Cellular Mechanisms of Melatonin Action

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Vanček, Jiri. Cellular Mechanisms of Melatonin Action. Physiol. Rev. 78: 687–721, 1998.—The pineal hormone melatonin is involved in photic regulations of various kinds, including adaptation to light intensity, daily changes of light and darkness, and seasonal changes of photoperiod lengths. The melatonin effects are mediated by the specific high-affinity receptors localized on plasma membrane and coupled to GTP-binding protein. Two different G proteins coupled to the melatonin receptors have been described, one sensitive to pertussis toxin and the other sensitive to cholera toxin. On the basis of the molecular structure, three subtypes of the melatonin receptors have been described: Mel1A, Mel1B, and Mel1C. The first two subtypes are found in mammals and may be distinguished pharmacologically using selective antagonists. Melatonin receptor regulates several second messengers: cAMP, cGMP, diacylglycerol, inositol trisphosphate, arachidonic acid, and intracellular Ca2+ concentration ([Ca2+]i). In many cases, its effect is inhibitory and requires previous activation of the cell by a stimulatory agent. Melatonin inhibits cAMP accumulation in most of the cells examined, but the indole effects on other messengers have been often observed only in one type of the cells or tissue, until now. Melatonin also regulates the transcription factors, namely, phosphorylation of cAMP-responsive element binding protein and expression of c-Fos. Molecular mechanisms of the melatonin effects are not clear but may involve at least two parallel transduction pathways, one inhibiting adenylyl cyclase and the other regulating phospholipide metabolism and [Ca2+]i.

I. INTRODUCTION

Most of the effects of the pineal hormone melatonin in vertebrates are connected with light, or rather with its absence, in one way or the other. Melatonin is directly involved in adaptation of the retinal photoreceptors and skin melanophores to the changing intensity of ambient light. In the melanophores, melatonin induces rapid aggregation of melanin-containing granules in perinuclear zone, which results in blanching of the cell (176, 178, 281).
the retina, melatonin mediates the adaptation of the photoreceptor to the ambient light intensity, causing cone photoreceptor elongation and rod photoreceptor contraction, aggregation of melanosome granules within retinal pigment epithelium, and change of sensitivity of horizontal cells to light (20, 77, 207, 252, 389).

In addition to mediating the direct effects of light, melatonin is also involved in the entrainment of circadian and seasonal rhythms by the light-dark cycle. Circadian rhythms are driven by endogenous oscillator and are sustained in constant conditions, i.e., in constant darkness and stable ambient temperature (254, 255). However, to run in phase with the external geophysical cycle, the rhythms need continuous entrainment, with the most important being photic entrainment by the light-dark cycle. In mammals, melatonin is involved in the entrainment of the endogenous clock and the overt circadian rhythms (52, 259). In nonmammalian vertebrates, the melatonin rhythm is necessary for the clock function; in several species, pinealectomy results in attenuation of free-running daily rhythms, and rhythmic melatonin infusions restore the rhythmicity (49, 100, 120, 344, 412, 413).

The most important role of melatonin in mammals is regulation of seasonal rhythms (30, 46, 47, 105, 343). In many species, melatonin has been shown to regulate the seasonal cycles of reproduction, fasting, thermoregulation, and hibernation (30, 46, 47, 105, 343). In some species, the pattern of the melatonin signal seems to drive the seasonal changes, whereas in others, the annual rhythms are endogenous (circannual) and are synchronized with the outside changes by the pattern of melatonin secretion (29, 127, 129, 262).

This review is focused on mechanisms of melatonin action in the cells that may mediate the above effects of melatonin. The distribution and features of the membrane-bound melatonin receptors are described. Recently cloned nuclear RZRb receptors are not included, because the evidence that they bind melatonin is not quite convincing (17, 308, 390). The melatonin effects on second and third messengers and the mechanisms of transduction of the melatonin signal in the cells are reviewed in detail. Finally, the attributes of the melatonin rhythm that may serve as a signal triggering the photoperiodic changes are discussed.

A. Daily Rhythm of Melatonin Synthesis and Its Modulation by Photoperiod

Melatonin synthesis in the pineal gland is controlled by light. Melatonin is synthesized from serotonin by a two-step pathway involving N-acetylation and O-methylation. The first step is catalyzed by the enzyme arylalkylamine N-acetyltransferase (NAT; EC 2.3.1.87) and the second by hydroxyindole-O-methyltransferase (EC 2.1.1.4) (8, 145, 165, 374). Synthesis of melatonin shows marked daily rhythm with low values during the day and large increase at night (163, 391). The rhythm is driven by the rhythm in NAT activity; it is endogenous, i.e., continues in animals kept in constant darkness with circadian period close to 24 h (163). The nocturnal melatonin increase is, however, abolished or substantially reduced in animals maintained in constant light. In animals exposed to light during the night, even to a short light pulse, melatonin synthesis is inhibited, and melatonin concentration declines rapidly (140, 164, 356). In addition to the immediate suppressing effect, the exposure to light at night shifts the phase of the rhythm of melatonin synthesis (142). The light-dark cycle thus entrains the rhythm of melatonin synthesis, as true of other circadian rhythms (254).

In all species, the melatonin increase occurs during the night, regardless of whether they are day active or night active. This makes melatonin rhythm an endocrine marker of night. Duration of the melatonin increase is controlled by photoperiod. On long photoperiods, such as in summer, duration of the melatonin increase is short, and on short photoperiods, the melatonin pulse is long (Fig. 1; Refs. 29, 138, 139, 141, 202). Although extension of the melatonin pulse after transfer of animals from long to short photoperiods is gradual, and it may take several days or even weeks to reach new steady state, the compression of the rhythm on long daylength is instantaneous (139, 141, 143, 144). The photoperiodic regulation of the melatonin rhythm makes the pattern of the hormone concentration an endocrine calendar. The target organs are informed of the year by the changing length of the melatonin pulse.

![FIG. 1. Rhythm of pineal melatonin concentration in Djungarian hamsters kept on short [light/dark (LD) 8:16] and long (LD 16:8) photoperiods. Horizontal bars indicate dark periods.](http://physrev.physiology.org/Downloadedfrom)
Apart from the pineal gland, melatonin is synthesized also in the vertebrate retina and Harderian gland, and its synthesis in these organs also shows daily rhythm (148, 251, 265, 346). Although the retina has high capacity to synthesize melatonin, it does not seem to contribute significantly to the plasma melatonin rhythm, because after pinealectomy, the amplitude of the melatonin rhythm is reduced by 60–100% (31, 66, 270, 342, 370). This is probably because of rapid catabolism of melatonin in the retina: it is deacetylated to 5-methoxytryptamine by the enzyme aryl-acylamidase (41, 113). Melatonin synthesized in the retina thus serves mainly the local purposes (see sect. 1B2).

Melatonin is a lipophilic compound and, as such, freely diffuses through biological membranes and readily crosses the hematooencephalic barrier. Therefore, the release of melatonin from the pinealocyte, where it is produced, does not seem to require any specialized mechanism. Melatonin concentration in circulating blood closely reflects the changes of pineal melatonin concentration (136, 391). Also, the indole concentration in cerebrospinal fluid reflects the changes in pineal and plasma (38, 267, 269, 369). However, in some species, higher concentrations of melatonin in cerebrospinal fluid than in blood have been found (124).

The half-life of melatonin in circulation is ~10 min (136, 347, 403). Melatonin is metabolized primarily in the liver by hydroxylation to 6-hydroxymelatonin, which is then converted to a sulfate or a glucuronide (166). Alternatively, melatonin is deacetylated to 5-methoxytryptamine, which is deaminated to 5-methoxyindoleacetic acid and 5-methoxytryptophol (41, 113, 280). This pathway is the most important in the retina, but it occurs also in liver. In the brain, choroid plexus, and pineal, melatonin is metabolized by indoleamine 2,3-dioxygenase to l-kynurenine by cleavage of the indole ring (98).

B. Endocrine Effects of Melatonin

1. Light adaptation in dermal melanophores

One of the best-characterized and the most-studied effects of melatonin is contraction of dermal melanophores in amphibians and fishes as adaptation to decreasing intensity of ambient light. Melanophores are the melanin-containing chromatofores that appear black. In adult amphibians, they represent a relatively small percentage of the total chromatofore population, but a much higher relative number is found in early developmental stages, in young tadpoles (11, 12, 96).

In the dermal melanophores, light may induce rapid pigment changes either through the direct action on light-responsive melanophores or indirectly by inducing hormonal changes that subsequently affect the melanophore appearance (13, 95, 281, 376). The pituitary hormone melano-cyte-stimulating hormone (MSH) mediates the light-induced dispersion of the pigment granules (376). Pinal hormone melatonin induces rapid aggregation of melanin-containing pigment granules in perinuclear zone, which results in blanching of the cell (178, 206). After exposure to light, the melatonin concentration declines, resulting in dispersion of the pigment granules and darkening of the cell. The physiological significance of the pigment changes seems to involve protection of deeper tissues from harmful radiation of the sun. Similar regulation of pigment movement by melatonin as in amphibians has been shown in fishes and some reptiles (26, 97, 157). No rapid effect of melatonin on pigmentation in mammals has been reported, although chronic melatonin administration has been shown to cause the change of the fur color in Djungarian hamsters (130). However, these pelage changes are because of the melatonin-induced decrease of prolactin levels rather than to direct action of melatonin on the follicle (86).

Melanosome movement is because of the changes in the melanophore cytoskeleton architecture and involves phosphorylation/dephosphorylation of specific proteins (188, 281, 284, 336). Dispersion of the pigment granules seems to be mediated by kinesin, the microtubule-dependent ATPase (279), and dynein is probably responsible for the aggregation (18, 64, 247).

2. Light adaptation in retina

Visual perception has remarkable dynamic range of sensitivity. This is enabled by multilevel regulation of visual sensitivity depending on the intensity of ambient lighting. Melatonin is involved in the regulation of light sensitivity, and its effects occur on several levels. One of the most prominent effects of melatonin is photomechanical movement, cone photoreceptor elongation and rod photoreceptor contraction, occurring in the retina of non-mammalian vertebrates as the adaptation to decreasing light intensity (207, 252, 309). The opposite photomechanical movements occur in response to increasing light intensity and may be mimicked by dopamine (309). The dopamine release in the retina increases after light exposure and decreases in darkness (35, 159). Melatonin suppresses the light-evoked dopamine release from retina, which may represent one of the mechanisms of melatonin effect on photoreceptor adaptation to darkness (77).

Melatonin, synthesized in the retina, is thought to participate in modulating cyclic day-night variations in photoreceptor and retinal pigment epithelium (RPE) function. Melatonin regulates the amount of light reaching the photoreceptor by controlling the movement of melanosome granules within the RPE (160, 167, 250). Melatonin has also been shown to alter the electrical activity of the RPE of the mammalian eye (232, 335).

In addition, melatonin is also involved in the regula-
tions of sensitivity of the horizontal cells to light (389, 397). The horizontal cells receive inputs from both cones and rods. During dark adaptation, rod input is favored, whereas light adaptation leads to the suppression of rod input and enhancement of cone input (21). Dopamine mimics the effect of light, whereas melatonin mimics the effect of darkness.

Finally, melatonin regulates the disk shedding and phagocytosis (20). Disk shedding by rod outer segments (ROS) is a degradative process balancing continuous assembly of the ROS disks and stabilizing ROS length. The complex process includes detachment of disks from ROS, their recognition by adjacent RPE, and their phagocytosis and degradation within RPE. Disk shedding is evoked by light; however, transition to light after several hours of dark adaptation activates disk shedding substantially more than long-term light exposure. Melatonin mimics the effect of darkness, because it activates the light-evoked disk shedding. However, a high concentration of melatonin is required for the effect.

3. Entrainment of daily rhythms

Melatonin is involved in synchronization of circadian rhythms. The effect is most prominent in birds and reptiles, where the pineal is an integral part of the circadian system (100, 330, 341). In these species, the rhythm of melatonin synthesis is driven by intrinsic pineal pacemaker and continues even after complete isolation of the gland in vitro (27, 72, 73, 174, 213, 328, 329). Moreover, their pinealocytes are light sensitive, and therefore the light-dark cycle directly entrains the melatonin rhythm (73, 213, 328). In passerine bird and iguanid lizard species, pinealocyte results in arrhythmic behavior, suggesting that the pineal pacemaker has the vital importance (25, 100, 119, 121, 330, 341). In the pinealectomized birds, implantation of the pineal into the anterior chamber of the eye results in reappearance of the melatonin rhythm; consequently, the daily rhythmicity of locomotor activity is restored and the rhythm continues with the phase of the donor of the pineal (412, 413). In addition, melatonin injections have been shown to entrain the activity-rest rhythm in iguanid lizards and passerine and columbiform birds (58, 120, 344).

In mammals, the circadian rhythms persist in pinealectomized animals without obvious interference. Nevertheless, melatonin has been shown to entrain the free-running circadian rhythms in mammals. In constant conditions, e.g., in continuous darkness, the endogenous rhythms run with their intrinsic circadian period (i.e., somewhat shorter or longer than 24 h), and therefore they occur every day a little phase shifted as compared with the geophysical cycle (254). Melatonin administered at 24-h periods synchronizes the free-running rhythms in rats so that the time of melatonin injection is locked to the beginning of subjective night (6, 51, 70, 259). The entrainment corresponds with the phase-response curve for melatonin; in this species, the phase shifts are induced only when melatonin is administered during a narrow window between circadian time (CT) 10 and CT 12, resulting always in phase advances (6, 7).

In humans, however, both phase delays and phase advances have been described after morning and afternoon administration of melatonin (179). The melatonin phase-response curve in humans is a mirror image of the light-induced response curve (180). Melatonin has been shown to phase shift the rhythms of sleep-wake cycle, body temperature cycle, and plasma melatonin onset (5, 71, 179). Because acute melatonin administration decreased and suppression of the endogenous melatonin increase attenuated the nocturnal decline of the core body temperature, it has been suggested that acute suppression of the core temperature may be a primary event of the phase-shifting mechanism of melatonin in humans (40, 71).

The physiological importance of the melatonin entrainment in mammals is not quite clear. In adult rats, melatonin phase shifts the circadian rhythms only when applied during the late subjective day at CT 10–12, but the endogenous melatonin does not increase until sometime after CT 12. Therefore, the physiological relevance is not very likely. The only time when a free-running clock might encounter melatonin at CT 10–12 is in utero before the fetal circadian pacemaker becomes synchronized to the maternal circadian system. Melatonin may thus have a role in maternal-fetal entrainment. Melatonin-induced entrainment may also be important in the early postnatal period, when the closed eyelids of the offspring do not allow the regular entrainment by the light-dark cycle. Melatonin rhythm has been detected in both maternal milk and placental blood (137, 405). Indeed, it has been shown that unborn fetuses and early postnatal pups of Syrian hamsters are entrained by timed melatonin administration (70, 118). Perhaps the general role of melatonin is to maintain entrainment in conditions not allowing the regular entrainment by the light-dark cycle.

Melatonin seems to act directly on the hypothalamic suprachiasmatic nuclei. These nuclei show daily rhythm in spontaneous neuronal activity with maximum in the middle of subjective day (114, 146, 208). With the use of the 2-deoxyglucose uptake method, the rhythm of metabolic activity of SCN has also been described (289, 290). Both these rhythms may be entrained by melatonin administration (53, 54, 204). Also, this effect of melatonin is confined to the late subjective day.

4. Regulation of seasonal rhythms

In mammals, the most important role of melatonin is to mediate the regulation of seasonal rhythms by photope-
riods. In moderate zones, the ambient temperature undergoes the annual cycle, which has a profound effect on living conditions. To adapt to the annual changes, the organisms display seasonal rhythms. Seasonal adaptation involves the change of reproductive status, feeding behavior, fur color and quality, and readiness to hibernation. These changes increase the viability of the organisms during the unfavorable seasons of the year. In the moderate zone, the change of photoperiod lengths is used by most of the organisms as a seasonal marker, because it is the most reproducible and predictable sign of the changing season. Nevertheless, the changes of ambient temperature or food access may also be used as seasonal cues. The environmental changes drive the organism's seasonal rhythms in some species, whereas in others, they merely synchronize the endogenous circannual rhythms with the season of the year (127, 129, 161, 262, 277, 402).

Some seasonal changes, e.g., in fur color or thermoregulation, may be locked to the same phase of a year in all species. Reproduction, however, may occur in various seasons of a year, depending on the species. The main strategy is obviously to deliver offspring in the most favorable time, i.e., usually during spring or early summer. This obviates the necessity to nurse and feed offspring during the winter and leaves enough time for their development before the next unfavorable season. Depending on the length of pregnancy, mating is locked in some species to the long days (e.g., rodents), whereas in the others, it occurs with decreasing daylengths (e.g., sheep or deer) (262, 402). The seasonal rhythm in fertility is driven primarily by changes in frequency of the gonadotropin-releasing hormone (GnRH) pulses, which regulate the release of gonadotrophic hormones from the pituitary and consequently the function of reproductive organs (32).

In all mammalian species studied so far, the adaptation to the seasonal change of ambient conditions depends on the presence of intact pineal gland. In pinealectomized animals, the seasonal changes either do not occur at all or lose their synchronization with the geophysical annual cycle (127, 129, 161). Melatonin infusions or injections may mimic the effect of photoperiods in pinealectomized animals. In pinealectomized adult Syrian hamsters, daily melatonin administration mimicking the pattern of melatonin secretion on short days (the long melatonin pulse) induces involution of gonads, cessation of the estrous cycle, and decrease of reproductive hormone concentrations (109, 111, 331–333). Similarly, in juvenile Siberian hamsters, long melatonin infusions result in a marked inhibition of gonadal growth (46, 110). Short melatonin pulses, which mimic the melatonin signal duration on long days, have the reverse effect: they stimulate the gonadal growth in this species (47). In sheep, on the contrary, the long melatonin infusions stimulate the reproductive activity and the mating behavior (30, 277, 404).

The melatonin target and the mechanism of its action are not known. The studies employing microimplantation or microinfusion of melatonin to various areas of the brain suggest that melatonin may act somewhere in the hypothalamus, probably in preoptic, suprachiasmatic, and/or mediobasal areas (9, 106, 107, 183, 184, 201). The selective lesions of mediobasal hypothalamic block the effect of melatonin infusion on gonadal size and concentrations of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in circulation in golden hamsters, whereas they do not affect the prolactin levels (201). This observation suggests that the melatonin target for seasonal regulation of LH and gonadal status is within mediobasal hypothalamus. The prolactin regulation seems to occur outside of this area. Because it has been observed that hypothalamopituitary disconnected rams retained the prolactin response to melatonin treatment, the target sites responsible for melatonin regulation of prolactin levels seem to be in the pituitary (183, 184). The recent experiments indicate that pars tuberalis cells secrete a peptidergic factor that induces prolactin release from pars distalis lactotrophs, and the release of this factor is increased by forskolin (123). It has been proposed that this unidentified factor named tuberalin may be responsible for seasonal regulations of prolactin release.

The mechanism of how the message encoded in the pattern of the melatonin rhythm is translated into the change of endocrine status remains to be determined. Two hypotheses are discussed. One is based on the observation that duration of the melatonin signal is prolonged on short days and that the long melatonin pulse induces the short-day response of the reproductive status. This “duration” hypothesis thus presumes that the length of the melatonin pulse is being measured by the target organ, and the response depends on the duration of the signal. The “phase coincidence” hypothesis is based on the observation that even in pinealectomized Syrian hamsters, the differential sensitivity to exogenous melatonin administration occurs throughout the daily cycle (310). It proposes that there is a distinct circadian rhythm of sensitivity to melatonin in the target organ(s) that may be entrained either by the light-dark cycle (but in a different manner from the melatonin concentration rhythm) or by the melatonin cycle. The response is determined by the phase relation between the melatonin rhythm and the sensitivity rhythm. So far, no definite arguments proving one or the other hypothesis have been presented. Even the most rigorous in vivo studies designed to separate these two hypotheses may not be conclusive, given the complexity of the regulated system and of the melatonin effects. The final decision awaits in vitro experiments on isolated organs or cells, which would allow eliminating some regulatory pathways and studying the remaining on the simplified model.

The phenomenon called the refractoriness to melatonin is even less understood. Photorefractoriness was first
described in Syrian hamsters, which show the seasonal reproductive involution driven by the decrease of the daylengths, but after ~20 wk of short days, the spontaneous regrowth of gonads occurs (261, 262). Spontaneous regrowth of gonads is observed also after prolonged administration of exogenous melatonin (264, 312, 313). To reestablish the sensitivity to melatonin, a several-week exposure to long photoperiods or short melatonin pulses resembling the melatonin profile on long photoperiods is required. Prolonged exposure to exogenous melatonin induces refractoriness also in sheep (2, 156, 278). These observations indicate that melatonin synthesis is not impaired, but rather, the target organ(s) become refractory to melatonin after a certain time. Interestingly, the transient nature of the melatonin effect has also been observed in melanophores (39, 282; see sect. IIIA for the details).

II. MELATONIN RECEPTORS

A. Distribution of the Melatonin Receptors

The effects of melatonin are mediated by specific high-affinity melatonin receptors. These receptors have been identified and characterized in a number of tissues by in vitro autoradiography and conventional binding assays using $^{[125]I}$iodomelatonin (I-MEL) as ligand (84, 223, 365, 380, 394). Although the pattern of distribution varies on a species-to-species basis, some features of tissue distribution are constant. In mammals, a very discrete distribution of the melatonin receptors has been shown, whereas in nonmammalian vertebrates, the melatonin receptors are much more abundant (Fig. 2) (90, 276, 365). Specifically, in most mammalian species, the high-affinity melatonin receptors are present in pars tuberalis (PT) of the pituitary and in suprachiasmatic nuclei (SCN) of hypothalamus (353, 383, 394). The specific I-MEL binding is often found also in medial preoptic area, anterior hypothalamus, dorsomedial and ventromedial hypothalamic nuclei, pars distalis (PD) of the anterior pituitary, paraventricular and anteroventral thalamic nuclei, hippocampus, cerebral and cerebellar cortex, area postrema, and retina (33, 34, 74, 87, 126, 185, 301, 304, 353, 358, 381, 383, 392, 394, 395).

Relatively abundant I-MEL binding has been detected in human brain; the specific binding is present not only in the SCN but also in cerebellum, especially in the external zone of the molecular layer, and in midbrain, pons, and cerebral cortex (94, 274, 408). The presence of saturable I-MEL binding has also been described in the arteries of the Willis circle, in the caudal artery, and in the spleen of several mammals (239, 373, 407).

More abundant distribution of the melatonin binding sites was described in lower vertebrates, especially in the structures of the visual, auditory, and limbic systems (37, 50, 68, 90, 195, 276, 386, 388). In many birds, reptiles, amphibians, and fishes, the intense specific I-MEL binding was found in the retina and retinorecipient structures of the hypothalamus, thalamus, and mesencephalon, in the thalamic and mesencephalic visual relay nuclei, in the visual integrative areas of the ectostriatum, and in the lobus parolfactorius. Excessive labeling was observed in the auditory system of chick and goldfish brain, e.g., in torus semicircularis and area dorsalis telencephali, and molecular layer of cerebellum (50, 90, 195).

The phenotype of the cells bearing the melatonin receptors is unknown in most cases. This is because of the inability to combine autoradiographic detection of I-MEL binding with in situ hybridization or immunocytochemistry in the same cells. Moreover, density of the melatonin receptors on individual cells is usually not high, making

![Fig. 2. Autoradiography of $^{[125]}$I-labeled melatonin binding to rat brain section. Binding of radioactive $^{[125]}$I-melatonin to suprachiasmatic nuclei (A) is displaced by excess of cold melatonin (1 μM; B). C and D: corresponding sections stained with toluidine blue. Arrows point to suprachiasmatic nuclei. (From Vanecek et al. (365), with kind permission of Elsevier Science-NL, Sara Burgerhartstraat 25, 1055KV Amsterdam, The Netherlands.)](#)
determination of the receptor distribution at the cellular level quite difficult. A serious effort has been invested to determine the melatonin-responsive cell type in the SCN. The results suggest that melatonin receptors are not present on retinorecipient cells in ventrolateral part of the nucleus but may be located on vasopressinergic cells in dorsomedial SCN (199). In the retina, the specific I-MEL binding has been found in the inner plexiform layer (34, 172, 386, 388), which contains the important synaptic connections, e.g., between amacrine and ganglion cells. Melatonin receptors may thus be localized on the neuronal cells. This hypothesis is supported by the finding that melatonin inhibits forskolin-induced cAMP increase in glia-free culture of photoreceptors and neurons prepared from chicken retina (149).

Indirect data suggest the localization of the melatonin receptors also in some other tissues. Because melatonin inhibits GnRH-induced LH and FSH release from the cultured pituitary cells (190, 191, 359), it seems likely that the receptors are present on the gonadotrophs. Moreover, melatonin inhibits the LH release also in enriched preparation of gonadotrophs (191). However, the direct evidence of I-MEL binding on gonadotrophs is still lacking. The well-described effects of melatonin on pigment distribution in dermal melanophores, shown also in the single-cell cultures, strongly suggest that the receptors are localized on these cells. Because the melatonin receptors have been cloned from the immortalized cell line derived from primary culture of amphibian dermal melanophores, the presence of the receptor in the melanophores is beyond doubt (89).

**B. Characterization of the Receptors**

The available data indicate that the melatonin receptor is membrane associated and coupled to GTP-binding protein (171, 172, 227, 275). The dissociation constant ($K_d$) of the receptors for I-MEL is in the range of 20–200 pM. In the receptors uncoupled from G protein, the affinity decreases below one-tenth of the above value. The uncoupling may be induced by GTP or its nonhydrolyzable analogs, but it may also occur as a result of normal signaling process, because of the conditions of the binding assay, storage of the tissue, or even spontaneously (62, 80, 227, 275, 324).

In natural cells, only one pharmacological subtype of the melatonin receptor, termed ML-1 by Dubocovich and co-workers (79, 81) or MEL, by Reppert and co-workers (268, 273), has been described in sufficient detail until now. The order of affinity for various indoleamines is 2-iodomelatonin > 6-chloromelatonin > melatonin > 6-hydroxymelatonin > 6-methoxymelatonin > N-acetyltryptamine > 5-methoxymelatonin >> 5-hydroxytryptamine (79, 324). The 5-methoxy group as well as the N-acyl side chain are important for high-affinity binding to the receptor, since the derivatives lacking these groups are orders of magnitude less potent. Nevertheless, it has been shown recently that replacement of N-acetyl group by longer alkyl group produces very potent analogs with affinity equal to or even higher than melatonin itself (321, 324).

In contrast to the side chains, the indole nucleus is not necessary for binding. When replaced by naphthalene or tetraline group, with methoxy and N-acyl groups retained at the appropriate positions, the derivatives were equipotent with the indole analogs (406). Therefore, it seems likely that the indole ring serves mainly to hold the functional groups in the correct position and orientation for binding to the receptor (322, 323).

Recently, the melatonin receptor cDNA has been isolated from *Xenopus* dermal melanophores, using an expression cloning strategy (89). The cDNA encodes a protein of 420 amino acids containing 7 hydrophobic segments, which likely represent the transmembrane regions. Estimated molecular weight is 47,424. On the basis of structural analysis, the melatonin receptor belongs to a distinct group within the large superfamily of G protein-coupled receptors; the highest identity found were ~25% with µ-opioid and type 2 somatostatin receptors. To date, three subtypes have been detected: MEL$_{1A}$ expressed in mammalian and bird brain, MEL$_{1B}$ expressed mainly in mammalian retina, and MEL$_{1C}$ found in amphibian melanophores, brain, and retina and also in bird and fish brain (268, 272, 273). The MEL$_{1A}$ and MEL$_{1C}$ types both have $K_a$ values of ~20–40 pM and are pharmacologically indistinguishable; the MEL$_{1B}$ subtype has a somewhat higher $K_a$ (160 pM), and a higher affinity for melatonin antagonists, e.g., luzindole or 4-phenyl-2-chloroacetamidotetraline, has been described (83).

On the basis of distribution studies that have detected the MEL$_{1B}$ receptor in the mammalian retina, this subtype is believed to mediate melatonin effects on photoreceptor sensitivity to light. On the other hand, because MEL$_{1A}$ forms the majority of melatonin receptors in mammalian brain including hypophysial PT and hypothalamic suprachiasmatic nucleus, it is presumed that this subtype may mediate seasonal and circadian effects of melatonin. This hypothesis is further supported by the natural knockout of MEL$_{1B}$ Receptor in Siberian hamsters (379). In this species, MEL$_{1B}$ Receptor gene cannot encode a functional receptor, because two nonsense mutations are present within the coding region. Therefore, MEL$_{1A}$ Receptor that does encode a functional protein has to be responsible for the circadian and seasonal effects of melatonin in this species (110, 130, 379). Also, the finding that only MEL$_{1A}$ but not MEL$_{1B}$ Receptor mRNA has been detected in human suprachiasmatic nucleus by in situ hybridization further supports this hypothesis (382).
C. Regulation of the Receptor Density

Density of the melatonin receptors not only varies with species and location, but in several tissues it is influenced by the lighting regime, time of the day, and developmental or endocrine status. The most dramatic developmental changes have been described in PD of the rat anterior pituitary, where the melatonin receptor density is ~30 fmol/mg protein on embryonic day 20 and postnatal day 1, but within 30 postnatal days decreases 10 times, i.e., <3 fmol/mg protein (352). The factors inducing the decrease or its physiological significance are unclear. With the consideration of the known inhibitory effects of melatonin on LH and FSH release (190), suggesting that melatonin receptors are located on the gonadotrophs, the decrease of the receptor density in early development may be connected with puberty. However, no data directly supporting this hypothesis have been published.

In contrast to the PD of the pituitary, the receptor density in PT does not change during development. No developmental changes were seen in either rat SCN or area postrema (173). However, the age-dependent decrease of the melatonin binding site density occurs also in anterior cerebral and caudal arteries. The mechanism of the decline has not been revealed.

Similar developmental changes as in rats have been described in Syrian hamsters. The concentration of the melatonin receptors in PD of the pituitary decreases in the course of postnatal development to about one-tenth of the neonatal value, whereas the concentration in the PT does not change significantly (363). In contrast to rats, Syrian hamsters show developmental changes of the melatonin receptor density in SCN. The highest concentration has been found in the perinatal period from embryonic day 15 to postnatal day 2, and decreased concentrations have been observed later in postnatal period starting on day 12 (85). The decrease of the receptor density correlates well with the observed disappearance of the entraining effect of melatonin in hamsters after postnatal day 6 (118). In adult Syrian hamsters, melatonin receptor density is regulated by photoperiod. Exposure to short photoperiods for 10 wk induces the decrease of the receptor density to about one-half in both PT and PD (358).

The melatonin binding site density in the PD of the anterior pituitary increases after castration in postpubertal rats (364). Within 2–4 wk after the surgery, about a twofold increase of the binding site concentration has been observed. The mechanism of this effect is unknown, but it is tempting to hypothesize that the receptor number may increase because of the removal of the suppressing effect of gonadal steroids, because reciprocal relation between estrogen concentration and the I-MEL binding has been described also in caudal and cerebral arteries (292). The I-MEL binding in the arteries is the highest during diestrus, when circulating estrogens are the lowest, whereas during proestrus and estrus, when estrogens are high, the I-MEL binding decreases.

Alternatively, the upregulation of the melatonin receptor number could be because of the postcastrational increase of GnRH secretion into the portal circulation (286, 294). Gonadotropin-releasing hormone induces the increase of gonadotroph number and of GnRH receptor density in the pituitary (65, 125). If gonadotrophs are the pituitary cells bearing the melatonin receptors, as suggested by multiple indirect evidence (194), then a mere increase of the cell number may be responsible for the postcastrational increase of melatonin receptor concentration in the pituitary.

This mechanism could also explain the developmental changes of the pituitary melatonin receptors, because concentration of GnRH in amniotic fluid increases ~10 times between embryonic day 12 and embryonic day 16 (152), which correlates with the time when the pituitary melatonin receptors are first detected on embryonic day 15 (393). After birth, the pups experience an abrupt decrease of GnRH, which might cause the postnatal decrease of the concentration of I-MEL binding sites (352).

Daily rhythm in the I-MEL binding has been shown in PT and PD of the pituitary as well as in SCN of rats (102, 103, 170, 364). In the evening before lights off, the binding site density was ~50–70% higher than in the morning (103, 364). The morning decrease of the receptor density in PT and PD of the pituitary may be because of downregulation induced by the endogenous melatonin synthesized during night. In agreement with this hypothesis, the morning decrease of the melatonin binding site density in PT is abolished in pinealectomized animals (103). The decrease of the receptor density may be also because of the inhibitory effect of melatonin on expression of its own receptor. In PT cells, melatonin inhibits the increase of MEL₁₄ mRNA and receptor protein induced by forskolin or occurring spontaneously during primary culture (14).

In the SCN, however, the regulation may be more complex. Gauer et al. (103) have shown that melatonin receptor density is reduced at night even in pinealectomized animals, whereas 1 h of light reverses the nocturnal decrease. Moreover, they could prevent the light-induced increase of the receptor density by the N-methyl-D-aspartate (NMDA) antagonist MK-801 (101). Based on these observations, they (103) suggest that the light-dark cycle directly regulates the receptor density in the SCN. The increase of the density of the melatonin receptors in the afternoon and the decrease during night have also been observed in the chicken brain (37). In contrast to these observations, the reverse rhythm, with high receptor concentration occurring in the morning and low in the evening at light-to-dark transition, has been described in the rat SCN by another laboratory (170). No daily changes in the receptor density have been found in salmon brain (90).
III. IN VITRO MODELS FOR STUDYING MELATONIN TRANSDUCTION

A. Amphibian Melanophore

Amphibian melanophore was the first in vitro system used for studies on the effects of pineal indoles. The isolated frog skin model has been developed by Lerner and co-workers (177, 178) for bioassay of the lightening substance of the pineal gland, and finally enabled isolation of melatonin and characterization of its structure. An alternative model using melanophores migrated from explanted amphibian neural tube and neural crest cultures has been introduced by Novales et al. (243, 245). More recently, the cells isolated from tadpole tissues (Xenopus tail fins) and the proliferating melanophore cultures have been introduced (99, 291). Before an assay of the effect of melatonin, melanin granules in the frog skin melanophores have to be dispersed by MSH, caffeine, forskolin, or ACTH (400). Melanophores from Xenopus tail fins show direct contracting response to light and may be dispersed by exposure to darkness (10, 291). The melanophore response may be monitored microscopically as the morphometric change and classified according to the degree of melanosome dispersion on the five-point scale (131); alternatively, the area occupied by the pigment in individual cells may be determined (321). The response may also be followed indirectly as the change of reflectance or transmittance (400).

Melatonin induces contraction of the melanin granules, acting via the high-affinity melatonin receptors (48, 89, 176, 177). Preincubation with pertussis toxin (PTX) blocks the blanching effect of melatonin, suggesting that the receptors are coupled to PTX-sensitive G protein (321, 385). The key role in the mechanism of melanosome movement has cAMP. Melanocyte-stimulating hormone, which induces the pigment dispersion, also elevates the intracellular concentration of cAMP (1). Melatonin inhibits the MSH-induced cAMP increase and reverses the darkening of the frog skin (1, 351, 385). Melatonin also reverses the darkening induced by high concentrations of KCl. The stimulation requires both forskolin or caffeine, which both increase cAMP, one by stimulating adenyl cyclase and the other by inhibiting phosphodiesterase (385, 400). However, melatonin has no effect on darkening induced by exogenous cAMP or by its dibutyryl derivative (1, 372). These findings indicate that cAMP increase is a sufficient signal for pigment dispersion and that decrease of cAMP induced by melatonin results in pigment aggregation. It has been suggested that the melatonin receptor is coupled to the adenyl cyclase, inhibiting its activity via a G protein (385).

An important feature is a transient nature of the melatonin’s effect on pigment distribution. The melatonin-induced pigment aggregation fully develops within 20–30 min of melatonin treatment, but then the melanophores gradually lose their sensitivity to melatonin and during the following 1–3 h disperse again (39, 61, 282). This refractory status is removed when the cells are maintained in melatonin-free conditions for some time (61). It would be interesting to know whether the melatonin-induced inhibition of cAMP accumulation in the melanophores has the same time course.

B. Retina

The model was first used for studies of melatonin effects by Dubocovich (77), who has reported inhibition of dopamine release by melatonin and its derivatives. The cultured rabbit retinas are preloaded with [3H]dopamine, and then the release is measured as the efflux of radioactivity. Spontaneous release of dopamine is quite low, but it is stimulated severalfold by electrical depolarization or high concentrations of KCl. The stimulation requires the presence of calcium in extracellular medium. The calcium dependency of the dopamine release suggests that the increase of [Ca²⁺], may be the signal inducing the release of dopamine, as is the case with many other neurotransmitters and hormones.

Melatonin has no effect on spontaneous dopamine release, but it is a very potent inhibitor of the evoked release, with the EC₅₀ being in 10⁻¹¹ M range (77). Many melatonin derivatives have been tested in this system. The structure-activity data and the I-MEL binding show that melatonin acts via the high-affinity melatonin receptors (34, 78). The intracellular mechanism of the melatonin effect is, however, unknown. The effect of PTX on melatonin inhibition of dopamine release has not been reported; therefore, it is not clear whether the indole effect is mediated by the PTX-sensitive G protein. Recently, melatonin has been shown to inhibit the forskolin-stimulated cAMP increase in glia-free monolayer culture of photoreceptors and neurons prepared from embryonic chick retina (149). This effect is abolished after PTX pretreatment, showing that melatonin acts via the receptors coupled with PTX-sensitive G protein. Whether the decrease of cAMP mediates the effect of melatonin on dopamine release is not known. Calcium sensitivity of dopamine release suggests that melatonin might act through modulation of [Ca²⁺], but no data are available.

The melatonin effect on dopamine release is significant for visual physiology. Dopamine is released during light adaptation and mediates several effects of light on retina (76). Therefore, the inhibition of its release by melatonin affects the light adaptation and photoreception. It is not known which cells are responsible for the melatonin effect. Melatonin is formed locally in the photoreceptor cells, whereas melatonin receptors are located at inner plexiform layer, where the connections are among the
dopamine-producing amacrine cells and ganglionic cells (34, 76, 172). It may thus be that melatonin acts directly on amacrine cells. Alternatively, melatonin receptors may be located on the axons of the ganglionic cells, and the indole may inhibit the dopamine release indirectly through the synaptic connections of the ganglionic with the amacrine cells.

C. Pars Tuberalis

The history of this model is quite recent. It has been used only after it was found that the most intense I-MEL binding in the mammalian brain occurs in the PT of the pituitary. Most of the work on this model has been done by Morgan and collaborators (223, 228) using the dispersed cells of sheep PT, which has the advantage of large size giving abundant supply of the cells.

Physiological significance of the PT is unknown. The PT is part of the adenohypophysis adjacent to the hypothalamic median eminence. It is composed of two main parenchymal cell types: glandular cells rich in dense-core vesicles, similar to the cells of PD, and nonsecretory cells with few or no dense-core granules (226, 314). Sheep PT has only 15% of gonadotrophs, with the remaining cells being nonimmunoreactive with any of the antisera against pituitary hormones (115, 337). In contrast, in the rat PT, 95% of the secretory cells are thyroid-stimulating hormone immunopositive and 5% LH/FSH positive (115).

Because the appearance of PT cells undergoes seasonal changes, it has been suggested that it may be involved in seasonal regulations (398). It has been shown recently that PT may serve as a melatonin target for seasonal regulation of prolactin release, although it is probably not responsible for the regulation of gonadotrophins or gonadal involution (183, 184, 201). With the use of [³⁵S]methionine labeling, it has been shown that forskolin stimulates the release of several radiolabeled proteins from the cells of PT, and the effect is blocked by melatonin (221). The results support the hypothesis that "melatonin may regulate the release of pars tuberalis-specific product, envisaged to convey photoperiodic information to a circannual center in the brain or directly to pars distalis of the pituitary" (221, 223). Because forskolin stimulates adenylyl cyclase, the data suggest that melatonin regulates the release of the proteins via a cAMP-dependent mechanism. The structure of the proteins has not been determined. Another drawback is that no natural stimulatory hormone or neurotransmitter of PT cells has been described.

Melatonin inhibits cAMP accumulation stimulated by forskolin, the well-known activator of adenylyl cyclase in all tissues (224). Melatonin acts via PTX-sensitive and -insensitive G proteins, because preincubation with the toxin prevents the melatonin effect only partially. However, preincubation with cholera toxin severely impairs the ability of melatonin to inhibit forskolin-stimulated cAMP accumulation (222). In the PT cells, thus melatonin receptors inhibit cAMP accumulation through two different G proteins, one of them belonging to the G_i/G_s family.

Recently, it has been reported that melatonin inhibits the forskolin-induced phosphorylation of cAMP-responsive element binding protein (CREB) in PT cells (209). Phospho-CREB is one of the transcription factors initiating the expression of secondary (late or structural) genes in many cells. Melatonin also inhibits forskolin-induced increase of c-fos and jun B mRNA as well as c-Fos protein (283). Melatonin may thus regulate expression of the late response genes in PT. This correlates with the observed inhibitory effect of melatonin on accumulation of [³⁵S]methionine-labeled proteins in PT cells (221).

D. Neonatal Rat Gonadotroph

The neonatal rat pituitary gland was set up by Martin and Klein (190) as an alternative bioassay system for testing antigonadotropic principle of the pineal gland. The neonatal tissue was selected because small immature tissues generally survive well in organ culture. This system worked in a highly reproducible manner. Pineaulectomies would be assayed by determining their ability to inhibit GnRH-induced LH secretion as an end point.

The release of LH from gonadotrophs is low under resting conditions, and it is stimulated by GnRH, the decapeptide synthesized in hypothalamus. Melatonin acts rapidly at low concentrations to block GnRH-induced release of LH in vivo and in vitro (Fig. 3) (189, 192). Effects of melatonin first reported using cultured glands have been confirmed using dispersed cells in culture (191, 359). Studies in organ and cell cultures have examined the relative potency of melatonin and related indoles. Melatonin blocks the effects of GnRH at a range of concentrations: a threshold concentration for inhibition of in vitro LH release is ~10⁻⁸ M, and the maximal inhibition is attained with 10⁻⁸ to 10⁻⁷ M concentration of melatonin (189). The order of potency of the indoles is iodomelatonin > melatonin > 6-hydroxymelatonin > N-acetylserylosorine > 5-methoxytryptamine >> 5-hydroxytryptamine.

Gonadotropin-releasing hormone stimulates the release of both LH and FSH. In most of the studies on the mechanism of action of melatonin on the neonatal pituitary gland, only LH has been studied. However, it has been established that melatonin also blocks the GnRH-induced release of FSH (194). Melatonin probably directly inhibits rat gonadotroph cells because it suppresses LH release in enriched gonadotroph fraction (191). In contrast to the effects of melatonin on the GnRH-induced release of LH and FSH, it has not been possible to demonstrate effects of melatonin on basal or releasing factor-
nin may exert the inhibitory effect on onset of puberty at least partially at the level of the pituitary. Removal of this inhibitory input because of the decrease of the melatonin receptors might be one of the events initiating puberty.

As in other tissues, melatonin acts via the high-affinity melatonin receptors coupled with the PTX-sensitive G proteins, because pretreatment with the toxin abolishes the effect of melatonin on LH release (359). The intracellular mechanisms of melatonin action in the pituitary have been described in more detail than in any other tissue, although they are still not fully understood. Melatonin affects several intracellular messengers in these cells. It inhibits the GnRH-induced accumulation of cAMP and cGMP, the increase of intracellular calcium, and also the synthesis of diacylglycerol and release of arachidonic acid (359, 366, 367). In stimulation of LH release by GnRH, the most important intracellular signal is the increase of $[Ca^{2+}]_i$ (134, 316). The inhibitory effect of melatonin on LH release is most probably because of the decrease of $[Ca^{2+}]_i$ (see sect. IV D).

In addition to its effect on the second messengers, melatonin also regulates the third messengers, the transcription factors. It inhibits the GnRH-induced expression of c-Fos (327). Melatonin involvement in the early gene expression suggests that it may regulate not only the release of LH and FSH but also synthesis of yet unknown protein(s) by the gonadotrophs.

E. Suprachiasmatic Nucleus

The SCN is an attractive model for studies on transduction of melatonin signal, because it has a high concentration of melatonin receptors throughout life and plays an important role in vertebrate physiology as a circadian pacemaker. Moreover, melatonin has well-described effects in this tissue (53, 295, 307). The SCN continues to show circadian rhythmicity when dissected and cultured in vitro. The rhythms of electric and metabolic activities have been shown to continue for several days or even weeks in cultured SCN explants or dispersed neurons (114, 296, 300, 384), and melatonin has a clear effect on these rhythms (53, 295, 307).

In SCN-containing slices of the rat or hamster brain cultured in vitro, the SCN neurons display spontaneous action potentials (197, 298). Frequency of the spontaneous activity shows daily rhythm, with the peak around the middle of subjective day (CT 6) and through during the night. It has been shown recently that the circadian rhythm in electric activity persists also in dispersed individual neurons; however, their rhythms are not running in phase (384). Melatonin decreases the frequency of the spontaneous electric activity in cultured rat SCN explants (196, 198, 295, 307). The effect is fast and reversible, occurs within a few seconds after melatonin administration,
and quickly dissipates after its withdrawal (Fig. 4). In the hamster but not in the rat SCN, an additional small population of the neurons was found, which responded to melatonin with an increased frequency of firing (198). The effects of melatonin are phase dependent, as ~90% of the neurons are sensitive to melatonin at late subjective day (CT 8–12), and only 20 or 40% at early subjective day or at night, respectively (307).

In addition to the acute inhibitory effect, melatonin also induces advancing phase shifts of the electric activity rhythm in cultured SCN slices of rats and Siberian hamsters (204, 306, 379). The melatonin effect is phase dependent, and the phase shifts occur only when melatonin is administered during sensitive periods, from CT 9 to 15 or from CT 22 to 2. The largest shifts are induced by melatonin administration at CT 10, inducing about a 4-h advance of the single-unit activity rhythm. The second sensitive window is from CT 22 to 2 with maximal advances of ~4 h at CT 23 (205). At other times, melatonin has no effect.

Uptake of radiolabeled 2-deoxyglucose, determining glucose utilization, is used as an estimate of the metabolic activity of neurons (289). In the SCN, the uptake of 2-deoxyglucose shows a robust daily rhythm that persists in vitro (296, 299, 300). In hamster hypothalamic slice preparations, 2-deoxyglucose uptake in the SCN is high during the subjective day and low during the subjective night. Melatonin inhibits 2-deoxyglucose uptake when administered in vivo between CT 6 to 10 or at CT 22 (53, 54). Melatonin administration at other times has no effect. The effect of melatonin on the 2-deoxyglucose uptake in vitro has not been determined.

What cell type responds to melatonin in SCN is not known. Rat SCN consists of ~10⁴ small cells (349). Most of the neurons in SCN produce GABA (248, 350), but the presence of cells producing vasopressin, vasoactive intestinal polypeptide (VIP), neuropehysin, bombesin, and somatostatin has also been described (44, 350). The nucleus may be subdivided into two regions: ventrolateral, receiving the direct input from retina by retinohypothalamic tract and consisting predominantly of VIP-producing neurons, and dorsomedial, which consists of vasopressinergic cells (55, 217, 218). In the hamster SCN, I-MEL binding is distributed predominantly in the rostral half of the nucleus extending across the full mediolateral range of the nucleus (199). In the caudal part of SCN, I-MEL binding is weaker and restricted to the medial division of the nucleus. The distribution of I-MEL binding is in close correlation with vasopressin mRNA. In contrast, the retinoreceptive cells are located in the middle and caudal thirds of the nucleus, predominantly in ventrolateral divisions. The available data thus suggest that melatonin receptors are not present on retinoreceptive cells but may be located on vasopressinergic cells. It is not clear, however, whether melatonin receptors are located on the neurons or on the glial cells, although the former should be more likely according to the effects of melatonin on neuronal firing.

Intracellular mechanisms of the melatonin action in SCN are intensely studied. Melatonin-induced phase shifts are abolished after preincubation with PTX, indicating involvement of G_i/G_o protein (205). Melatonin (1 μM) administration increases protein kinase C activity. Importantly, melatonin has the stimulatory effect only at the time when it induces the phase shifts, i.e., around CT 10 or CT 22, but not at any other time (205). Furthermore, protein kinase C inhibitors block the melatonin-induced phase shifts, and the kinase activator mimicks the shift. Together, these observations suggest that melatonin induces phase shifts of SCN clock via the activation of protein kinase C. This activation may be because of diacylglycerol hydrolyzed from phosphatidylinositol bisphosphate by phospholipase C (242). Indeed, PTX-sensitive activation of phospholipase C mediated by βγ-subunit of G_i protein has been described in many other cells (93, 230, 411). The second product of the phosphoinositide hydrolysis, inositol trisphosphate (InsP₃), would mobilize calcium from endoplasmatic reticulum, which would further increase protein kinase C activity (19). Alternatively, protein kinase C may be activated by increased [Ca²⁺], which might be caused by melatonin-induced Ca²⁺ influx through G protein-coupled channels.

However, another recent report indicates that melatonin may induce the phase shifts of the circadian clock by activation of nitric oxide synthesis (305). Inhibitors of nitric oxide synthase blocked the melatonin-induced phase shifts and the nitric oxide donors mimicked the melatonin effects. Nitric oxide synthase is a calcium-sensitive enzyme; therefore, the increase of nitric oxide may be secondary to Ca²⁺ mobilization or influx as discussed above.

Finally, melatonin has been shown to induce hyperpolarization of the SCN neurons by activating an outward
current carried probably by $K^+$ (153). Because frequency of the spontaneous neuronal discharge in SCN is dependent on membrane potential (212, 375) and membrane depolarization by KCl or activation of $Na^+$ channels by veratridine phase shifts the rhythm of electric activity (288), the melatonin-induced hyperpolarization might cause inhibition of the spontaneous neuronal activity in SCN as well as the phase shift of the rhythm. However, high concentrations (1–10 $\mu$M) of the indole have been used in the study. Moreover, the changes of membrane potential and of outward currents are not big and develop slowly in a range of minutes. More experiments are needed to ascertain whether the hyperpolarizing effect of melatonin in SCN is because of the activation of ionic channel or to the stimulation of some electrogeneic carrier or pump.

Melatonin also affects the third messengers in SCN. Administration of melatonin to rats has been reported to induce expression of c-Fos, the protein product of the c-fos protooncogene (162). The expression of the Fos protein is dependent on circadian phase; melatonin injections in the late subjective night (CT 22) induce Fos expression in cells within the ventral SCN, whereas injections during subjective day are ineffective. However, this observation has not been replicated and is somewhat conflicting when compared with the phase-shifting effects of melatonin. Melatonin applied at the end of subjective day phase-advances the circadian rhythm of locomotor activity but has no effect on c-Fos in SCN (162, 204, 259). If true, these results would indicate that the melatonin-induced phase advances of the circadian system can occur without increased expression of c-Fos protein in the SCN, at least at levels detectable by immunohistochemistry.

F. Caudal Artery

The effect of melatonin on contraction of caudal artery has been tested after the description of high-affinity I-MEL binding in the smooth muscle layer of the anterior cerebral and caudal arteries (373). The first report has described potentiation of norepinephrine-induced contraction of the caudal artery by melatonin. Later studies have shown that melatonin itself induces concentration-dependent contraction of segments of the distal caudal artery pressurized to 60 mmHg (92). These findings indicate that pressurized segments of the isolated distal caudal artery may provide a simple and convenient functional model of melatonin receptors.

The melatonin effect is age dependent. In vessels isolated from juvenile rats at 4–5 wk of age, melatonin itself caused robust vasoconstriction. In arteries of adult rats, melatonin showed no direct vasoconstrictor activity, but in the presence of phenylephrine-induced tone, melatonin produced a small and variable constrictor response in some vessels. This age dependency correlates well with an observed loss of the melatonin binding sites in the caudal and anterior cerebral arteries in the course of postnatal development (173). These observations suggest that in juvenile rats melatonin may cause circulatory adjustments in the arteries, which are believed to be involved in thermoregulation. The intracellular mechanisms of the melatonin effect have not been studied in great detail.

G. Transfected Cells

The recent cloning of the melatonin receptors enabled the study of melatonin transduction pathways in transfected cells. In NIH 3T3 cells stably expressing the human MEL1A or MEL1B receptor, melatonin inhibits forskolin-induced increase of cAMP accumulation, suggesting that the receptor is coupled to inhibition of adenylyl cyclase (108, 268). The effect is dose dependent, reaching ~80% inhibition with 10 nM melatonin. Pretreatment with PTX abolishes the inhibitory effect of melatonin, indicating that the receptor acts via G/$\alpha_i$ protein.

In addition to the inhibitory effect on cAMP, MEL1A receptor regulates phosphoinositide hydrolysis (108). In the transfected cells, melatonin potentiates InsP$_3$ accumulation induced by prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$), although it has no effect alone. Moreover, melatonin also potentiates arachidonate release induced by PGF$_{2\alpha}$ or ionomycin, and both these effects are sensitive to PTX. The effect of melatonin on arachidonate release is independent of the effects of melatonin on cAMP, but it is abolished after inhibition of protein kinase C. The authors (108) suggest that melatonin signal is transduced by parallel pathways involving inhibition of adenylyl cyclase by $\alpha$-subunit of G, protein and potentiation of phospholipase C activation by $\beta\gamma$-subunits. The resulting diacylglycerol may activate protein kinase C, which in turn may induce phosphorylation and activation of phospholipase A$_2$ and increase of arachidonate release as shown in other cells (238, 257, 258). Alternatively, arachidonate may increase because of the InsP$_3$-mediated calcium mobilization.

This model is unique because it enables one to work with a homogeneous population of cells, all of them expressing melatonin receptors. This is never the case with natural cells. However, there are also some drawbacks of the system, with the most dreadful being the possibility that overexpressed receptors may couple with other effectors than in the natural cells. Another disadvantage is that the transfected cells have only the machinery native to the cell line used, and some transduction or effector mechanism important for melatonin function may be missing. Therefore, the observations on the transfected cells should be interpreted with some caution.

In *Xenopus* oocytes expressing the MEL1A receptors, melatonin activates inward-rectifying potassium channels.
Kir3 (237). The melatonin effect is sensitive to PTX preincubation, indicating that the receptor is coupled to G\(\alpha\)/G\(\beta\) protein. This observation might explain the melatonin-induced hyperpolarization seen in SCN and in neonatal rat pituitary cells (153, 360).

IV. TRANSDUCTION OF MELATONIN SIGNAL

On the basis of studies of several tissues, there are several constant features of the effects of melatonin, which are mediated by high-affinity G protein-linked receptors. These include effectiveness at the range of \(10^{-11}\) to \(10^{-8}\) M concentrations, similar rank orders of binding to the melatonin receptor and in most cases sensitivity to PTX. Because the receptors are located on plasma membrane, melatonin regulates the function of the cell through intracellular second messengers. For example, in many tissues, melatonin has been found to decrease intracellular concentration of cAMP (45, 228, 366). Melatonin effects on other second messengers such as \([Ca^{2+}]_i\), cGMP, diacylglycerol, protein kinase C, or arachidonic acid have been described in the neonatal rat pituitary cells and in SCN but rather rarely in other tissues (Table 1) (205, 359, 366, 367).

Most of the melatonin effects are inhibitory and require previous stimulatory input, because basal levels are often not affected by the indole. However, this is not a rule, e.g., melatonin increases protein kinase C activity and induces c-Fos expression in SCN and hyperpolarizes the resting membrane potential in neonatal rat pituitary cells (162, 205, 360).

Melatonin affects several second messengers. Because three different subtypes of the melatonin receptors have been recognized based on their molecular structure (273), it may be possible that these subtypes are coupled to different effectors. The finding that all three subtypes when overexpressed in NIH 3T3 cells have inhibitory effect on cAMP accumulation does not necessarily imply that this is true also in natural cells (108, 268, 271, 273). Alternatively, one receptor subtype could be coupled via different G proteins to the different effectors. The finding that in ovine PT cells melatonin inhibits forskolin-induced cAMP accumulation through both PTX-sensitive and -insensitive G proteins may support the hypothesis (224). Although most of the described effects of melatonin on second messengers are PTX sensitive, the signal may still be transduced by different G proteins. Five distinct G proteins from G\(\alpha\)/G\(\beta\) family coupled to six different effector systems (adenylyl cyclase, phospholipase C, potassium channel, calcium channel, amiloride-sensitive cation channel, and ATP-sensitive potassium channel) have been shown to be PTX sensitive (28). Still another possibility may be coupling of the melatonin receptor with one effector (e.g., adenylyl cyclase) via \(\alpha\)-subunit of G\(\alpha\) protein and with another (e.g., phospholipase C) via \(\beta\gamma\)-subunit, as described for other receptors (93, 230, 411). Finally, the possibility exists that melatonin receptor might be

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<th>InsP(3)</th>
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AA, arachidonic acid; PKC, protein kinase C; \([Ca^{2+}]_i\), intracellular free calcium concentration; P-CREB, phospho-cAMP-responsive element binding protein; DAG, diacylglycerol; InsP\(_3\), inositol trisphosphate; ↑, increase; ↓, decrease; no, no effect. Reference numbers are given in parentheses.
coupled to a single, so far unrevealed, effector, which would mediate all melatonin effects (e.g., inhibition of cAMP and cGMP accumulation, decrease of \([\text{Ca}^{2+}]_i\), and inhibition of diacylglycerol formation).

A. Melatonin Effects on cAMP

Adenosine 3',5'-cyclic monophosphate has been suggested to transduce the lightening effect of melatonin in frog skin melanophores (1, 385). In cultured melanophores or in melanophore-rich skin, melatonin decreases cAMP concentration induced by MSH, by phosphodiesterase inhibitors, or by the adenylyl cyclase activator forskolin (1, 69, 351, 385). Later it has been shown that melatonin inhibits cAMP accumulation also in other tissues or cells and in other species. In PT of several mammals, melatonin inhibits the forskolin-induced stimulation of cAMP accumulation (228, 366). Melatonin also inhibits cAMP in dopamine- or forskolin-stimulated monolayer cultures of photoreceptors and neurons prepared from chicken retina (149, 150) and in forskolin-stimulated cAMP accumulation in rabbit ciliary body (249), rat retinal pigment epithelium (236), rat circle of Willis arteries (43), optic tectum explants of frog (387), and parietal cortex explants of rabbit (303). In cultured explan or dispersed cells from PD of immature rat pituitary, melatonin has been found to inhibit basal and GnRH-induced cAMP accumulation (355, 366, 368). In adult rat pituitary, melatonin is without effect on basal and GnRH-induced cAMP accumulation, in correlation with the age-dependent decrease of the melatonin receptors in PD (352, 368).

The melatonin effects on cAMP are prevented after preincubation with PTX. The exception has been found in the cells from ovine PT, where the toxin was only partially effective, although the maximal concentrations were used (224). Because preincubation with cholera toxin blocks the inhibitory effect of melatonin on forskolin-stimulated cAMP accumulation and reduces the number of I-MEL binding sites in PT membranes by \(\sim 50\%\) (222), the melatonin receptors in PT cells thus may be coupled to two different G proteins.

The most likely intracellular target of melatonin is adenylyl cyclase. The PTX sensitivity of \(G_i\), the G protein inhibiting the activity of adenylyl cyclase, is well known (158). However, no effect of melatonin on basal or forskolin-induced adenylyl cyclase activity has been observed in cell-free preparations from several tissues (228; B. H. White and J. Vanecek, unpublished data). The exception is the observation of Niles and co-workers (240, 241), indicating that melatonin inhibits activity of adenylyl cyclase in a dose-dependent manner in the membrane homogenate from the chicken brain or hamster hypothalamus. The melatonin effect is prevented by PTX pretreatment (241). However, this effect is somewhat unusual, because the effective melatonin concentration extends over 6 log units (from \(\sim 10^{-13}\) to \(10^{-7}\) M) and does not saturate even at the highest concentration.

Another possibility, i.e., that melatonin activates phosphodiesterase and decreases cAMP concentration by increasing its catabolism, is not likely because melatonin acts even in the presence of high concentration of 3-isobutyl-1-methylxanthine. This is a potent inhibitor of phosphodiesterase and as 1 mM should completely inhibit its activity, making any further effect through this pathway impossible. Moreover, melatonin has the same inhibitory effect on cAMP accumulation in the presence and absence of 3-isobutyl-1-methylxanthine (220, 366).

These observations suggest that melatonin may regulate cAMP synthesis indirectly, through mechanisms depending on the integrity of the cell. One of these mechanisms could be intracellular calcium. Nearly all of the eight cloned adenylyl cyclases are regulated by one or the other arm of the phospholipase C pathway (67). Five of them are calcium sensitive; two are inhibited by calcium and three are stimulated. Because in the rat gonadotrophs melatonin has been shown to decrease GnRH-induced \([\text{Ca}^{2+}]\) (359, 361), the indole may inhibit the calcium-sensitive adenylyl cyclase activity via this mechanism. However, our recent data do not support this hypothesis. In sodium-free medium, melatonin has no effect on \([\text{Ca}^{2+}]\), but it still significantly decreases forskolin or GnRH-induced cAMP accumulation (355). Although the calcium sensitivity of cAMP accumulation in the pituitary cells is clearly documented by the data, because in calcium-free medium the forskolin-induced increase of cAMP is much smaller than in normal medium, these observations indicate that the decrease of \([\text{Ca}^{2+}]\), is probably not the primary mechanism of melatonin-induced decrease of cAMP. The inhibitory effect of melatonin on cAMP accumulation is not dependent on the changes of plasma membrane potential (211, 355).

There are some other possibilities of how melatonin could regulate cAMP. Recently, the products of phospholipase C- or D-mediated breakdown of membrane phospholipids (phosphatidylcholine, phosphatidic acid, and lysophosphatidic acid) have been shown to inhibit adenylyl cyclase in a number of cells (75, 287, 348). Moreover, some of these pathways are PTX sensitive. However, melatonin does not stimulate phospholipases A or D in PT cells (208). Therefore, the inhibition of cAMP accumulation by melatonin has to involve other mechanisms. After all, coupling of the melatonin receptor with adenylyl cyclase seems to be the most likely from all mechanisms.

Adenosine 3',5'-cyclic monophosphate has been shown to mediate the effects of melatonin in several cells. Most likely, the lightening effect of melatonin in frog skin melanophores is transduced by cAMP (1, 385). Derivatives of cAMP and agents increasing cAMP accumulation as forskolin or 3-isobutyl-1-methylxanthine all induce mela-
Melatonin dispersion in several amphibian and fish species (1, 244, 385). In angelfish, which does not respond to melatonin but its melanophores aggregate in response to α2-adrenergic agonists, it was shown that reduction of intracellular cAMP is both necessary and sufficient for pigment aggregation (285); inhibitors of protein kinase A induce contraction of melanosomes, and cAMP analogs blocked the α2-adrenergic agonist-induced aggregation. Although these agents also increased [Ca2+], this increase was not essential for contraction, because the ionophore-induced [Ca2+] increase did not have such an effect, nor was the movement blocked when the increase was suppressed by withdrawal of extracellular calcium or by loading the cells with the calcium chelator BAPTA. These observations suggest that decrease of cAMP is both a sufficient and necessary signal for contraction of melanosomes.

In Xenopus melanophores, melatonin contracts melanosomes dispersed by MSH, forskolin, or 3-isobutyl-1-methylxanthine, and it is paralleled by the decrease of intracellular cAMP (1, 385). Melatonin is, however, unable to concentrate melanosomes dispersed by nonhydrolyzable cAMP derivatives (1). Together with the described inhibitory effect of melatonin on cAMP accumulation, the finding strongly suggests that melatonin acts via cAMP in melanophores. However, other data indicate the possible involvement of calcium or other intracellular messengers in the process. In calcium- or sodium-free medium, the dispersing effect of MSH or ACTH is blocked (243, 400). Removal of extracellular calcium or addition of various divalent cations able to inhibit Ca2+ influx blocks the melatonin-induced pigment aggregation (215, 321). Moreover, treatments of isolated Xenopus melanophores with activators of protein kinase C, including phorbol esters, mezerein, and diacylglycerol, prevent and reverse melatonin-induced pigment aggregation (325). In addition, in these melanophores, the cell-permeable inhibitor of protein kinase A induces only very slow (~5 h) aggregation of the pigment, whereas melatonin induces contraction within minutes.

An explanation of these conflicting data is difficult. One possibility is that in melanophores of different species, the pigment movement is regulated in parallel by different intracellular messengers. Another possibility is that dispersion of the pigment granules may be induced either by protein kinase A or C (325). Alternatively, melanosome movement may be regulated by cAMP-mediated protein kinase A, but calcium (or protein kinase C) may be involved in the mechanism of regulation of cAMP synthesis. The decrease of cAMP accumulation may thus be caused by the melatonin-induced change of [Ca2+], or protein kinase C, as nowhere has it been shown that the melatonin inhibition of cAMP accumulation in the melanophores is because of a direct effect of the indole on adenylyl cyclase.

Adenosine 3′,5′-cyclic monophosphate may also mediate the effects of melatonin in PT. Forskolin has been shown to stimulate the release of several radiolabeled proteins from cultured PT pretreated with [35S]methionine, and this was inhibited by melatonin (221). On the other hand, cAMP does not seem to be of primary importance in regulation of LH release from gonadotrophs. The hypothesis that melatonin inhibits the GnRH-induced release of LH by inhibiting the generation of cAMP was tested by determining whether the effects of melatonin were blocked in the presence of cAMP antagonists. The presence of forskolin, or 3-isobutyl-1-methylxanthine, or nonhydrolyzable cAMP analogs 8-bromo-cAMP or dibutyryl cAMP did not prevent inhibition of LH release by melatonin (362). The results of these investigations indicated that the inhibitory effects of melatonin were not transduced by cAMP, i.e., the decrease of cAMP was not the necessary signal for inhibition of LH release in the neonatal rat gonadotrophs.

B. Melatonin Effects on cGMP

In cultured neonatal rat pituitary, GnRH increases intracellular concentration of cGMP, and melatonin inhibits the effect (366). The effect of melatonin is dose dependent, and the maximal inhibition is reached at ~10 nM. The mechanism of the melatonin effect is unknown, but because membrane as well as soluble guanylyl cyclases are Ca2+ sensitive, the decrease of [Ca2+]i in the pituitary cells might be involved (112, 175, 399).

In contrast to the inhibitory effect of melatonin in the pituitary, stimulatory effects of melatonin on cGMP concentration have also been described in other tissues. In cultured hypothalamic explants cultured in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, melatonin increased cGMP concentration (345). Melatonin also potentiated VIP-induced increase of cGMP accumulation in human lymphocytes (187).

It is not clear whether cGMP plays an important role in transduction of any of the effects of melatonin. In cultured melanophores prepared from Xenopus embryo neural crest, cGMP induced pigment aggregation, similar to the effect of melatonin (215). In neonatal rat gonadotrophs, addition of 8-bromo-cGMP had no effect on the inhibitory effect of melatonin on LH release (unpublished data). On the other hand, in adult gonadotrophs, cGMP potentiated the release of LH (233). The involvement of cGMP in melatonin-induced phase shifts of SCN pacemaker is also possible (305).

C. Melatonin Effects on Phospholipids

Melatonin affects also the second messengers derived from membrane phospholipids. In gonadotrophs, GnRH acting via phospholipase C stimulates the formation of
Diacylglycerol and InsP₃ from phosphatidylinositol bisphosphate (4, 133, 134, 410). In addition, GnRH stimulates arachidonic acid production, acting probably through both phospholipase A₂ and the diacylglycerol lipase (60, 234). Melatonin has been shown to inhibit the GnRH-induced increase of diacylglycerol formation in cultured neonatal rat pituitary and to attenuate the release of arachidonic acid from the gland (367). The effects of melatonin are transduced by PTX-sensitive G protein, because preincubation with the toxin prevents the melatonin effect.

The mechanism of the effect is unclear. Gonadotropin-releasing hormone is known to stimulate phospholipase C activity in gonadotrophs (4, 133, 134, 410), but melatonin does not seem to influence the GnRH-induced InsP₃ formation in neonatal rat gonadotrophs, making the involvement of phospholipase C in its effect unlikely (V. Pelisek, R. Novotna, and J. Vanecek, unpublished data; Z. Naor, J. Vanecek, and D. C. Klein, unpublished data). Another possibility is through the action of phospholipase D, which is also stimulated by GnRH and catalyzes the formation of choline and phosphatidic acid from phosphatidylcholine (410). Phosphatidic acid then may be dephosphorylated to diacylglycerol by phosphatidate phosphohydrolase (186). Melatonin thus might decrease diacylglycerol formation via effect on phospholipase D or phosphohydrolase.

A stimulatory effect of melatonin on phosphoinositide breakdown has been observed in chick brain slices prelabeled with myo-[2-³H]inositol (256). In the presence of lithium, melatonin and its derivative 6-chloromelatonin induced rapid and transient increase of InsP₃, inositol bisphosphate, and inositol monophosphate. The effect was dependent on the presence of calcium in the medium and required rather high concentrations of the inodexes (~10⁻⁵ M). Furthermore, melatonin potentiated the PGF₂α-induced InsP₃ accumulation in the NIH 3T3 cells transfected with MEL₁₄ receptor (108). This is most probably because of the phosphoinositide hydrolysis by phospholipase C. The melatonin-induced increase of protein kinase C activity described in SCN may also be because of activation of phospholipase C (205).

Diacylglycerol acting via activation of protein kinase C has been shown to potentiate the calcium-stimulated LH release (128, 317). However, melatonin inhibition of GnRH-induced LH release from neonatal rat gonadotrophs is probably not mediated by the decrease of diacylglycerol concentration. Melatonin inhibits the LH release even in the presence of phorbol esters, which stimulate protein kinase C directly (362). On the other hand, the melatonin-induced phase shifts of the circadian pacemaker in the SCN are most probably mediated by the activation of protein kinase C (205). In contrast, in isolated Xenopus melanophores, the activators of protein kinase C, including phorbol esters, mezerein, and synthetic diacylglycerol, prevented and reversed melatonin-induced pigment aggregation, and the effect was blocked by inhibitors of protein kinase C (325). The observation thus suggests that protein kinase C, like protein kinase A, may be involved in dispersion of the pigment granules.

The possibility that melatonin may act through decrease of the kinase C activity should also be considered, but first the effect of melatonin on diacylglycerol or protein kinase C activity in melanophores would have to be established.

**D. Melatonin Effects on [Ca²⁺]**

The effects of melatonin on [Ca²⁺], were thoroughly studied in dispersed cells from neonatal rat pituitary, because it has been established that Ca²⁺ plays a central role in regulation of LH release (59, 63, 133, 151, 182, 316). The effects of melatonin on [Ca²⁺], were studied using calcium-sensitive fluorescent dyes in cell suspensions or in monolayer cell cultures (155, 359, 361). The latter allow one to monitor [Ca²⁺], changes in individual cells, which is of great importance when dealing with such nonhomogeneous cell population as the pituitary cells. With this approach, it has been discovered that melatonin inhibits GnRH-induced increase of [Ca²⁺] in neonatal rat gonadotrophs (Fig. 5A) (359, 361). Melatonin has no effect on basal [Ca²⁺].

Gonadotropin-releasing hormone induces the [Ca²⁺], increase primarily by InsP₃-mediated mobilization of calcium from intracellular stores (4, 59, 182, 235, 334). This is followed by cyclic changes of membrane potential: hyperpolarization followed by few action potentials (318, 339). During the depolarization period, calcium influx through voltage-sensitive channels occurs (334). Melatonin inhibits both these pathways, influx and mobilization of calcium. When added after GnRH-induced [Ca²⁺], increase, melatonin decreases the calcium concentration obviously by blocking the influx of calcium through the voltage-sensitive channels, because its effect may be mimicked by the channel blocker verapamil or by removal of calcium from extracellular medium (359, 360). In calcium-free medium, GnRH also induces increase of [Ca²⁺], but after the spike, the concentration rapidly declines, and the plateau phase is missing in most of the neonatal rat gonadotrophs; melatonin added after the GnRH-induced spike has no effect on [Ca²⁺], (Fig. 6B, right trace; Refs. 355, 361). These findings strongly support the conclusion that melatonin blocks influx of calcium. The melatonin inhibition of Ca²⁺ influx through voltage-sensitive channels may involve membrane potential, because melatonin hyperpolarizes plasma membrane of neonatal rat pituitary cells (see below), and hyperpolarization blocks calcium influx through the voltage-sensitive channels (359, 360).

The effect of melatonin on [Ca²⁺], in neonatal rat...
Recent findings indicate that melatonin may inhibit not only Ca\(^{2+}\) influx but also GnRH-induced mobilization of Ca\(^{2+}\) from intracellular stores (409). Melatonin added before or together with GnRH blocks or attenuates the initial spike of [Ca\(^{2+}\)], and the resulting oscillations of calcium-dependent potassium current in a subpopulation of neonatal gonadotrophs (Figs. 6, A and B, middle traces). These effects are independent of calcium influx and the membrane potential changes, because they occur in calcium-free medium and in voltage-clamped cells. These effects of melatonin are abolished in PTX-pretreated cells.

Melatonin inhibition of GnRH-induced [Ca\(^{2+}\)], is probably the main intracellular signal transducing the inhibitory effect of melatonin on LH release. The melatonin effect on LH release is mimicked by the calcium channel antagonists verapamil or nifedipine, which block calcium influx (362; Fig. 7). In their presence, GnRH-induced LH release is grossly attenuated, the residual stimulation of pituitary cells is dose dependent, with EC\(_{50}\) of $\sim 10^{-9}$ M (359, 361). The relative potency of a number of melatonin agonists is similar to their affinity for the melatonin receptor, indicating that melatonin is acting through these receptors. Melatonin inhibits GnRH-induced stimulation of [Ca\(^{2+}\)], only in neonatal, but not in adult, rat gonadotrophs, and the effect is blocked by pretreatment with PTX. These findings indicate that melatonin acts through the high-affinity melatonin receptors linked to effectors via G\(_i/G_o\) protein. However, not all gonadotrophs in the neonatal rat pituitary are sensitive to melatonin (361) (Fig. 5C). In 8-day-old rats, melatonin inhibited the GnRH-induced [Ca\(^{2+}\)], increase in $\sim 40\%$ of gonadotrophs. The relative number of melatonin-sensitive gonadotrophs is higher in neonates and decreases with age (unpublished data).

**FIG. 5.** Effect of melatonin (20 nM) on GnRH-induced (2 nM) increase of intracellular free calcium concentration ([Ca\(^{2+}\)]) in individual gonadotrophs from neonatal rats. Drugs were applied through times marked by horizontal lines. Melatonin was added after GnRH in all experiments. Two types of [Ca\(^{2+}\)], responses to GnRH in 3 different cells are shown. A, biphasic, with spike and plateau which are decreased after administration of melatonin. B and C: oscillatory with melatonin inhibiting oscillation in cell B but not in cell C. [Ca\(^{2+}\)], was measured by fura 2-AM. (From O. Slanar, H. Zemkova, and J. Vanecek. Biol. Signals 6: 284–290, 1997, with kind permission of Karger, Basel.)

**FIG. 6.** Effects of melatonin (20 nM) on GnRH-induced (2 nM) increase of [Ca\(^{2+}\)], in individual gonadotrophs cultured in normal or calcium-free media. Three consecutive experiments on each of 2 different cells (A and B) are shown. Drugs were applied through time marked by horizontal lines. In first experiments on both cells, melatonin administration started after GnRH administration to determine the effect of indole on late phase of GnRH-induced [Ca\(^{2+}\)], increase (calcium influx), which is dependent on presence of calcium in medium. In second experiments on both cells, melatonin administration started before GnRH to determine the indole effect on initial spike of [Ca\(^{2+}\)], (calcium mobilization), which is independent of extracellular calcium. [Ca\(^{2+}\)], was measured by fura 2-AM.
the LH-release by GnRH being probably because of mobilization of intracellular calcium. Melatonin has no effect on LH release in the presence of the calcium channel antagonists. Melatonin has also no effect on GnRH-induced LH release, when high [Ca\(^{2+}\)], is maintained by calcium ionophore. Therefore, the decrease of [Ca\(^{2+}\)\)] seems to be both a necessary and sufficient signal for inhibition of GnRH-induced LH release.

No effect of melatonin on [Ca\(^{2+}\)] has been reported in other cells. However, the only serious attempt published has studied the cells of ovine PT (225). In the PT cells, a general pharmacological activator of G protein-coupled systems, aluminum fluoride (24) has been used to increase [Ca\(^{2+}\)], because their natural stimulatory hormone is unknown. Melatonin had no effect on basal or aluminum fluoride-induced [Ca\(^{2+}\)]. However, this observation should not be interpreted as the proof that melatonin has no effect on [Ca\(^{2+}\)], in the PT cells, because aluminum fluoride activates not only G proteins coupled with phospholipase C, which increase [Ca\(^{2+}\)], through InsP\(_3\)-mediated mobilization, but also the PTX-sensitive G protein(s) coupled with the melatonin receptor. Therefore, it is not surprising that melatonin does not decrease [Ca\(^{2+}\)], in these studies, because the signaling pathway used by the melatonin receptor is already activated by aluminum fluoride.

On the basis of available knowledge, calcium might mediate the effects of melatonin also in other cells. The release of dopamine from retina is a calcium-dependent event (77, 82); melatonin thus might inhibit the dopamine release by decreasing [Ca\(^{2+}\)]. Similarly, because the frequency of spontaneous electric activity of SCN neurons is reduced in calcium-free medium (297), decrease of [Ca\(^{2+}\)], may be involved in the mechanism of melatonin action on the pacemaker firing rate. In addition, the contracting effect of melatonin in the caudal artery could also be mediated by a change of [Ca\(^{2+}\)], because the increased [Ca\(^{2+}\)], has been shown to induce vasoconstriction (42, 326, 371). However, in the above tissues, no effect of melatonin on [Ca\(^{2+}\)], has been reported so far.

**E. Hypothetical Mechanism of Melatonin Inhibition of [Ca\(^{2+}\)], Increase**

As shown above, melatonin inhibits mobilization of calcium from intracellular stores as well as the influx through the voltage-sensitive channels. It is quite possible that both effects are because of a single action of melatonin on a single effector. However, at present, it is difficult to propose a plausible unified hypothesis. Therefore, we discuss the melatonin effects on calcium mobilization and influx as two separate issues.

Membrane potential is important in controlling [Ca\(^{2+}\)], in gonadotrophs (168). The best recognized link between membrane potential and [Ca\(^{2+}\)], is through voltage-sensitive L-type calcium channels (151, 320, 334). The conductance of these channels increases when the cell is depolarized, i.e., when the membrane potential is decreased (334). Accordingly, it is possible that the effects of melatonin on [Ca\(^{2+}\)], might be mediated by an increase in membrane potential, a hyperpolarization. This would inhibit inward flow of calcium through these channels, resulting in a decrease of [Ca\(^{2+}\)].

To test this possibility, the membrane potential was measured indirectly using a membrane potential-sensitive
Melatonin treatment alone caused a small hyperpolarization, both in the presence and absence of calcium in the extracellular medium. In addition, GnRH had the opposite effect, a depolarization, which was reversed by melatonin. In calcium-free medium, the GnRH-induced depolarization was markedly augmented, and the repolarizing effect of melatonin was potentiated (Fig. 8A). The finding that melatonin was effective in the absence of calcium in the medium, which lowers [Ca\textsuperscript{2+}], in neonatal gonadotrophs and prevents the melatonin effects on Ca\textsuperscript{2+} influx, was especially important because it demonstrated that the effects of melatonin on membrane potential are not mediated by calcium. This has led to the conclusion that the hyperpolarization is a primary effect of melatonin, which inhibits calcium influx through the voltage-dependent channels rather than a secondary consequence induced by the changes of [Ca\textsuperscript{2+}].

Additional studies have revealed that melatonin induces hyperpolarization via PTX-sensitive G proteins, and the effect is age dependent (360). The relative potency of 6-hydroxymelatonin, 2-iodomelatonin, melatonin, and N-acetylserotonin in causing hyperpolarization is the same as that for inhibition of the GnRH-induced increase in [Ca\textsuperscript{2+}], and LH release. This indicates that all these effects are mediated by the same high-affinity melatonin receptor and the PTX-sensitive G protein.

Later it was found that the melatonin effects on membrane potential and on [Ca\textsuperscript{2+}], are both prevented when sodium in the medium was replaced with N-methylglucamine or choline (Fig. 8B; Ref. 360). Furthermore, the preliminary studies using the fluorescent dye sodium binding benzofuran phthalate (SBFT) have shown that GnRH induces an increase of intracellular concentration of sodium, and melatonin blocks the effect (unpublished data). Moreover, the measurements of \textsuperscript{86}Rb and \textsuperscript{36}Cl fluxes have indicated that the melatonin-induced changes of [Ca\textsuperscript{2+}], and membrane potential are not mediated by potassium or chloride. Together, the data suggest that GnRH-induced depolarization may involve sodium influx and that melatonin may hyperpolarize the cell by inhibiting sodium influx (Fig. 9). It is, however, not clear which channels or transporting mechanisms are responsible for the sodium fluxes. The presence of tetrodotoxin-sensitive sodium channels has been described in gonadotrophs (36, 340). These channels could mediate the effect of melatonin, but no possible mechanism has been described to date, and this possibility is therefore quite hypothetical. Alternatively, melatonin may inhibit sodium influx through cyclic nucleotide-gated cationic channels, which have been detected in a number of tissues including the pituitary (91, 231, 414; Fig. 9). These channels are opened by cAMP, and melatonin decreases the nucleotide concentration in the pituitary cells (366). Finally, melatonin might inhibit sodium influx through Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (Fig. 9). The exchanger is activated by increased concentration of intracellular calcium and transports out 1 Ca\textsuperscript{2+} in exchange for 3 Na\textsuperscript{+} coming in (or rather 1 Ca\textsuperscript{2+} and 1 K\textsuperscript{+} coming out and 4 Na\textsuperscript{+} coming in) (56, 401). The exchange is electrogenic; it increases the concentration of sodium inside the cell and depolarizes the cell membrane and could thus explain the changes of the membrane potential and of intracellular sodium concentration observed after GnRH treatment (see above). Depolarization is known to open voltage-sensitive calcium channels. Melatonin, by inhibiting the exchanger, thus could prevent the depolarization of the plasma membrane and inhibit further influx of calcium through L channels.

At present, the possibility cannot be excluded that melatonin primarily regulates permeability of the voltage-regulated calcium channels, e.g., by changing their phosphorylation-dephosphorylation status via cAMP-dependent kinase (3, 147); the decreased calcium influx would then attenuate the sodium/calcium exchange, resulting in decreased sodium influx and relative hyperpolarization of the cell. However, this possibility would not easily explain why the hyperpolarizing effect of melatonin is observed even in calcium-free medium, when no effect on [Ca\textsuperscript{2+}], could be seen (359, 361).

The mechanism of how melatonin blocks the GnRH-induced mobilization of intracellular calcium is even less clear. The mechanism of GnRH-induced calcium mobilization has been well described in adult rat gonadotrophs. Gonadotropin-releasing hormone acting via the specific receptor coupled with G\textsubscript{q}/G\textsubscript{11} protein stimulates phospholipase C activity and formation of InsP\textsubscript{3}, which binds to the InsP\textsubscript{3} receptor channel on endoplasmatic reticulum and releases calcium from the intracellular stores (132, 151, 214, 319, 334). Intracellular free calcium concentration in the gonadotrophs may hyperpolarize the cell by inhibiting sodium influx through cyclic nucleotide-gated cationic channels. Melatonin, when added before or together with GnRH, inhibits the GnRH-induced spike of [Ca\textsuperscript{2+}], and the following oscillations in ~21% of the neonatal rat gonadotrophs and partially reduces the GnRH-induced [Ca\textsuperscript{2+}], or
FIG. 8. Changes of membrane potential and \([\text{Ca}^{2+}]_i\) induced by GnRH (2 nM) and melatonin (100 nM) in suspension of neonatal rat pituitary cells cultured in normal, calcium-free (A) or sodium-free (B) media. Drugs were applied at time marked by arrows and remained present until end of trace. Melatonin was added after GnRH in all experiments. In calcium-free medium (A, right traces), GnRH induced only minor increase of \([\text{Ca}^{2+}]_i\), whereas depolarization was enhanced as compared with control medium (A, left traces). Melatonin induced repolarization but had no effect on \([\text{Ca}^{2+}]_i\) in calcium-free medium. In sodium-free medium (B, right traces), GnRH had no effect on membrane potential and induced only transient increase of \([\text{Ca}^{2+}]_i\), not followed by a plateau. Melatonin had no effect on either membrane potential or \([\text{Ca}^{2+}]_i\) in sodium-free medium. \([\text{Ca}^{2+}]_i\) was measured by fluo 3-AM. Changes of membrane potential were estimated by oxonol fluorescence. [Adapted from Vanecek and Klein (360).]
hyperpolarization of the plasma membrane may occur. According to this hypothesis, the melatonin-induced changes of intracellular sodium concentration and of membrane potential are regarded as secondary consequences, induced by the primary effect of melatonin on calcium mobilization and \([Ca^{2+}]_i\). The observation that in calcium-free medium melatonin added after GnRH has no obvious effect on \([Ca^{2+}]_i\) but still affects the membrane potential might be perhaps explained by buffering capacity of fura 2 and fluo 3 dyes. However, the hypothesis is quite speculative and based on some presumptions not supported by experimental data. To determine the causal relationship of the melatonin-induced changes, parallel recording of \([Ca^{2+}]_i\) by microfluorometry and of membrane potential by electrophysiological techniques should be used. Some parallel studies have been done already, but they have used fluorescent dyes for measurement of both \([Ca^{2+}]_i\), and membrane potential changes (361). Although in these studies the effect of melatonin on \([Ca^{2+}]_i\), seems to occur before the change of membrane potential, this cannot be taken for granted, because the potential-sensitive dyes need a much longer time for redistribution across the plasma membrane then needed for fura to bind calcium (15, 302, 396).

**F. Effects of Melatonin on Third Messengers**

Melatonin has also been shown to regulate activity of transcription factors. In ovine PT cells, forskolin induces phosphorylation of CREB, and melatonin inhibits the effect (209). The melatonin effect is dose dependent and time dependent. Interestingly, melatonin also inhibits the CREB phosphorylation induced by addition of 1% lamb serum (210). This effect is probably not mediated by cAMP, because no changes of the cyclic nucleotide concentration decreases the frequency of the oscillations in additional ~50% of the cells (409). Because the inhibition is observed in both calcium-free or calcium-supplemented media, the melatonin effect may not be ascribed to the inhibition of calcium influx. The melatonin effect may be because of either inhibition of phospholipase C and InsP\(_3\) formation, inhibition of calcium release from InsP\(_3\)-sensitive stores, stimulation of calcium removal from cytosol via uptake into intracellular stores, or transport to extracellular fluid by calcium pumps (Fig. 10).

Because no effect of melatonin on phospholipase C activity and InsP\(_3\) formation has been detected in our experiments, the hypothesis suggesting the primary site of the melatonin action on calcium pump or InsP\(_3\) receptor channel appears to be more attractive (Fig. 10). The channel is regulated not only by InsP\(_3\) but also by the concentration of calcium on cytoplasmic side (22, 23), and melatonin might affect the calcium sensitivity or other feature of the channel. The ATP-dependent Ca\(^{2+}\) pump removes calcium from cytoplasm and pumps it back to the intracellular stores in the endoplasmic reticulum. By activating the uptake, melatonin could decrease calcium concentration near the InsP\(_3\) channel and remove the potentiating effect of the cation.

The attenuated calcium mobilization may be responsible for reduced response of Na\(^+\)/Ca\(^{2+}\) exchanger as a result of the smaller increase of \([Ca^{2+}]_o\), and consequently, less Na\(^+\) may be transported into the cells and relative

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**FIG. 9.** Hypothetical model of melatonin action on GnRH-induced influx of calcium in neonatal rat gonadotrophs. GnRH induces \([Ca^{2+}]_i\), increase primarily by inositol triphosphate (InsP\(_3\))-mediated mobilization of calcium from intracellular stores followed by calcium influx through voltage-sensitive channels. Melatonin may inhibit calcium influx by hyperpolarizing plasma membrane induced by attenuated influx of Na\(^+\) via sodium/calcium exchanger or cyclic nucleotide-gated cationic channels. Alternatively, melatonin may affect permeability of voltage-regulated calcium channels by inhibiting their phosphorylation by cAMP-dependent kinase (see text for details). AC, adenyl cyclase; CNG, cyclic nucleotide-gated channel; ER, endoplasmic reticulum; \(K_{Ca}\), calcium-sensitive \(K^+\) channel; PLC, phospholipase C; VSCC, voltage-sensitive calcium channel; VSSC, voltage-sensitive sodium channel; +, stimulatory input; −, inhibitory input; SERCA, smooth endoplasmic reticulum Ca\(^{2+}\)-ATPase.

**FIG. 10.** Hypothetical model of melatonin action on GnRH-induced mobilization of calcium in neonatal rat gonadotrophs. Melatonin may inhibit PLC activity and InsP\(_3\) formation, block calcium release from intracellular stores, or stimulate calcium uptake (see text for details). Definitions are as in Fig. 9.
centration have been observed after the treatment. This observation lends some support to the possibility that melatonin may act not only via cAMP but also through other second messengers in PT cells.

Melatonin also inhibits forskolin-stimulated induction of c-fos and jun B mRNA as well as of c-Fos protein in ovine PT cells (283). Furthermore, melatonin inhibits the GnRH-induced c-Fos immunoreactivity in neonatal rat pituitary cells cultured in vitro (327). Expression of c-Fos is controlled by the upstream regulatory elements within the promoter that are controlled by multiple second messengers, including serum responsive and Ca$$^{2+}$$/cAMP response elements (Ca-CRE) (104, 219, 293). According to the recent finding, GnRH-induced c-fos expression in gonadotrophs appears to be mediated by protein kinase C and calcium (57). Melatonin inhibits the GnRH-induced increase of [Ca$$^{2+}$$], as well as the increase of diacylglycerol concentration, which is known to regulate protein kinase C activity (359, 367). Moreover, transcription through Ca-CRE requires the induced phosphorylation of the CREB (104), and melatonin inhibits GnRH-induced increase of cAMP in neonatal pituitary (366). Any of these second messengers may thus transduce the melatonin effect on GnRH-induced c-Fos.

The transcription factors are initiating the transcriptional activity of the cell. The above findings thus suggest that melatonin may regulate expression of structural genes in neonatal rat gonadotrophs, PT cells, or SCN neurons. In this respect, it would be interesting to study the effect of melatonin on synthesis of LH and FSH in the gonadotrophs, because the indole might regulate not only their release but also their synthesis. The secondary genes that could be regulated by melatonin in the SCN or PT cells are unknown. Nevertheless, melatonin might act via CREB phosphorylation when inhibiting the forskolin-induced synthesis of several proteins (221).

V. IMPLICATIONS FOR MECHANISM OF MELATONIN ACTION

A. Two Hypotheses Explaining the Mechanism of Melatonin Action on Seasonal Rhythms

The in vitro modeling may be used to solve one of the central questions of melatonin physiology: how the pattern of melatonin secretion triggers the photoperiodic response. Several years ago it was recognized that the response does not depend on the total amount of melatonin synthesized, because melatonin implantations giving constant increase of the indole concentration in circulation have not induced the short-day response in Syrian hamsters. In fact, the implants prevented the short day-induced response (263, 266). The use of timed melatonin infusions then revealed the importance of alternating the periods of high and low circulating concentrations of the hormone (46, 47, 109, 331, 333). However, the melatonin-free interval may be important in hamsters (203) but not in sheep or mice (246).

Presently, the two hypotheses are discussed, one emphasizing duration of the melatonin pulse and the other its phase relationship as the trigger of photoperiodic response. On transition to short days, the duration of the melatonin pulse is prolonged (139, 141). Concomitantly, the change of photoperiod may also alter the phase relationship and temporal order of circadian rhythms (253).

The experimental strategy testing most directly which features of the melatonin signal trigger photoperiodic responses is daily melatonin infusion. The animals are pinealectomized to prevent interference by endogenous melatonin. Melatonin is infused for a specific number of hours usually once daily and at a predetermined time for several days. With the use of this paradigm, it has been shown that the long (8–12 h) melatonin pulse to pinealectomized hamsters induces the gonadal involution regardless of which phase of the daily cycle it is applied, whereas the short pulses of <7 h have no such effect (117, 200). Most of the experimental data indicate that duration of the melatonin stimulation is the critical feature of the melatonin signal for both inhibitory and stimulatory effects of the hormone on the reproductive development in juvenile Siberian hamsters and for reproductive and metabolic responses in adult Siberian and Syrian hamsters and sheep (30, 46, 47, 109, 200, 277, 404). One of the most convincing pieces of evidence is the observation that long melatonin pulses infused randomly at one of the three different phases of the day induce the short-day gonadal response in pinealectomized Syrian hamsters (116, 117).

The phase coincidence model presumes the existence of endogenous rhythm in sensitivity to melatonin, which is driven by circadian oscillator different from that driving the rhythm of melatonin synthesis (311). With a change of photoperiod, the phase relations of the rhythms may change. The photoperiodic response is triggered when both rhythms coincide, i.e., when high melatonin concentration occurs during the window of sensitivity. The hypothesis has been originally based on observations that in hamsters kept on long days, melatonin injections induce gonadal involution when given daily in late afternoon or very early in the morning, whereas injections in other times are ineffective (311). Because these experiments have been performed on pineal-intact animals, the obvious criticism has been that the rhythm of sensitivity is rather due to apposition of the injected melatonin to the rhythm of endogenous melatonin synthesis, and therefore, it would rather fit the duration hypothesis. When melatonin is injected shortly before or immediately after the period of endogenous melatonin increase, the duration of high melatonin levels is prolonged and reproduc-
tive change is triggered. Melatonin injections at other times are perceived as isolated pulses that do not add to the duration of endogenous melatonin increase and therefore have no effect. In agreement with this view, single daily injection of melatonin in late afternoon to pinealectomized hamsters did not trigger the response (331, 378). The response was induced only when triplicate melatonin injections 90 min apart were given every day.

However, the recent experiments on pinealectomized Siberian hamsters may somewhat complicate the view. These show that very short (1-h) daily melatonin infusions may be effective as the photoperiodic signal when applied during sensitive windows, just after the lights are turned off (310). It is argued that the melatonin pulse may have duplicate effects. First, it entrains the circadian rhythms, among others also the rhythm of sensitivity to melatonin, and second, it induces the photoperiodic response when high melatonin concentration coincides with the sensitive window.

Although most of the in vivo experiments support the duration hypothesis, the question can hardly be definitively solved, because, due to the complexity of the system, the in vivo experiments leave usually enough space for alternative explanation of all data. Therefore, the final proof for the duration hypothesis should be finding of an in vitro system responding differently to the “long” and “short” melatonin pulses and description of the cellular mechanism, enabling one to recognize them.

B. Modeling the Photoperiodic Signal In Vitro

The location of the melatonin target for photoperiodic regulations is not determined quite precisely, but according to the recent studies, melatonin might act through more than one structure even within one species. In the Syrian hamsters, the melatonin target for seasonal regulation of LH and gonadal status in the hamsters is within mediobasal hypothalamus, but the target for prolactin regulation seems to occur outside of this area, most likely directly in the pituitary (183, 184, 201). Another target site may be the SCN, because the microinfusions of 20 pg melatonin/day into the nucleus inhibited the gonadal maturation in Siberian hamsters, whereas melatonin infused to any other brain site had no effect (9).

The finding showing that more than one target site may mediate the photoperiodic response to melatonin signal is important, because it suggests that ability to read the information encoded in the pattern of melatonin secretion may exist in several different cells equipped with melatonin receptor. However, this assumption may not necessarily be correct.

To test the duration hypothesis, the effect of long-term melatonin treatment has been examined in cultured cells from ovine PT and from neonatal rat PD of the pituitary. The available data from both cultures do not indicate a clear effect of the duration of the melatonin treatment on the response of the cells. With the use of cultured hemipituitaries from neonatal rats, potentiation of the inhibitory effect after long-term pretreatment with melatonin has been described (354). Gonadotropin-releasing hormone-induced LH release was inhibited more severely when the explants were pretreated with melatonin for 6 h than when the indole was added just 20 min before GnRH (354). However, these observation could not be replicated using dispersed cells from the neonatal rat pituitary (V. Pelisek and J. Vanecek, unpublished data). In these cells, the length of incubation with melatonin up to 18 h had no effect, because melatonin always produced ~50% inhibition of GnRH-induced LH release. Nevertheless, melatonin attenuated the development of desensitization induced by long treatment with GnRH.

In cells of ovine PT, the prolonged pretreatment with submaximal dose of melatonin potentiated the stimulatory effect of forskolin on cAMP accumulation (122). After the acute withdrawal of melatonin after 10- to 16-h pretreatment, forskolin-stimulated cAMP production was amplified two- to eightfold compared with untreated cells. This sensitization is time dependent, with no effect apparent after 4 h, but reaching the maximal effect after 16 h. However, similar sensitization has been observed for other receptors that have an inhibitory effect on cAMP, e.g., \( \alpha_{1c} \) receptor, D receptor, and somatostatin (16, 154, 260). The maximal inhibitory effect of melatonin on forskolin-stimulated cAMP production is not decreased after the prolonged preincubation with melatonin, but the response desensitizes and melatonin EC\(_{50}\) is shifted by about an order from ~19 to 150 pM (122, 223).

The coincidence hypothesis receives somewhat more support from the in vitro experiments. The inhibitory effect of melatonin on spontaneous electric activity of cultured SCN explants has clear daily rhythm, with maximum during the late subjective day (196, 295, 307). The SCN is important because it may be the melatonin target site for photoperiodic signaling in Siberian hamsters. In other cells, however, the high-amplitude daily rhythm of sensitivity to melatonin has not been observed. Melatonin's inhibitory effect on GnRH-induced LH release does not show daily rhythmicity. However, the low-amplitude daily rhythm in the melatonin effect on GnRH-induced cAMP accumulation has been observed. Although melatonin has significant inhibitory effect on cAMP accumulation regardless of the time, a somewhat greater inhibitory effect of melatonin on GnRH-induced cAMP accumulation has been observed in the evening than in the morning (366). These changes may be because of the changes in the receptor number, which also increases in the evening. The daily variations are, however, too small to explain the profound changes induced by melatonin in vivo depending on the timing of the application.
VI. CONCLUSIONS

Two things should be kept in mind, however. First, the hypothetical daily rhythm of sensitivity to melatonin may be generated outside of the cultured tissue and imposed on the cells bearing melatonin receptors through the endocrine or neural connections. In such a case, the ordinary in vitro system would be too simplified to detect the rhythm of sensitivity to melatonin. Another thing that might affect the responsiveness and obstruct the melatonin signaling is altered intercellular communication due to the cell dispersion or partial degeneration of neural connection in long-term organotypic cultures. Although a synchronized rhythm of neuronal activity has been observed in cultured SCN slices for the first 3 days (114, 204, 298, 306), in organotypic cultures kept in vitro for more than 1 wk the rhythm in individual neurons is desynchronized (216). Also, in dispersed SCN neurons cultured in vitro, the circadian rhythm of electric activity in individual cells is maintained even after several weeks, but the neurons are out of phase (384). This may be because of the missing intercellular communication. Interestingly, the rhythm of arginine vasopressin release is running in phase for several weeks in both organotypic cultures and dispersed neurons (88, 229, 377). One explanation might be that both rhythms are generated in different subpopulations of cells, or the endocrine products are able to synchronize the rhythm in individual cells.

VI. CONCLUSIONS

Melatonin regulates the function of various cells involved in photoreception and light adaptation, but also in some secretory and vascular cells. The melatonin effects are mediated by the specific high-affinity receptors localized on the plasma membrane. On the basis of the molecular structure, three subtypes of the melatonin receptor have been described, but only MEL_{1A} and MEL_{1B} subtypes may be distinguished pharmacologically according to the affinity for the specific antagonists.

Melatonin receptors are coupled to G proteins belonging to the G_{i/o} family in the vast majority of cells. In agreement with this, most of the melatonin effects are prevented by PTX pretreatment. The only known exception has been found in ovine PT cells, where melatonin receptors couple through both PTX-sensitive and cholera toxin-sensitive mechanisms to inhibit cAMP accumulation. This suggests the involvement of two G proteins in transduction of melatonin signal in the PT cells.

The melatonin receptor regulates several second messengers: cAMP, cGMP, diacylglycerol, arachidonic acid, InsP_3, and [Ca^{2+}]. In many cases, its effect is inhibitory and requires previous activation of the cell by a stimulatory agent. Forskolin-induced cAMP accumulation is inhibited by melatonin in most of the cells bearing the melatonin receptors. In contrast, the inhibitory melatonin effects on diacylglycerol, arachidonic acid, and [Ca^{2+}], have been described in neonatal rat pituitary only, whereas the stimulatory effect of the indole on InsP_3 has been described in chicken brain and on protein kinase C in rat SCN only. However, because the interest in the transduction mechanisms of the melatonin signal was aroused quite recently, well after the description of the high-affinity melatonin receptors in 1987, more findings on the effect of the indole on various second messengers might be expected in the future.

The same holds true for the molecular mechanisms of the melatonin effects on individual second messengers. Presently these are not clear. Because the melatonin effects on all second messengers are sensitive to PTX, it cannot be excluded that they are mediated by common primary effector. However, no direct data supporting this hypothesis are available. On the contrary, at least two melatonin effects on second messengers seem to be independent and separable, mediated by parallel transduction pathways. This holds true about the melatonin effects on cAMP accumulation and [Ca^{2+}], in neonatal rat pituitary cells as well as for the indole effects on cAMP and arachidonic acid in the NIH 3T3 cells transfected with MEL_{1A} receptor. The available data indicate that inhibition of forskolin- or GnRH-induced cAMP accumulation is most likely because of the inhibition of adenyl cyclase, whereas stimulation of arachidonate release is likely mediated by the increase of phospholipase C activity. Decrease of GnRH-induced [Ca^{2+}] is probably because of the increase of membrane potential mediated by a yet unknown sodium-dependent mechanism.

Melatonin also regulates the third messengers, namely, the phosphorylation of CREB and expression of c-Fos. Interestingly, the stimulatory effect of melatonin on c-Fos immunoreactivity has been found in SCN, whereas in the pituitary cells, melatonin inhibits the forskolin or GnRH-induced c-Fos. Melatonin may thus regulate the expression of late response genes. Indeed, the effect of melatonin on the proteosynthetic activity in PT cells has been described.

Although an effort has been made to use the in vitro models to study the mechanism of melatonin action in photoperiodic regulation, no conclusive data have been available until now.

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