DIG RNA probes for chick Mel. GCL staining is strong in both sense and antisense and INL staining is visible. Arrows indicate cell bodies, likely amacrine cells, in the INL along the IPL boundary. Note that the INL staining is virtually absent in the sense slide. Tissues were extra-fixed (PFA (4%) for 5 hrs) after perfusion. Probe concentrations had been reduced by half to 1 μg/50 μl hybridization solution.
Figure 2.13
Figure 2.14

*In situ* hybridization in chick retina with a) antisense and b) sense DIG RNA probes for chick Mel18. Note the decrease in GCL staining in the sense slide and overall reduction in background.
Figure 2.14
Figure 2.15

*In situ* hybridization in chick retina with a) antisense and b) sense DIG RNA probes for chick Mel₁β. Additional adjustments to the protocol resulted in specific staining as can be seen by the virtual lack of signal in the sense slide. Final adjustments are reflected in the slides shown in Results.
**Figure 2.16**

Immunostaining in chick retina with monoclonal anti-GABA Ab (diluted 1: 50). Tissue was fixed in PFA (4%) for 1 hr prior to freezing. Tissue stained dark all over; impossible to determine specificity of signal.

**Figure 2.17**

Immunostaining in chick retina with monoclonal anti-serotonin Ab (diluted 1:5). Tissue was perfused with PFA (4%). An all-over, nonspecific reaction with high background covers the tissue.
**Figure 2.18**

Immunostaining in chick retina with monoclonal anti-substance P Ab (diluted 1:200). Tissue was perfused with PFA (4%). The antisera was not reactive in this tissue.

**Figure 2.19**

Immunostaining in chick retina with monoclonal anti-enkephalin Ab (diluted 1:100). Tissue was perfused with PFA (4%). Again, the antisera appears nonreactive in the tissue.
RESULTS

3.1. Cellular Localization of Melatonin Mel receptor 1st Hypothesis

3.1.1. Detection of melatonin receptor Mel presence in chick tissues by Western Blot

Autoradiographic studies showing melatonin binding in the inner plexiform layer of chick retina (Laitinen & Saavedra, 1990, and Dubocovich et al., 1989) was the basis of our first hypothesis, that actual melatonin receptors would be localized to the IPL of chick retinal tissue. To investigate the presence of melatonin Mel receptor, we performed Western Blots, using an IgG purified anti-human Mel Ab, in four chick tissues: retina, cerebellum (Cb), tectum and thalamus (figure 3.1). A clear, blockable band is visible at a molecular size of 37 kDa; the expected size for this receptor (Song et al., 1997). The antibody-antigen reaction is specific, as seen by the blocking of the signal with the human peptide in each of the four chick tissues (figure 3.2). All blots were performed in triplicate. Because there is 77% homology between the human Mel receptor peptide and the corresponding TIL3 region of the receptors for both chick subtypes Mel and Mel, Western Blots were done using the corresponding chick peptides to confirm the blocking of the signal. Specificity of the anti-human Mel Ab for the chick subtype Mel is strongly supported by the ability of this corresponding peptide to block the signal, and the absence of blocking after preincubation with the chick peptide for the Mel subtype (figures 3.3 & 3.4). Hence, these results suggest the presence of the chick Mel receptor subtype in all four of the chick tissues investigated. Additionally, these Western Blots suggest a specific immunoreaction between the human Mel Ab and the chick Mel receptor antigen.
All results shown were obtained with samples prepared by the TCA / urea-Triton method (final protocol), which had been diluted as described in Methods. Optimization of the Ab concentration was necessary during detection, and for the final urea-Triton samples, a low concentration of 1:4000 (3.4 μg of IgG pure Ab in 10 ml of blocking solution) was required for the signals observed; less than half of what had been needed for the PMSF/EDTA and N-P40 method.

3.1.(b) Specific localization of subtype Mel₁₈ in chick retina by immunocytochemistry

In order to specifically localize the melatonin Mel₁₈ receptor at the cellular level in chick retina, immunocytochemistry was performed using the anti-human Mel₁₈ Ab, which had been successful in detecting the melatonin receptor in the Western Blots. The Ab was affinity purified for the immunocytochemical investigations. As hypothesized, specific binding occurs exclusively in the IPL of the retina (figure 3.6 a & b). The IPL staining localizes the receptor to the dendrites of the target cells (reference figure 3.5). No cell bodies are visible in the staining. Background is low and the staining, which is distinct and continuous throughout both strata 2 and 4 of this retinal layer, is completely blocked by the human peptide (figure 3.6 c). Both chick peptides were capable of blocking the signal (figure 3.7 a & b). This differs from the Western Blot study using the same antiserum, in which only the Mel₁₈ signal was blocked. Ab was diluted 1:50 for this immunostaining, compared to 1:4000 in Western Blotting. All immunostaining was performed in triplicate. These results confirm the presence of receptor subtype Mel₁₈ in chick retina and support the hypothesis of IPL localization.
3.2. **Melatonin Receptor Subtype Mel₁β Expression (2nd Hypothesis)**

3.2.(a) *In situ* hybridization localizes chick Mel₁β mRNA expression to retina

The second objective of these investigations was to determine the presence of the melatonin receptor subtype Mel₁β in chick retina. Reppert et al (1995b) had reported the expression of chick subtypes Mel₁α and Mel₁c mRNA in chick retina. Furthermore, they had reported mammalian Mel₁β expression in mammalian retina (Reppert et al, 1995a). We had, therefore, hypothesized that the chick Mel₁β subtype would also be localized to retinal tissue. To investigate this, we used *in situ* hybridization in order to both detect receptor mRNA expression and to specifically localize the signal at the cellular level. For the *in situ* hybridization, a DIG labeled RNA probe for chick subtype Mel₁β was used, as described in Methods.

*In situ* hybridization reveals specific staining in distinct amacrine cells along the innermost boundary of the retina INL. As well, specific staining is seen throughout the GCL; implying that perhaps all ganglion cells are positive for the chick Mel₁β receptor subtype (figure 3.8 a & b). The specificity of the probe is confirmed by the absence of staining with the sense probe (figure 3.9). Low background in the absence of any probe confirms a specific Ab-DIG reaction in the detection phase (figure 3.10). The probes were purified (see Methods), and the tissue shown here had been extra-fixed after dissection.

The delicate nature of the tissue required a reduction in the amount of proteinase K; 0.5 µg/ml was used. A high probe concentration was needed to obtain a stronger signal (1 µg probe/ 50 µl hybridization solution). It was also necessary to eliminate an incubation of the slides in 0.2 N HCl, and all *in situ* hybridization shown had been prepared without this
step. Likewise, the treatment of RNase A was eliminated from the final protocol and no formamide was used in wash solutions during the processing of these tissue slides. Details of the optimizations are provided in Methods. These in situ hybridization studies support the second hypothesis, localizing chick Mel₁b to distinct retinal amacrine cells in the INL at the INL/IPL boundary, as well as to ganglion cells.

3.3. Colocalization with Dopaminergic Cells (3rd Hypothesis)

The third goal of these studies was to determine whether melatonin receptors colocalize with chick retinal dopaminergic cells, as melatonin inhibits dopamine synthesis in chick retina (Dubocovich, 1983). Dopamine is synthesized in amacrine cells at the INL/IPL boundary, the same retinal region where Reppert et al reported melatonin receptor Mel₁a and Mel₁c mRNA expression (1995b). We therefore hypothesized that melatonin receptors would colocalize with these dopaminergic amacrine cells. We used immunocytochemistry and antisera against tyrosine hydroxylase (T.H.) to identify dopaminergic cells. The chick retina was highly responsive to this antisera, as a dilution of 1:5000 was sufficient for results. Also, fixed tissue was used to ensure a well-preserved cell structure.

Specific binding occurs in a subset (or sets) of amacrine cells in the INL of the retina (figure 3.11 a). Staining occurs along the innermost region of this cell layer. Cell bodies are visible and dendrites stain along the INL/IPL boundary. The dendritic staining pattern is distinct from that of chick Mel₁a staining, which was localized to strata 2 and 4 of the IPL only (figure 3.11 b). Due to the lack of Mel₁a cell body staining with the human Mel₁a Ab, a lack of colocalization can only be postulated through dendrite patterns.
Additionally, this lack of colocalization refers only to the Mel1a receptor subtype. No colocalization studies using anti-T.H. Ab and *in situ* (for melatonin receptor positive cells) were performed.

Immunocytochemistry with antibodies to various known retinal neurotransmitters was also performed, without success. Antibodies to enkephalin, substance P, and neurotensin produced no staining at all or very high background (neurotensin). Notably, Abs to GABA and serotonin resulted in staining over the entire retina, leaving it impossible to determine either specificity or colocalization of these reactions (data shown only in Methods figures). No positive controls were performed for these neurotransmitters.

These investigations refute the hypothesis that a direct colocalization exists between the Mel1a receptor positive cells and retinal dopaminergic cells. However, the hypothesis is not fully investigated, with additional studies still required for all three chick receptor subtypes.
FIGURES FOR RESULTS
Figure 3.1

Western Blot of chick retina, cerebellum, tectum, and thalamus with Ab against human Mel₁₅ (IgG pure, diluted 1:4000). A specific band is observed in all four tissues at approximately 37 kDa, the molecular size of the melatonin receptor.
<table>
<thead>
<tr>
<th>Ret</th>
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<td>51 kDa</td>
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Figure 3.1
Figure 3.2

Peptide blocking of the Western Blot with human Mel\textsubscript{1a} peptide. The specificity of the Ab-antigen interaction was confirmed by preincubation with this peptide, resulting in a complete block of the 37 kDa band.
Figure 3.2

34 kDa -
Peptide blocking of the Western Blot with chick Mel₁₈ peptide. The specificity of the Ab to the chick Mel₁₈ receptor subtype was supported by this complete blocking of the signal after a preincubation of the Ab with this peptide. (Chick peptide blocking was not performed on thalamus.)
Figure 3.3
Figure 3.4

Lack of peptide blocking after preincubation of Ab with chick Mel₁₉ peptide. The inability of this peptide to block the band suggests specificity of the Ab for the chick Mel₁₉ peptide.
Figure 3.4
Figure 3.5

Chick Retina: Reference staining with hematoxylin and eosin. Uppermost part of the photograph is the outermost part of the retina. The cell layers are identified in a progression toward the GCL (ganglion cell layer), which borders the innermost part of the eye, the vitreous body.

PE= pigment epithelium, PRL= photoreceptor layer, ONL= outer nuclear layer, OPL= outer plexiform layer, INL= inner nuclear layer, IPL= inner plexiform layer, GCL= ganglion cell layer. Note that there are no cell bodies in either the outer or inner plexiform layers.
Figure 3.5
Immunostaining of chick retina with anti-human Mel, Ab (affinity purified, diluted 1:50) using fresh tissue. Specific binding occurred in what appears to be strata 2 and 4 of the IPL. Only processes (dendrites) could be stained; no cell body staining occurred in any of the cell layers. Magnification 112X.
Figure 3.6 (b)

Magnification of the chick retina immunostaining (magnification 200X).

Figure 3.6 (c)

Peptide blocking of the Ab with human Mel$_{1a}$ peptide. Preincubation of the anti-human Mel$_{1a}$ Ab with this peptide resulted in a complete block of the IPL immunostaining.
(Magnification 200)
Figure 3.6 (b & c)
Figure 3.7 (a)
Peptide blocking of the immunostaining with *chick* Mel₁₆ peptide. Preincubation of the Ab with chick receptor peptide Mel₁₆ also blocked the staining. (Mag 200X)

Figure 3.7 (b)
Peptide blocking of the immunostaining with *chick* Mel₁₆ peptide. Preincubation of the Ab with chick receptor peptide Mel₁₆ resulted in a block of the staining, in contrast with the Western Blot results (figure 3.4). (Mag 200X)
**Figure 3.8 (a)**

*In situ* hybridization in chick retina with the DIG-labeled, RNA antisense probe for chick Mel₁. Specific staining occurred in distinct amacrine cell bodies (arrows) along the innermost boundary of the INL. Specific staining is also strongly visible throughout the GCL, implying that perhaps all ganglion cells are Mel₁ receptor-positive. (Mag 112 X)

**Figure 3.8 (b)**

Magnification of the same. Arrowheads indicate cell body staining in the INL. Leftmost arrowhead marks what appears to be a cluster of cell bodies, visible also in 3.8 (a). (Mag 200 X)
Figure 3.8
**Figure 3.9**

*In situ* hybridization in chick retina with sense probe for Mel₁₅. Sense probe failed to elicit a signal, confirming the specificity of the antisense signal. (Mag 112 X)

**Figure 3.10**

*In situ* hybridization in chick retina without probe. *In situ* without any probes showed no background staining, and therefore confirmed the specificity of the anti-DIG Ab for the probes. (Mag 112 X)
Figure 3.11 (a)

Immunostaining in the chick retina with monoclonal anti-tyrosine hydroxylase (anti-T.H.) Ab (diluted 1:5000). Specific binding occurred in amacrine cells of the INL, revealing dopaminergic cells. Cells bodies stain dark brown with processes visible as linear staining along the INL/ IPL boundary (arrows). Note that the anti-T.H. staining reveals different processes than those revealed by the anti-MelA staining, implying distinct cell bodies. (Mag 200X)

Figure 3.11 (b)

Reprint of figure 3.6 (b) for comparison. Arrowheads show location of dopaminergic dendrites from 3.15 (a).
Figure 3.11
DISCUSSION

4.1. Cellular Localization of the Melatonin Mel₁₄ Receptor (1st Hypothesis)

4.1(a) Identification of melatonin receptors in chick tissue using Western Blots

The first hypothesis, that melatonin receptors would be localized to chick retinal IPL, had been based on previous evidence of melatonin binding in this region by autoradiographic studies (Laitinen & Saavedra, 1990, and Dubocovich, 1989). The use of Western Blots in these investigations provided the first support of this hypothesis. Using the human Mel₁₄ Ab to detect the presence of the chick melatonin receptor subtype Mel₁₄, we obtained preliminary evidence of this subtype's presence in chick retinal tissue. Final results were obtained from tissue samples that had been prepared by the TCA / urea-Triton protocol and, after optimization of this protocol, blots were very clear and the signals were reproducible. Chick retina, cerebellum, tectum, and thalamus all showed bands at the expected 37 kDa size; indicating the presence of the melatonin receptor in these tissues. Although these are the first Westerns to report the presence of melatonin receptors in chick tissue, there is serious doubt as to the validity of the observed signal. A signal was observed in each of the four chick tissues investigated, displaying a single band at 37 kDa, and indeed the specificity of the human Mel₁₄ Ab binding in these chick tissues is shown by the fact that the signal is clearly blockable with peptide in all tissues. However, protein loading concentrations, as determined by the Lowrey method (see Methods section) estimate protein loads as excessive and incredulous in producing specific, valid signals. Loads of greater than 300 μg’s are not likely to produce more than smearing or false
positive artifacts. Indeed, upon closer examination of the Western blots, faint bands appear near the 37 kDa band, producing an almost triple band appearance. This does indeed bring the specificity of the reaction into question as these faint bands are also blocked by the peptide and therefore cannot be ruled out as nonspecific. Negative controls would be necessary to confirm the Ab antigen reaction as specific and to strengthen the data supporting the first hypothesis. No negative controls were included in these studies, partly because all tissue revealed bands in the expected 37 kDa region (data not shown). However, further experiments to include negative controls are certainly required. Additionally, the need to further dilute the samples prior to loading is essential and will be interesting to see if the bands are preserved.

The binding of Ab with the receptor in these tissues was expected, as previous binding studies and autoradiographic investigations had already suggested their presence (as cited above). However, these previous studies could be, at best, speculations regarding the presence of an actual receptor through this indirect evidence. Reppert et al.'s work (1995b) with \textit{in situ} hybridization confirmed the presence of receptor mRNA expression for the chick subtypes \textit{Mel}_{ia} and \textit{Mel}_{ie} in these four regions (although \textit{Mel}_{ia} was not reported in Cb), but these Westerns still mark the first, if only preliminary, evidence of the actual, expressed receptor in chick.

4.1.(b) Specificity of the human \textit{Mel}_{ia} Ab for the chick \textit{Mel}_{ia} receptor

As mentioned in the Introduction, the human \textit{Mel}_{ia} Ab shows 77% homology to both chick subtypes \textit{Mel}_{ia} and \textit{Mel}_{ib}, but only 46% homology to chick subtype \textit{Mel}_{ie}. Consequently, the human \textit{Mel}_{ia} Ab could potentially cross react with either, or both, of
these chick subtypes. If the chick Ab trials had been successful, they would have easily identified the specific subtype(s) in these tissues, but unfortunately the task of receptor subtype discrimination falls to the human Ab alone.

Although the human Mel₁₈ peptide showed the Ab's specificity for the chick receptor by blocking the signal, specific subtype blocking with chick peptides helped to discriminate between chick Mel₁₈ and Mel₁₉. These chick peptides, used in order to block the human Ab, were not the same peptides that were used to block the original chick Abs, as the chick Abs were raised against a sequence within the TIL₂ region (2nd intracellular loop) of the receptor, and the human Ab recognizes a region of the TIL₃ (3rd intracellular loop). So chick peptides for the two subtypes were synthesized from the chick TIL₃ region, corresponding to the human TIL₃ region of the Mel₁₈ peptide.

As seen in the Results, only the chick peptide for the Mel₁₈ subtype blocked the human Ab. The lack of blocking by the chick Mel₁₉ peptide strongly suggests specificity of the human Ab for the chick Mel₁₈ subtype. Of course this does not rule out the possibility that Mel₁₉ is also localized to any or all of these tissues; it merely provides evidence for the presence of Mel₁₈ and the specificity of this Ab for that subtype. In situ hybridization and Northern blots would have to determine the presence of subtype Mel₁₉ as well as to localize the receptors at the cellular level. The first step in receptor localization, however, was achieved by further work with this human Mel₁₈ Ab and the use of immunocytochemistry, but the Western data has been valuable in confirming the presence of Mel₁₈ melatonin receptors in chick retina, Cb, tectum and thalamus, as well as determining the specificity of the reaction between Ab and antigen.
4.1.(c) Chick melatonin receptor localization by immunocytochemistry

In order to further investigate the presence of melatonin receptors in chick retinal tissue, as well as to specifically localize the receptor within the tissue, we used immunocytochemistry and the anti-human Mel, antisera which had been successful in the Western Blot investigations. As mentioned above, we hypothesized that melatonin receptors would specifically localize to the IPL of the chick retina based on the previously cited autoradiographic studies.

4.1.(c). i. A brief review of retinal structure

For the purpose of the following discussion, it is helpful to review the cell layers within retina (figure 3.5 in the Results section, page 110). Briefly, the top of the photograph represents the outermost layer of the retina; the bottom, the innermost layer, adjacent to the vitreous body. The pigment epithelium (PE) is the outermost region of the retina and appears as the darkest band at the figure's top end. The photoreceptor layer (PRL) appears next, with the cell bodies of the photoreceptor cells located in the outer nuclear layer (ONL). Photoreceptor cells synapse with horizontal and bipolar cells, whose cell bodies make up the outermost region of the inner nuclear layer (INL). The synapses occur in the outer plexiform layer (OPL), which is composed only of these dendritic processes. The bipolar and horizontal cells serve to transmit and modify neural information within the retina.

The inner nuclear layer also contains amacrine cells located mainly along the vitreal (innermost) region of the INL, as previously mentioned, which, along with the above
mentioned bipolar cells, synapse with ganglion cells (and with each other) in the inner plexiform layer (IPL). The ganglion cell bodies make up the innermost region of retina; the ganglion cell layer (GCL), and only their dendrites localize to the IPL, as their axons converge to form the optic nerve. Therefore, the IPL is composed solely of ganglion cell dendrites and their synapses with amacrine dendrites (these cells lack an axon) and the axons of bipolar cells which also project down into the IPL. Little is known about the precise functions of amacrine cells, or their various subsets, except for an apparent role in the transfer and modification of information between cells (see reviews by Luvone, 1986, Djamgoz et al, 1995, and Fawcett, 1986). As each cell type and even subset of cell types has its own role to play in the physiology of retinal tissue, and more is being learned about their functions almost every day, the potential for important information with an exact cellular melatonin receptor localization is enormous.

4.1.(d) Melatonin Mel₁₈ receptors localized to retinal IPL with human Mel₁₈-Ab

The final results in chick retina with immunostaining and the human Mel₁₈ Ab produces two very strong signals in the IPL. A magnification of the staining reveals that the bands appear to localize to strata 2 & 4 (out of 5 strata) of the IPL. The specificity of the Ab - antigen interaction is confirmed, as both bands are clearly blockable with the human peptide.

These results are the first localization of the receptor itself within chick retina. This data supports the findings of Laitinen & Saavedra (1990) who had reported autoradiographic binding of melatonin in the chick retinal IPL. However, only now is the receptor presence seen as two distinct bands, apparently localized to two separate strata
within this retinal layer. As the Western data in these investigations had suggested a chick Mel$_{1a}$ subtype specificity on the part of the Ab, and therefore suggest it is this subtype in the IPL, the same chick peptides were again used to confirm the specific blocking by this subtype.

However, although the chick Mel$_{1a}$ peptide does block the IPL signals, the signals are also blocked by the chick Mel$_{1b}$ peptide in the immunostained slides. This contradicts the subtype specificity findings of the Western blots.

4.1.(e) Possible explanations for Mel$_{1b}$ peptide blocking

There are a few possibilities that may explain this chick Mel$_{1b}$ peptide blocking of the human Mel$_{1a}$ Ab, and therefore explain the discrepancy between the Western blot and immunocytochemical peptide blocking. The first possibility is based on an initial presumption that the human antisera might actually be a mixture of two melatonin receptor specific Abs; one specific only for subtype Mel$_{1a}$ (call it Ab-A), and the other Ab recognizing either subtype 1a or 1b (call it Ab-AB). If this were the case, these Abs would bind competitively with the chick Mel$_{1a}$ peptide, resulting in the observed signal block, while Ab-A would not recognize the 1b peptide (which would block Ab-AB) and Ab-A would still bind to the Western blot. The reason for the complete block by chick Mel$_{1b}$ peptide might have been due to the fact that there was insufficient amounts of Ab-A to successfully produce a signal in the immunostained slide. However, the weakness of this explanation lies with the fact that the amount of human Ab used in these immunostaining experiments was, in fact, quite high (a 1:50 dilution), and this excessive amount should have been enough to elicit a signal despite the Ab-AB block.
A second explanation is derived from the differences in the human Ab used for the Westerns and immunostaining. The Western blots used IgG purified antisera, whereas the immunostaining used the highly specific, affinity purified antisera. The idea behind this explanation is based on a different presumption; that the human Ab would have been equally responsive to either chick Mel_{1a} or Mel_{1b} (call the antibody Ab-AB). Furthermore, if the less pure IgG fraction contained something nonspecific that could absorb the chick Mel_{1b} peptide, the Westerns would then show blocking with only the chick Mel_{1a} peptide, as the nonspecific element would have absorbed the 1b peptide and Ab-AB would still bind to the blot. Then, with the process of affinity purification, which uses the human Mel_{1a} peptide to isolate the Ab-AB, the nonspecific component would have been cleared from the antisera, leaving the Ab-AB to then be blocked by either peptide during the immunostaining.

This would seem to be at least a possible explanation for the data discrepancy, and could ultimately be tested by running Westerns with affinity purified antisera to see if the Western blots would now block with the chick Mel_{1b} peptide. Alternatively, the antisera itself could be affinity purified by using the chick peptides, instead of the human Mel_{1a} peptide, coupled to the sepharose in the purification column. Then, if the Ab does not recognize subtype Mel_{1b}, it would pass through the column (i.e. washed away) and no signal at all would be possible by the antisera with either technique. The major weakness with this second explanation is its dependence on the questionable presence of the nonspecific, Mel_{1b} peptide absorbing factor within the IgG pure antisera. All the same, the use of chick peptides in affinity purification of the human antisera might very well be a useful means of subtype specificity determination.

Finally, and likely the most reasonable explanation for the immunostaining blocking by Mel_{1b} peptide, is one based on the specificity of the Ab - antigen interaction. Even if the
Ab is highly specific for the chick Mel_{1a} subtype, and shows this specificity at the low Ab levels used in Western blots by selective blocking (Mel_{1a} only), the presence of excess Ab (used to elicit a signal with immunocytochemistry) might force a nonspecific reaction between the Ab and the chick Mel_{1b} peptide. As the chick Mel_{1a} peptide is able to block with either technique, we can safely assume this high specificity in the Ab - Mel_{1a} peptide binding, especially since the Western blots required so little Ab (a 1:4000 dilution). This would mean that on a hypothetical affinity binding curve, where the percentage of bound Ab is related to the concentration of Ab used (see figure 4.1 next page, as an example), the high specificity for the chick Mel_{1a} subtype would be reflected in a steep slope which begins even at low concentration (figure 4.1 a). The Ab’s affinity for chick Mel_{1b} would be much lower, and the nonspecific binding would follow a curve that was ‘right-shifted’ on the graph (figure 4.1 b); reflecting the fact that only much higher Ab levels could cause binding with this subtype. The Western blots would have, due to low levels of Ab (1:4000), occurred ‘further left’ on the curve, allowing the Ab to bind only Mel_{1a} receptor and therefore be blocked by the peptide. Immunostaining, with Ab dilutions of 1:50 would have ‘right shifted’ the curve to allow for the Mel_{1b} peptide to block the Ab. Note that a block by this peptide does not allow for speculation on the actual presence of Mel_{1b} in the tissue; this would simply be an Ab - peptide reaction.

This may best explain the observed peptide blocking, as one can expect nonspecific binding to Mel_{1b} under the excessive conditions used in immunostaining. The need to secure a signal with this technique required such high Ab amounts, and although still a blockable reaction, the Ab could no longer effectively discriminate between the somewhat similar subtypes. It is reasonable to expect, then, that lowering the Ab amounts would ultimately result in a more selective and specific reaction, with chick Mel_{1b} peptide eventually becoming incapable of Ab blocking. Titration experiments would ultimately
Mella
concentration of Ab

% Ab bound

Mella a

Mella b

concentration of Ab

Figure 4.1 Hypothetical Binding Affinity for the Human Mella Ab

Curves are theoretical only. Each curve shows a possible affinity of the Ab for the chick subtype shown. w = western blot Ab conc. i = immunostaining.
determine the level of Ab needed for selective binding. However, lowering the amount of Ab would result in an absolute loss of signal, so these titration experiments could be done by lowering the amount of peptide used to block the signal. If a gradual reduction in peptide amount result in a level at which both the human and chick Mel₁₆ peptide still block, but the chick Mel₁₆ peptide now ceases to block, the immunostaining would, like the Western blots, show a specificity of the Ab for the chick Mel₁₆ receptor subtype. These experiments have yet to be done, but would be of great interest in confirming (or even still refuting) the Ab specificity and, therefore, direct revelation of the Mel₁₆ receptor localization to the IPL of chick retina.

4.1.(f) Human Mel₁₆ Ab fails to elicit a signal in immunostaining studies

Even if the observed bands in the chick retinal IPL are confirmed to be Mel₁₆, this does not, as previously cautioned, deny the possibility of the presence of chick Mel₁₆ receptor in retina as well. Unfortunately, all attempts at immunostaining with the human Mel₁₆ Ab were unsuccessful, as there was a lack of reactivity with this antisera. A 46% amino acid sequence variation between human and chick for subtype Mel₁₆ is the most probable cause in the lack of staining. Therefore, a final attempt to localize chick Mel₁₆ in retina by immunostaining was performed with the chick specific Abs, despite the lack of results with Western blotting.
4.1.(g) Chick specific Abs fail to produce signals in immunostaining studies

Figure 2.8 from Methods shows the fresh chick retina with affinity purified chick Mel_1a Ab, as representative of the trials with all three chick subtype antisera. As seen in this figure, no staining is visible, and even 1:10 Ab dilutions were unsuccessful. As mentioned in Methods, poor immunogenic response, with a resultant low Ab titer in the antisera, is the likely cause of this lack of staining. Nonetheless, perfused retinal tissue was also tried (as was perfused brain) but this was also unsuccessful; only background increased. Even crude antisera (which may have had a higher Ab titer) was unable to evoke any signal in chick retina.

4.1.(h) Chick Mel_1a is localized to retinal IPL

These investigations provide evidence for the specific localization of the chick Mel_1a receptor subtype in the IPL of the chick retina. These results, therefore, support the first hypothesis and concur with autoradiographic evidence of previous research (Laitinen & Saavedra, 1990, and Dubocovich, 1989). This is the first direct evidence of melatonin receptor presence in the IPL and the first visualization of the two strata pattern of receptor distribution. The receptor subtype revealed in the immunocytochemical studies is most likely the chick Mel_1a subtype, as suggested by the lack of Mel_1b peptide blocking in Western Blots. However, as mentioned, further work is needed to confirm Ab specificity, due to the observed Mel_1b blocking in the immunostaining studies. Ultimately, these studies do reveal receptor presence in the IPL. As Reppert et al (1995b) had localized chick Mel_1a and Mel_1c mRNA expression to amacrine cells of the INL at the INL / IPL boundary, as
well as Mel₁₆ mRNA expression to the ganglion cell layer (GCL), regions which border the IPL, it is likely that the double strata staining pattern of the receptors may be due to dendrites projecting from cells in these layers into the IPL. Lack of cell body staining in these immunostaining studies prevent a direct identification of the melatonin receptor-positive cells responsible for the observed staining. However, we present here evidence of Mel₁₆ receptors in the chick retinal IPL, in agreement with the first hypothesis, as supported by the immunocytochemical investigations.

4.2. Melatonin Receptor Subtype Mel₁₆ Expression (2nd Hypothesis)

4.2.(a) Localization of chick Mel₁₆ mRNA expression in retina by in situ hybridization

The second objective of these investigations was to determine the presence of the melatonin receptor subtype Mel₁₆ in chick retina. As stated in the Introduction, little is known about the localization of this subtype in chick, and previous work in this lab had produced a clone of the chick Mel₁₆ receptor subtype (Liu et al., 1995). As Reppert et al. (1995b) had localized chick Mel₁₆ and Mel₁₄ mRNA expression to the INL and GCL (Mel₁₄), and had shown mammalian Mel₁₆ to localize to retina (Reppert et al., 1995a), we hypothesized that chick Mel₁₆ would also localize to retinal tissue. We investigated this hypothesis through the use of chick specific antibodies and immunocytochemistry, which would localize the individual subtypes within the retina. These investigations, as discussed in the above section, were not successful due to low antisera titer. We further investigated this hypothesis by in situ hybridization, using the above-mentioned Mel₁₆ clone to produce
a DIG labeled RNA probe. This technique reveals mRNA expression in tissues and identifies the actual cell types which express the Mel1b receptor.

As seen in figure 3.8 (a & b), specific in situ staining with the chick Mel1b antisense probe reveals mRNA expression in chick retina. This is the first report of chick Mel1b localization in this tissue. This data also establishes the presence of all three receptor subtypes in chick retina, as the Mel1s and Mel1e subtypes had been previously reported by Reppert et al (1995b). The lack of signal with the sense probe confirms the specificity of the antisense signal. Interestingly, the pattern of mRNA expression for Mel1b is similar to that previously reported for Mel1s and Mel1e. That is, Mel1b positive cells are continuous throughout the GCL and intermittent along the INL at the IPL boundary. This suggests that all ganglion cells are expressing this subtype, while only a subset of amacrine cells are Mel1b positive. The INL staining is most likely amacrine cells, due to the proximity to the INL/IPL boundary; characteristic to this cell type. (Bipolar cells have their soma localized more in the center of the INL and horizontal cells are even further outward; along the INL / OPL layer.)

It appears that the Mel1b in situ staining may, in fact, be clusters of cell bodies. Small groups containing four or five cells of one (or more) subsets of amacrine cells appear sporadically dispersed along this vitreal region of the INL. Reppert's data with Mel1s and Mel1e was not repeated in these studies beyond preliminary stages, and 'clustering' of the cells was therefore not detectable with these subtypes (nor is it mentioned in the work by Reppert et al, 1995b). The reason for cell clustering in the signal positive areas is unknown, but may be due to cell-cell interactions of one or more amacrine subsets.

Human retina also expresses the Mel1b receptor subtype, as shown by RT-PCR (Reppert et al, 1995a). The fact that chick expresses all three subtypes in retinal tissue, and in a similar, if not exact, distribution is curious. If further colocalization studies reveal that
all three subtypes localize to the same cells, one explanation may be that the subtypes perform a similar functional role. If so, redundancy in function may have resulted in the loss of the Mel₁酶 subtype in higher vertebrates as the dependency on melatonin decreased. However, differences in Mel₁₉ and Mel₁₆ distribution in chick brain as reported by Reppert et al (1995b), suggest otherwise. If these subtypes have specific roles within distinct brain regions, perhaps these specific roles are also performed in retina within the same target cells. Subtypes may be localized to different regions of the cell, one subtype to dendrites while another expresses only on cell bodies. Also, the different subtypes could be responsible for different signal transduction pathways. The amacrine and ganglion cells may serve as relay stations for the multiple messages conveyed by the receptor subtypes.

Ultimately, the in situ hybridization with the Mel₁₆ RNA probe supports the hypothesis that this subtype localizes to retinal tissue in chick and provides the first evidence of this in this species, and the pattern of distribution. It also, for the first time, establishes the presence of all three subtypes in chick retina. The fact that ganglion cells express melatonin receptors is now certain, and the specific amacrine cell subset(s) positive for melatonin receptors is likely soon to be identified.

4.2.(b) Mel₁₆ mRNA expression in chick

The second hypothesis was supported by the findings of the in situ hybridization studies. The Mel₁₆ melatonin receptor subtype is expressed in chick retina, and localizes to the same regions as Mel₁₉ and Mel₁₆, as reported by Reppert et al (1995b). Further studies are required to specifically localize the Mel₁₆ receptor in brain, and functional studies are needed to elucidate the role of each melatonin receptor subtype.
The similar expression pattern in chick retina between the receptor subtypes suggests possible cellular colocalization. Further studies are needed to investigate this possibility. However, these investigations have provided important information on Mel₁₈ expression in chick retina and establish the presence of all three receptor subtypes in this tissue.

4.3. Colocalization with Dopaminergic Cells (3rd Hypothesis)

4.3.(a) Colocalization studies and the melatonin Mel₁₈ receptor

The third goal of these investigations was to determine whether melatonin receptors colocalize with chick retinal dopaminergic cells. The human Mel₁₈ Ab was able to localize the melatonin receptor to the retinal IPL by immunocytochemistry, but the question still remains regarding which cell type expresses this receptor. As previously mentioned in the Introduction, and briefly reviewed in this Discussion, three retinal cell types are known to extend dendrites (or bipolar cell axons) into the IPL; bipolar and amacrine cells from the INL, and ganglion cells from the GCL. Additionally, dopaminergic cells, which are directly or indirectly inhibited by melatonin (Dubocovich, 1983), are localized to a subset of amacrine cells at the IPL/INL boundary. Reppert et al (1995b) had also localized chick Mel₁₈ and Mel₁₆ mRNA to cells in this region, as well as Mel₁₈ expression in the GCL, using radiolabeled probes and in situ hybridization. It is very probable that the immunostained bands seen in these studies are revealing melatonin receptors in the dendrites from these cells. The localization to the boundary of the INL/ IPL as reported by Reppert is characteristic of amacrine cells. It is possible that the melatonin receptor positive
cells are the same amacrine cells that are dopaminergic. We hypothesized that melatonin receptors would colocalize with chick retinal dopaminergic cells.

We used anti-Tyrosine Hydroxylase antisera and immunocytochemistry to identify the dopaminergic cells, and then compare the staining patterns with the melatonin receptor immunostaining. Abs against the dopamine biosynthesis enzyme, tyrosine hydroxylase (T.H.), reacted well with chick tissue, and results show that cell bodies stained strongly, marking the dopaminergic cells. Their dispersed staining pattern implies that only a subset of amacrine cells are dopamine positive. Furthermore, it is possible to see the dendrites faintly stained just along the INL/ IPL boundary in this figure (figure 3.11). Compared to the melatonin Mel, receptor staining, which clearly stains deep in the IPL (strata 2 & 4), there does not appear to be a colocalization between the two.

If the Ab recognizing the melatonin receptor is specific for subtype Mel, only, it is still possible that either Mel, or Mel, could colocalize with dopaminergic cells based on the fact that Reppert et al (1995b) had reported this subtype’s expression in the INL, and based on the findings of Mel, expression in retina from these studies. A combination of in situ hybridization, to reveal melatonin receptor positive cells, and immunocytochemistry, to reveal the dopaminergic cells on the same slide, would ultimately reveal any direct interaction between the two. Furthermore, it may show a specific subtype to be solely responsible for the effect on dopamine, supporting different functional roles for the melatonin receptor subtypes. At the end of this project, these experiments had not yet been done, and leave some exciting experiments for future work.
4.3.(b) Indirect dopamine inhibition studies

A direct melatonin - dopamine interaction is not the only possibility. Alternatively, the other subtypes may also, eventually, be reported as not colocalizing with the dopamine cells, arguing for an indirect melatonin effect. GABA-containing amacrine cells have also been implicated in the inhibition of dopamine release, with this inhibition being restricted to darkness (Morgan & Kamp, 1980). As suggested in the Introduction, GABA's inhibition of dopamine could be regulated by melatonin. A colocalization between melatonin receptors and GABAergic cells would support the alternative hypothesis that melatonin exerts its dopamine inhibition through this neurotransmitter. Kalloniatis and Fletcher (1993), among others, localized GABAergic cells to amacrine cells along the INL/IPL boundary as well as in the GCL. If melatonin receptors colocalize to these cells, it would explain the melatonin receptor immunostaining pattern in the IPL. One band could originate from GABAergic amacrine cells, while the other could stem from the GABAergic ganglion cells. However, the lack of success with these GABA staining trials leaves this question unanswered. The tissue used in these experiments had been fixed for 1 hour in 4% PFA and an anti-GABA antibody was applied as a probe. This was based on successful staining by Reis et al, 1997, and Agardh et al, 1987, in chicken retina. It was since recommended that an antibody to GABA's biosynthetic enzyme, GAD, would likely be more successful, in combination with retina fixed strongly by glutaraldehyde. These experiments remain to be performed. And just as for the dopaminergic cells, immunostaining with antisera against GAD, for the GABAergic cells, in combination with the in situ hybridization for melatonin receptor mRNA, would reveal any colocalization between these two messengers.

Poor results were also obtained with the anti-serotonin Ab, as described in Methods. Although serotonin is the precursor to melatonin, the latter's synthesis appears to
be localized to photoreceptor cells alone (Pang et al, 1977). However, serotonin itself is also expressed in amacrine cells without any apparent melatonin precursor role here (Thomas et al, 1993). The amine may behave as a neuromodulator in amacrine cells, although conclusive evidence is still lacking, and therefore a possible melatonin - serotonin interaction was investigated. Only nonspecific background was obtained with the anti-serotonin Ab. Even extra-fixation with paraformaldehyde after perfusion did not elicit a signal (although Ab was excessive at 1:5 dilution). The tissue preparation protocol was per Cuello et al, 1979, Millar et al, 1987, and Karten & Brecha, 1980, with specific staining of serotonin and substance P in chick retina. Further work is required to establish staining, and therefore, any possible colocalization with this amine.

Troubles with antisera reactivity in chick tissue continued with the other neurotransmitters. Antisera against substance P, VIP, enkephalin, neurotensin, and choline acetyltransferase (for acetylcholine) all failed to produce a detectable signal in the retina. All of these neurotransmitters are known to localize to amacrine cells in chick retina (Kalloniatis & Fletcher, 1993, and Stell et al, 1980). Previous studies have shown substance P, VIP, and acetylcholine with a stratified dendritic staining pattern in the IPL (Wassle & Boycott, 1991, and Stell et al, 1980), similar to the stratified immunostaining of the melatonin Mel₁α receptor from these studies. Additionally, enkephalin also displays two strata of dendrites in the IPL, and Dubocovich and Weiner (1983) showed that enkephalin analogs can inhibit dopamine release from isolated rabbit retina. After the failure of the anti-enkephalin antisera in these studies, anti-neurotensin was used in an attempt to reveal the enkephalin cells in our tissue, as the two neurotransmitters are known to colocalize in chick retinal amacrine cells (Watt & Florack, 1994). However, it is suspected that poor species cross reactivity was the reason for the lack of staining with these antisera. All of the mostly monoclonal antisera had been raised in mammals, yet as they are ubiquitous
neurotransmitters, cross reactivity can be expected in all species. The only exceptions
would be antisera against the enzymes choline acetyltransferase and tyrosine hydroxylase,
which might differ between species in their antigen peptide sequences. The antisera against
choline acetyl transferase was raised in mice against porcine brain enzyme extracts which
may have had high antigen sequence variation from chick. However, antibodies against
tyrosine hydroxylase were also raised in mice, against a rat T.H. immunogen and were
successful in staining. Failure to obtain specific reaction with the antibodies against the
other neurotransmitters was most likely due to fixation problems with the chick tissue.
Stronger fixation may be required in order for any of these antibodies to reveal specific
staining. Alterations in the tissue fixation protocol remain to be done in order to identify
any colocalization between melatonin and these neurotransmitters.

4.3.(c) Lack of colocalization between dopaminergic cells and \( \text{Mel}_{1\alpha} \) receptors

This preliminary evidence suggests that there is no direct interaction between
melatonin \( \text{Mel}_{1\alpha} \) receptors and dopaminergic cells. This refutes the hypothesis of revealing
melatonin receptors on dopaminergic cell dendrites for this receptor subtype. Further
colocalization studies are still needed to corroborate this. Both subtypes \( \text{Mel}_{1\beta} \) and \( \text{Mel}_{1\epsilon} \)
still need to be investigated for a possible direct link with dopamine, which will be
elucidated with further \textit{in situ} hybridization work and anti-tyrosine hydroxylase staining.
Therefore, the third hypothesis remains to be investigated further, and no final conclusion
regarding colocalization can yet be made. Also, the investigations into the use of an
intermediate neurotransmitter to inhibit dopamine have only just begun. There are an estimated 30 different subsets of amacrine cells in this region of INL (Wassle & Boycott, 1991) and so discriminating the melatonin positive amacrine cells will be very interesting work. However, the first step has been taken with melatonin Mel₂ receptor immunostaining in these studies.

4.4. Future Work

These investigations have resulted in some valuable information regarding the expression of the Mel₂ receptor subtype in chick retina, as well as providing insight into the localization of Mel₂ receptors in the IPL of retina. However, there remains a great deal of research to be performed. The specificity of the anti-human Mel₂ antibody for the chick Mel₂ subtype can be reasonably argued from the lack of chick Mel₂ peptide blocking in Western Blots and the explanation provided for its blocking in the immunostaining. Still, this specificity needs to be confirmed by the peptide titration studies mentioned in section 4.1.(e) of the Discussion. Specificity could also be established through affinity purification of the anti-human Mel₂ antisera using the chick peptides in the purification column.

The colocalization studies also present a large body of work yet to be accomplished. The use of in situ hybridization, now optimized in chick retina for DIG-labeled probes, in combination with the anti-T.H. immunostaining for dopaminergic neurons may establish or refute a colocalization with any of the three chick receptor subtypes. Additionally, these double staining studies need to be performed with the other potential neurotransmitters. It
may be possible to determine colocalization between the three receptor subtypes by using fluorescence microscopy and labeling each subtype with a different colour marker.

*In situ* hybridization studies also need to be performed in chick brain with the chick Mel₁b RNA probe which would be the first such study in chick brain with this subtype. Finally, it will be of great interest to begin functional studies on these receptors. Subtype selective antagonists have been developed for mammalian melatonin receptors (Dubocovich et al, 1997, and Dubocovich et al, 1998) and are currently being used to investigate functional roles for the subtypes in mice and rabbits. The development of subtype selective antagonists for chick would be invaluable in assessing receptor function for each subtype. These experiments are all of great interest to the understanding of melatonin function in all target tissues, and therefore the animal as a whole. With such knowledge, a better understanding of melatonin function would be of great value in the care of livestock and for therapeutic use in humans.

4.5. **Final Conclusions on the Findings of these Studies**

In order to better comprehend the functional role of melatonin in tissues in general, and in target cells in particular, a specific localization of the melatonin receptor is a vital first step. Additionally, now that it is known that the melatonin receptor is expressed as three distinct subtypes in avian species, the individual localization for each subtype becomes of great interest. These experiments were successful in advancing the knowledge of receptor localization and thereby contributing to a more complete picture of melatonin function.

The pattern of melatonin Mel₁b receptor immunostaining in the chick retina confirms the autoradiographic binding reported by Laitinen & Saavedra (1990).
immunostaining reveals two strata of continuous signal in the IPL. But this work does not confine the staining only to this subtype and leaves open the possibility that the other two subtypes may also display this receptor localization, based on the similarity in mRNA expression (Reppert et al, 1995b).

The in situ hybridization studies performed in these investigations establishes Mel₁₆ expression in chick retina, and therefore confirms the hypothesis that all three subtypes are expressed in chick retina. The obvious next step is to investigate the function of each subtype in this tissue. This will first be achieved by colocalization studies as described for dopaminergic cell studies as well as possible interactions with other neurotransmitters.

Preliminary evidence from the colocalization studies appears to refute the third hypothesis with regards to the Mel₁₆ subtype, as the immunostaining did not appear to colocalize with dopaminergic cells. However, immunostaining of tyrosine hydroxylase in combination with in situ hybridization of Mel₁₆ (on the same slide) is still needed to corroborate this. It is possible that a subtype other than the Mel₁₆ may colocalize with the dopaminergic cells, leaving this hypothesis still open to investigation. In fact, it is quite possible that the melatonin receptor subtypes may show diverse expression patterns in various amacrine cell subpopulations. One subtype may colocalize to more than one type of amacrine cell in the INL, and may even show partial or occasional colocalization with the other melatonin receptor subtypes. All three receptor subtypes are of high affinity, and act to inhibit cAMP through a G-protein coupled reaction. This does not, however, restrict the resultant signal to the same function in the cell. Different effects of this cAMP inhibition may occur downstream in the intracellular signal pathway with each subtype, triggered by the deviation in the receptor's structure. This may be the case with ganglion cells, as all three subtypes appear to be expressed throughout this retinal layer. Furthermore, even if each receptor subtype does perform a similar role within a target cell, there may be
variations in the efficiency or strength of each subtype's effect in that cell. Ultimately, once
the precise amacrine cells positive for the receptors are known, cellular functional studies
can be performed to establish the role of the melatonin hormone in these cells. In vitro
studies may be helpful with the use of receptor specific antagonists.

Little is known about the function of ganglion cells, other than relaying retinal
information to the brain. Dendrites from these cells synapse with amacrine cells in the IPL.
The more vitreal stratum of immunostaining may be receptors on ganglion cell dendrites.
Retinal information regarding photoperiod may be conveyed to the brain by melatonin
binding at these sites. Synapses with dopaminergic amacrine cells may be a possible route
of inhibiting the melatonin message in these cells during the day. Alternatively, ganglion
cells may also assist in the inhibition of dopamine. GABA has been localized to ganglion
cells in the chicken retina (Kalloniatis & Fletcher, 1993) and could be released as
melatonin's indirect modulator of dopamine. Antagonists against melatonin could elucidate
any possible melatonin - GABA coupling in dopamine inhibition.

Despite the difficulties encountered during the optimization of the techniques, some
valuable information has been acquired from these studies. Receptor subtype Mel₁₉
localizes to the INL and GCL of chick retina. The Mel₁₉ subtype does not appear to
colocalize with dopaminergic amacrine cells, providing some support to an indirect route of
inhibition. Although further work is needed to finally elucidate the role of the melatonin
hormone at the cellular level and to discriminate between receptor subtype functions, these
investigations provide important information on Mel₁₉ receptor localization and novel
evidence of Mel₁₉ expression in chick retina.
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