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Melatonin Action in Neonatal Gonadotrophs

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Summary

Neonatal pituitary cells express MT_1 and MT_2 subtype of melatonin receptors that are coupled to pertussis toxin-sensitive G proteins. Their activation by melatonin leads to a decrease in cAMP production and activity of protein kinase A, and attenuation of gonadotropin-releasing hormone (GnRH)-induced gonadotropin secretion. Single cell calcium and electrophysiological recordings have revealed that a reduction in gonadotropin release results from melatonin-induced inhibition of GnRH-stimulated calcium signaling. Melatonin inhibits both calcium influx through voltage-dependent calcium channels and calcium mobilization from intracellular stores. Inhibition of calcium influx, probably in a cAMP/protein kinase C-dependent manner, and the accompanying calcium-induced calcium release from ryanodine-sensitive intracellular pools by melatonin results in a delay of GnRH-induced calcium signaling. Melatonin-induced attenuation of GnRH-induced and inositol (1,4,5)-trisphosphate-mediated calcium release from intracellular pools attenuates the amplitude of calcium signal. The potent inhibition of GnRH-induced calcium signaling and gonadotropin secretion by melatonin provides an effective mechanism to protect premature initiation of pubertal changes that are dependent on plasma gonadotropin levels. During the development, such tonic inhibitory effects of melatonin on GnRH action gradually decline due to a decrease in expression of functional melatonin receptors. In adult animals, melatonin does not have obvious direct effects on pituitary functions, whereas the connections between melatonin release and hypothalamic functions, including GnRH release, are preserved, and are critically important in synchronizing the external photoperiods and reproductive functions through still not well characterized mechanisms.

Key words

Melatonin • Melatonin receptors • Gonadotrophs • GnRH • Calcium oscillations • Reproduction

Introduction

A majority of wild species have seasonal reproduction in order to give birth at the optimal time of year, usually spring, allowing the newborn to grow and develop under favorable temperature and food availability conditions. Decreasing photoperiod lengths indicate that winter is approaching and allows species to prepare in advance. Annual cycle of external light thus plays an important role in the reproduction and causes seasonal changes in reproductive behavior, weight gain, appetite, body fat storage, energy metabolism, growth of fibers and horns or hibernation (Bartness *et al.* 1993).

In this very complex process that reflects on activity of numerous pathways, the production of melatonin plays a central role. Melatonin is necessary and sufficient for entrainment of seasonal photoperiodic responses to the annual cycle of day length (Kennaway

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and Rowe 1995). The activity of melatonin-producing enzyme in pineal gland, arylalkylamine N-acetyltransferase (serotonin N-acetyltransferase, EC2.3.1.87), precisely reflects duration of the night (Hoffmann *et al.* 1981). During the day, melatonin level is decreased due to inhibitory neuronal outputs from suprachiasmatic nuclei (SCN) (Kalsbeek *et al.* 2000). In addition to controlling the seasonal biological rhythms in mammals, melatonin also participates in the coordination of circadian rhythms (Cassone 1990, McArthur *et al.* 1991, Svobodová *et al.* 2003). Humans also secrete melatonin in a pattern that reflects the environmental light-dark cycle, but the seasonal melatonin information is not an integral part of their reproductive cycle.

The extracellular messenger functions of melatonin are mediated by its high-affinity membrane receptors expressed in target tissues. In mammals, melatonin receptors are expressed in high density in the hypothalamic neurons localized in the SCN and gonadotropin-releasing hormone (GnRH)-secreting neurons within the preoptic area and/or the mediobasal hypothalamus, depending on the species. Melatonin receptors are also present in pars tuberalis and pars distalis regions of anterior pituitary (Fig. 1). Melatonin receptors in the SCN are responsible for the phase-shifting effects on the circadian rhythms (Armstrong *et al.* 1986, McArthur *et al.* 1991, Liu *et al.* 1997). Hypothalamic GnRH neurons (Roy *et al.* 2001, Roy and Belsham 2002) and pituitary (Vaněček and Klein 1992a, Zemková and Vaněček 1997) are the main sites of the reproductive actions of melatonin. However, the actions of melatonin in anterior pituitary are time-limited, as melatonin receptor expression progressively decreases over the perinatal period (Vaněček 1988b, Williams *et al.* 1991).

The primary structures of two high-affinity melatonin receptor subtypes, termed MT_1 and MT_2, have been elucidated (Reppert *et al.* 1994, 1995a). The principal effects of native melatonin receptors in many cell types are mediated by inhibiting the activity of adenylyl cyclase, leading to a decrease of intracellular cAMP levels (White *et al.* 1987, Carlson *et al.* 1989, Vaněček and Vollrath 1989) and inhibition of cellular processes regulated by this intracellular messenger (Barrett *et al.* 1998, Ross *et al.* 1998). Functional studies with recombinant MT_1 and MT_2 receptors confirmed that melatonin inhibits agonist- and forskolin-stimulated adenylyl cyclase (Reppert *et al.* 1994, 1995b, Liu *et al.* 1997, Teh and Sugden 1999). In cells expressing MT_1 receptors, inhibition of adenylyl cyclase pathway is occasionally accompanied by simultaneous facilitation of phospholipase C activity, an enzyme that produces two intracellular messengers: inositol (1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG) (Godson and Reppert 1997, Brydon *et al.* 1999). Dual effect of melatonin, i.e. inhibition as well as stimulation of GnRH-induced calcium signaling, has been confirmed in neonatal pituitary cells expressing endogenous melatonin receptor (Zemková and Vaněček 2001). Activation of the MT_2 receptors also leads to inhibition of soluble guanylyl cyclase activity (Petit *et al.* 1999), but to which extent this is a receptor-specific function is unclear.

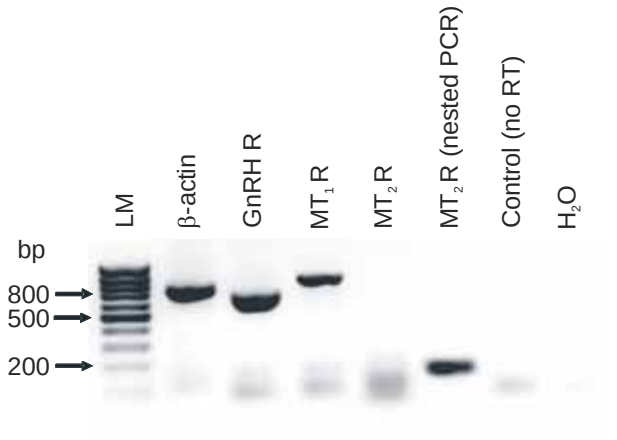

Fig. 1. RT-PCR analysis of transcripts for MT_1 and MT_2 receptors in pituitary cells. In samples of total RNA isolated from pituitary cells of 6- to 10-day-old rats, the mRNAs for GnRH receptor and MT_1 melatonin receptor were easily detected, whereas nested-PCR had to been applied on PCR products from RT-PCR reaction to detect the message for MT_2 melatonin receptor. These results indicate that MT_1 receptor provides the major pathway by which melatonin inhibits gonadotropin secretion (Balík and Zemková, unpublished).

Melatonin control of reproduction

The role of melatonin in animal reproduction was confirmed in experiments with its exogenous infusion, which successfully mimics the seasonal effects of changing photoperiod (Badura and Goldman 1992).

Melatonin probably regulates reproduction at three levels: the hypothalamic GnRH neurons, pituitary and gonads and reproductive tissues. Melatonin micro-implants into the area of preoptic and mediobasal hypothalamus of mice (areas that contain GnRH neurons) elicit complete gonadal involution, whereas its injection in other areas was ineffective (Glass and Knotts 1987). It has been hypothesized that melatonin acts directly on synapses of hypothalamic neurons and inhibits reproduction by decreasing GnRH synthesis and release

(Glass and Knotts 1987, Kennaway and Rowe 1995). However, the effect of melatonin is apparently more complex and in accordance with this, it has been found that melatonin regulates GnRH gene transcription in the immortalized GnRH-secreting neurons in a cyclic manner (Roy *et al.* 2001, Roy and Belsham 2002). The possibility that melatonin directly modulates pituitary function was established in 1987 with the finding that melatonin receptors are expressed in pituitary (Reppert *et al.* 1988, Vaněček 1988b). The subsequent investigations in neonatal gonadotrophs revealed that melatonin inhibits GnRH-induced increases in several intracellular messengers, including cAMP (Vaněček and Vollrath 1989), DAG (Vaněček and Vollrath 1990) and intracellular calcium concentrations ($[Ca^{2+}]_i$) (Vaněček and Klein 1992a). Melatonin also acts at the level of the gonads, where it modulates androgen production by Leydig cells (Valenti *et al.* 1997, Li *et al.* 1998). In prostate epithelial cells, melatonin suppresses cGMP levels (Gilad *et al.* 1997).

Localization and structure of melatonin receptors

The radioligand ^{125}I-melatonin has been commonly used to localize binding sites in central and peripheral tissues. Autoradiographic studies indicated that melatonin receptors are expressed in brain, with high density of receptors in hypothalamic SCN (Vaněček *et al.* 1987, Reppert *et al.* 1988, Duncan *et al.* 1989, Siuciak *et al.* 1990), as well as outside the brain, including retina (Dubocovich 1985), anterior pituitary (Reppert *et al.* 1988, Vaněček 1988b, Williams and Morgan 1988), some arteries (Viswanathan *et al.* 1990), and cells of immune system (Lopez-Gonzalez *et al.* 1992). The pars tuberalis of anterior pituitary contains the highest concentration of melatonin receptors of all mammalian tissues, whereas in pars distalis melatonin binding is restricted to gonadotroph fraction of secretory cells (Williams *et al.* 1991, 1997, Skinner and Robinson 1995). Melatonin receptors are expressed more widely in fetal and newborn animals, both in the CNS (Williams *et al.* 1991) and pituitary (Vaněček 1988b, Laitinen *et al.* 1992). However, within the pituitary, melatonin receptor persists until adulthood in the pars tuberalis only (Morgan and Williams 1996), whereas in the pars distalis melatonin receptor number gradually declines over the perinatal period (Vaněček 1988b, Laitinen *et al.* 1992) to about 10 % of the neonatal number of melatonin receptors in adult pituitary (Vaněček 1988b).

The structure of two melatonin receptor subtypes, called MT_1 and MT_2, has been identified in mammals by cloning (Reppert *et al.* 1994, 1995a, Roca *et al.* 1996, Gauer *et al.* 1998). Both receptors belong to the class of G protein-coupled receptor superfamily that signals through pertussis toxin-sensitive pathways (White *et al.* 1987, Carlson *et al.* 1989, Morgan *et al.* 1994). Similarly as other G protein-coupled receptors, melatonin receptors contain seven transmembrane domains connected by three extracellular and three intracellular loops. Transmembrane domains V, VI and VII are suggested to be involved in specific interaction between receptor and ligand (Navajas *et al.* 1996). MT_1 binds melatonin with K_d of < 200 pM, whereas MT_2 binds this agonist with K_d between 1 and 10 nM (Duncan *et al.* 1989). There are very few pharmacological tools for the study of MT_1 and MT_2 receptors (Sugden *et al.* 1998, Spadoni *et al.* 1999). Luzindole is the only known effective antagonist that has slight selectivity for the MT_2 receptor subtype (Dubocovich 1988). Structural analysis and site-directed mutagenesis of helical part of human MT_2 receptor revealed that Val^{204}, Leu^{272} and Tyr^{298} in transmembrane domains V, VI and VII are critical for the melatonin binding (Fig. 2) (Mazna *et al.* submitted).

The mammalian MT_1 subtype seems to be widely expressed and functionally important subtype, whereas the expression of MT_2 subtype is more localized (Weaver *et al.* 1996, Liu *et al.* 1997). The MT_1 receptor is expressed in hypothalamic SCN and hypophyseal pars tuberalis (Reppert *et al.* 1994). Quantification of MT_1 mRNA expression by PCR and melatonin binding revealed that developmental decrease in melatonin receptor number in the pars tuberalis and SCN of Syrian hamster could not be attributed to the inhibition of the mRNA expression, but rather could be related to a post-transcriptional blockade of the MT_1 receptor expression (Gauer *et al.* 1998). There are also circadian variations in melatonin receptor density in pars tuberalis, which is directly regulated by the daily variations of melatonin itself (Gauer *et al.* 1993). During the long photoperiod, melatonin receptor density in Syrian hamster pars tuberalis reaches its maximum in the first half of the light period and its minimum at the end of the night (Recio *et al.* 1998).

The mammalian MT_2 melatonin receptors have been found in retina and brain (Reppert *et al.* 1995a). The role of MT_2 melatonin receptor appears to mediate the melatonin inhibition of dopamine release in retina (Reppert *et al.* 1995a, Liu *et al.* 1997, Dubocovich *et al.* 1998). In MT_1 receptor-deficient mice, the MT_2

melatonin receptor substitutes the role for MT_1 in the phase-shifting response (Liu *et al.* 1997).

Other forms or states of the mammalian melatonin receptor may also exist in mammalian brain. This includes the expression of low-affinity melanin receptor, called MT_3 receptor, identified in central and peripheral hamster tissues (Paul *et al.* 1999, Nosjean *et al.* 2000).

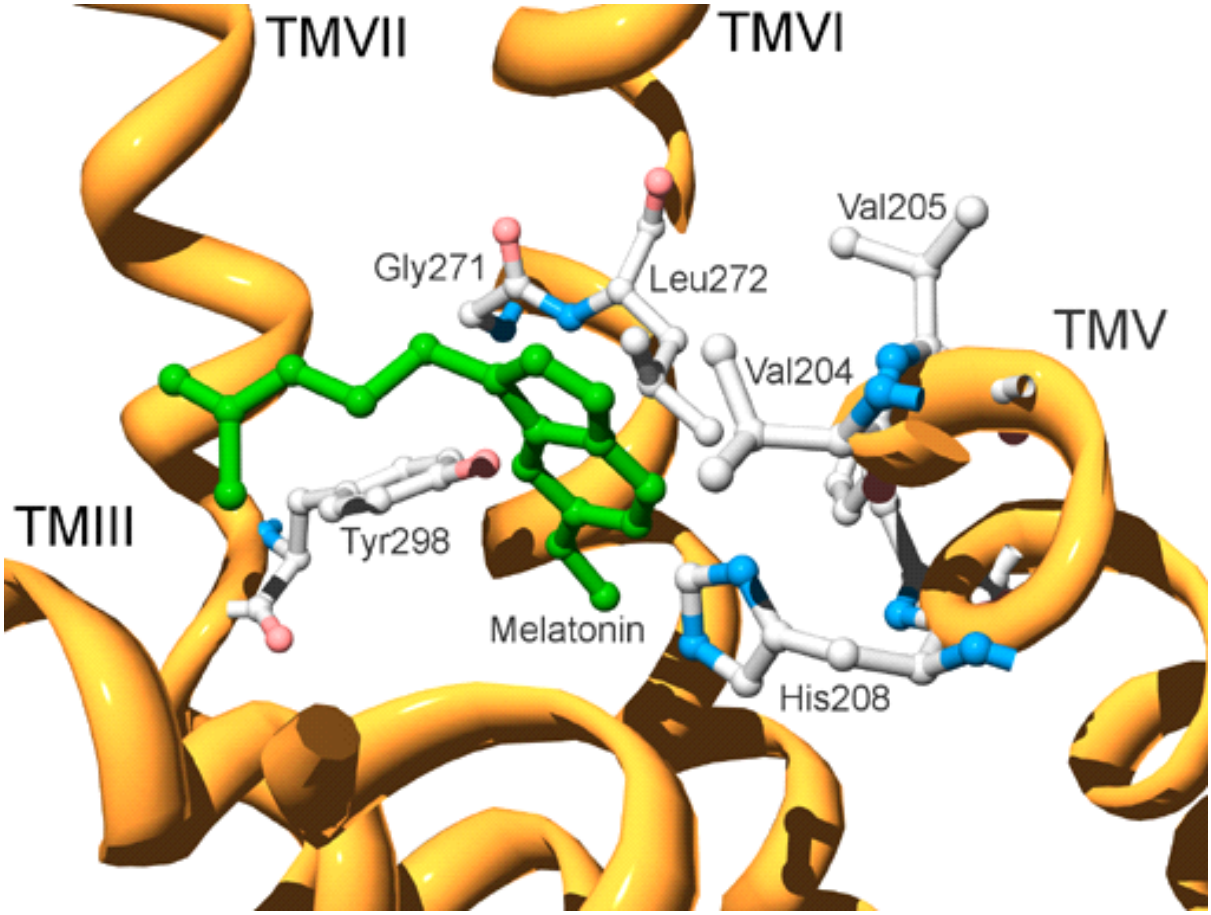

Fig. 2. The model of the hMT_2 receptor with bound melatonin. Three dimensional model of helical part of human MT_2 melatonin receptor with presumptive position of melatonin molecule. Only amino acid residues in transmembrane domains (TMs) V, VI and VII of the hMT_2 receptor found to be specifically involved in the melatonin-receptor interaction are shown. The molecule of melatonin is shown in gray (Mazna *et al.* submitted).

Expression of melatonin receptors in pituitary

The MT_1 melatonin receptor mRNAs has been found in pituitary pars tuberalis of the rat (Reppert *et al.* 1994, Gauer *et al.* 1998, Guerrero *et al.* 2000). *In situ* hybridization showed that gonadotrophs, which represent a very small fraction of pars tuberalis cells, do not express MT_1 receptor (Klosen *et al.* 2002). MT_1 receptor is also expressed in pars distalis and is localized in gonadotroph fraction. The expression of MT_2 subtype is more questionable. So far studies indicate the presence of both subtypes in teleost fish pituitary (Gaildrat and Falcon 2000). Our recent RT-PCR analysis suggested that both subtypes of melatonin receptor mRNAs are also expressed in the anterior pituitary from neonatal rats, but the expression of MT_1 melatonin receptor is more robust (Fig. 1). During the attempt to clone melatonin receptors from the human pituitary, a melatonin receptor-related protein was identified. This protein does not bind iodo-melatonin and its endogenous ligand and physiological roles are to be established (Reppert *et al.* 1996).

In the anterior pituitary gland, melatonin mediates the effect of photoperiod acting primarily at two secretory cell types: lactotrophs that secrete prolactin, and gonadotrophs that secrete two gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The mechanisms of melatonin actions in lactotrophs are unknown at large (Morgan 2000), whereas its actions in gonadotrophs are well documented. Gonadotrophs of pars

distalis express melatonin receptors and their activation leads to the inhibition of GnRH-controlled gonadotropin release (Lincoln and Clarke 1994). However, these effects were observed only in neonatal gonadotrophs (Martin and Klein 1976, Vaněček and Klein 1992a,b, 1993, Zemková and Vaněček 1997). This coincides with an early expression of high-density melatonin receptors and their gradual decline in the pituitary during the postnatal development (Vaněček 1988b). At the present time, it is not clear what underlies the lack of effects of melatonin in adult gonadotrophs: the low expression level of receptors and/or the lack of effective coupling of residual receptors to intracellular signaling pathways.

Signal transduction pathways of GnRH melatonin receptors

Reproductive functions in vertebrates are controlled by neuropeptide GnRH, also known as luteinizing hormone-releasing hormone (LHRH). GnRH is synthesized by hypothalamic GnRH neurons and is secreted in a pulsatile manner into the hypophyseal portal system. So far, sixteen forms of GnRH have been isolated from the brain of vertebrates. In the vast majority of species, several forms occur in anatomically and developmentally distinct neuronal populations. In mammalian brain, two GnRH forms coexist. The second type, GnRH-II, is the most evolutionarily conserved form of GnRH (White *et al.* 1998), but its function is not yet known, and up to now type II GnRH receptor has been cloned from Marmoset monkey only (Millar *et al.* 2001).

GnRH binds with a high affinity to plasma-membrane GnRH receptors in gonadotroph cells (Perrin *et al.* 1989). Like melatonin receptors, GnRH receptor belongs to the rhodopsin-like family of seven transmembrane domain receptors (Reinhart *et al.* 1992, Tsutsumi *et al.* 1992). In contrast to melatonin receptor, GnRH receptor is coupled to a pertussis toxin-insensitive G_q/G_{11} proteins that stimulate phospholipase Cβ (PLCβ) pathway, leading to the generation of IP_3 and DAG production (Morgan *et al.* 1987, Chang *et al.* 1988) and activation of phospholipase D pathway (Zheng *et al.* 1994). However, a growing number of information also indicates that GnRH receptor cross-couples to G_s and $G_{i/o}$-signaling pathway (Krsmanovic *et al.* 2003).

The signaling by GnRH receptors is relatively well established in gonadotrophs from adult rats (Stojilkovic *et al.* 1994, Hille *et al.* 1995), but less is known about agonist-induced signaling in immature gonadotrophs (Tomic *et al.* 1994). In adult gonadotrophs, generated IP_3 binds to specialized tetrameric IP_3 receptor-channel complex that spans the endoplasmic reticulum membrane (Berridge 1993) and triggers oscillatory release of Ca^{2+} from the endoplasmic reticulum. DAG activates Ca^{2+}-dependent protein kinase, which in turn affects several pathways, including the extracellular Ca^{2+} entry *via* voltage-dependent calcium channels. Such influx is necessary for the long-lasting maintenance of oscillations in intracellular calcium concentrations ($[Ca^{2+}]_i$) (Shangold *et al.* 1988, Stojilkovic *et al.* 1991). The cross-coupling of GnRH receptors to G_s signaling pathway may also participate in modulating voltage-gated Ca^{2+} influx and Ca^{2+} release from IP_3-sensitive intracellular pool.

GnRH-stimulated increase in the $[Ca^{2+}]_i$ is oscillatory and gonadotropin release is also oscillatory (Hille *et al.* 1994). The recovery to the baseline $[Ca^{2+}]_i$ levels in oscillating cells is accomplished by Ca^{2+} sequestering into the endoplasmic reticulum and mitochondria and by efflux outside the cell by Ca^{2+} pump-ATPase and Na^+/Ca^{2+} exchange system in the plasma membrane (Zemková *et al.* 2004). GnRH-stimulated increase in $[Ca^{2+}]_i$ together with activated protein kinase C regulates many other cellular functions, including ion channel activity and gene expression (Cesnjaj *et al.* 1994). The GnRH-induced $[Ca^{2+}]_i$ transients trigger oscillatory changes of membrane potential driven by rhythmic opening and closing of apamin-sensitive, Ca^{2+}-activated K^+ channels. In voltage-clamped cells, measurements of Ca^{2+}-activated K^+ current can substitute for calcium measurements (Fig. 3A) and were frequently used as an additional method in characterization of the nature of calcium signaling in immature and adult gonadotrophs (Kukuljan *et al.* 1992, Tse and Hille 1992, Zemková and Vaněček 1997). There are three obvious advantages for such measurements. First, this current monitors the $[Ca^{2+}]_i$ changes in the plasma membrane domain. Second, the possible chelating effects of calcium dyes are eliminated. Third, the membrane potential is controlled, which provides an elegant method to eliminate voltage-dependent calcium influx or to substitute the periodic calcium influx with the steady calcium influx. However, this current depends on $[Ca^{2+}]_i$ in a nonlinear and saturable manner, indicating that the amplitude of responses should be interpreted with reservation.

Using such measurements, we found that the oscillatory pattern of GnRH-induced $[Ca^{2+}]_i$ responses in immature gonadotrophs are sensitive to melatonin (Fig. 3B). Neonatal gonadotrophs exhibit some additional characteristics not present in gonadotrophs from adult animals (Zemková and Vaněček 1997, 2000, 2001).

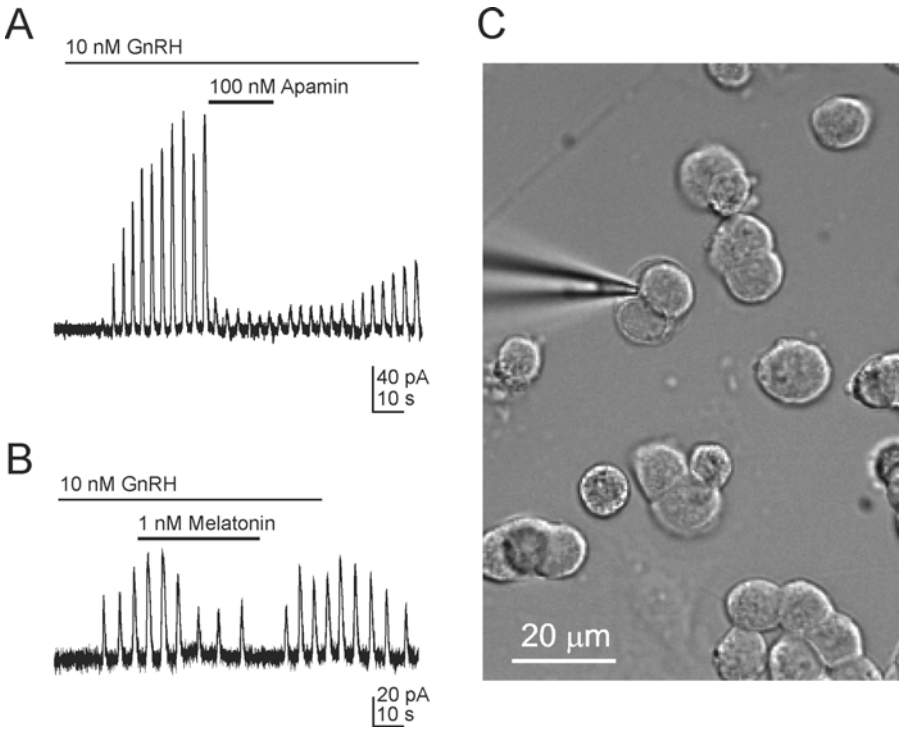

Fig. 3. Electrophysiological monitoring of GnRH-induced $[Ca^{2+}]_i$ oscillations by recording $I_{K(Ca)}$ in neonatal gonadotrophs.
A. Effects of apamin, a specific blocker of SK-type of calcium-controlled potassium currents ($I_{K(Ca)}$) on GnRH-induced current oscillations. **B.** Attenuation of GnRH-induced $I_{K(Ca)}$ oscillations by melatonin. The time of GnRH, apamin and melatonin application is indicated by horizontal bars. **C.** Patch-clamp electrode and neonatal pituitary cells in culture. For details see Zemková and Vaněček (1997).

When stimulated with GnRH, neonatal gonadotrophs exhibit more often non-oscillatory responses during the initial and sustained stimulation. More importantly, the coupling of voltage-dependent calcium influx with Ca^{2+} release from ryanodine-sensitive intracellular stores plays a critical role in initiation of GnRH-induced $[Ca^{2+}]_i$ oscillations in neonatal gonadotrophs, but the coupling was lost in gonadotrophs from adult animals. In cells with blocked voltage-gated calcium influx (by removal of extracellular calcium or by the addition of nifedipine, a blocker of L-type voltage-dependent calcium channels), the latency preceding the GnRH-induced response is prolonged more than three times. Similar effect has ryanodine in concentrations that blocks Ca^{2+} release from intracellular pools. In neonatal gonadotrophs, calcium-induced calcium release from ryanodine-sensitive intracellular calcium pool is very small, but is sufficient to amplify voltage-dependent calcium influx to the level needed to influence IP_3-controlled Ca^{2+} release (Bezprozvanny *et al.* 1991). The ryanodine receptor has been found in other anterior pituitary cells, including GH3 immortalized pituitary cells (Kramer *et al.* 1994). Thus, the expression and functional coupling of ryanodine-sensitive channels in neonatal gonadotrophs coincides with expression and function of melatonin receptors.

It is also known that GnRH-induced $[Ca^{2+}]_i$ oscillations of neonatal gonadotrophs rapidly disappear in the absence of extracellular calcium (Tomic *et al.* 1994), whereas they last for prolonged period in adult gonadotrophs (Kukuljan *et al.* 1992, Tse and Hille 1992). The short-lasting Ca^{2+} oscillations in neonatal gonadotrophs indicated that their intracellular stores contain less amount of Ca^{2+} and recently it has been confirmed that their refilling is highly dependent on calcium influx (Zemková *et al.* 2004). The mechanism by which GnRH receptor activation leads to opening of dihydropyridine-sensitive Ca^{2+} channels is not clear. L-type Ca^{2+} channels could be modulated directly by G protein in a membrane-delimited way by βγ subunit released from activated G protein (Herlitze *et al.* 1996). Alternatively, GnRH could stimulate Ca^{2+} influx by phosphorylation of channel protein *via* protein kinase C (Bosma and Hille 1992).

In picomolar to low nanomolar concentration range, melatonin completely inhibits GnRH-induced $[Ca^{2+}]_i$ increase or prolongs latency of responses (Fig. 4) in 40-70 % of rat neonatal gonadotrophs (Vaněček and Klein 1992a,b, 1993, Zemková and Vaněček 1997, 2000). Latency prolongation by melatonin seems to involve melatonin-induced inhibition of extracellular Ca^{2+} entry, since the same effect was observed in Ca^{2+}-depleted medium or in the presence of nifedipine (Vaněček and Klein 1992a, 1993, Zemková and Vaněček 1997). The effects of melatonin and Ca^{2+}-depleted media on GnRH-induced calcium signaling were not additive (Zemková

and Vaněček 2000). Dihydropyridine-sensitive L-type Ca^{2+} channels are known to be expressed in adult gonadotrophs (Stutzin *et al.* 1989). Provided that in neonatal gonadotrophs GnRH-stimulated cAMP/PKA pathway (Stojilkovic and Catt 1995, Grosse *et al.* 2000) activates Ca^{2+} entry through voltage-dependent L-type Ca^{2+} channels, melatonin could inhibit Ca^{2+} entry by inhibiting cAMP production, the later being demonstrated in neonatal gonadotrophs (Vaněček and Vollrath 1989). This effect of melatonin is probably mediated *via* α-subunit of PTX-sensitive G_i proteins (see model in Fig. 5) as indicated in amphibian melanophores (White *et al.* 1987) or pars tuberalis cells from Djungarian hamsters (Carlson *et al.* 1989), and confirmed in cells expressing recombinant receptor (Brydon *et al.* 1999).

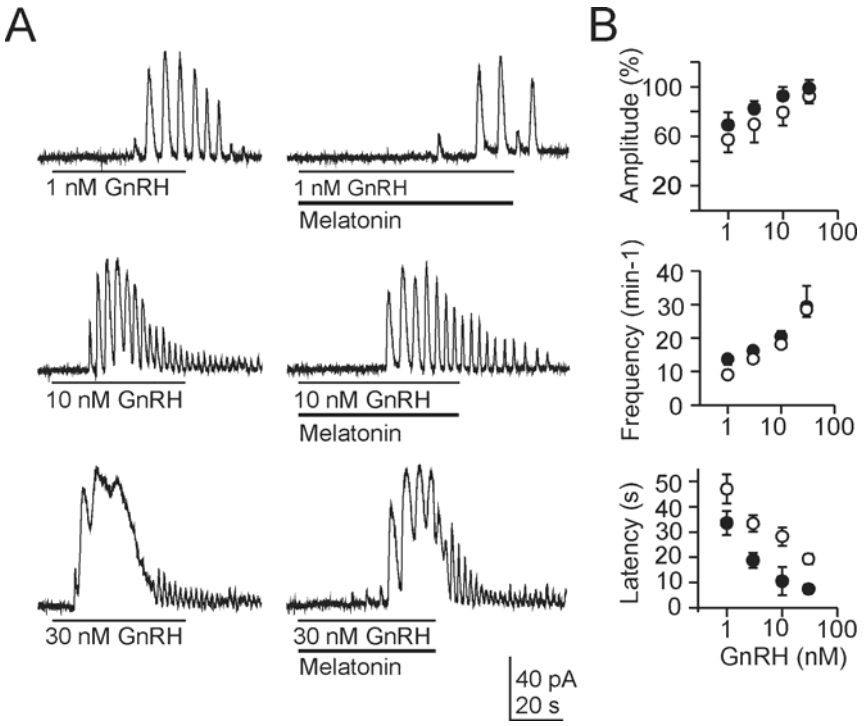

Fig. 4. Inhibitory effect of melatonin on GnRH-induced calcium oscillations. **A**. Responses to 1, 10 and 30 nM GnRH recorded in the absence (left) or presence (right) of 1 nM melato-nin. Melatonin prolonged the latency, reduced the frequency and abolished the non-oscillatory part of GnRH-induced calcium responses. The inhibitory effect of melatonin is more evident at lower GnRH concentrations. **B**. The relationship between GnRH con-centration and amplitude (upper panel), frequency (middle panel) and latency (bottom panel). Open circles, GnRH-treated cells, filled circles, GnRH+melatonin-treated cells. For details see Zemková and Vaněček (2000).

The inhibitory effects of melatonin on GnRH-induced Ca^{2+} oscillations were also observed in cells bathed in Ca^{2+}-deficient medium (Slanař *et al.* 1997, Zemková and Vaněček 1997, 2000). This indicates that melatonin not only inhibits voltage-gated calcium influx, but also inhibits Ca^{2+} release from intracellular Ca^{2+} stores. The mechanism by which melatonin inhibits IP_3-dependent calcium signaling is largely unknown. The finding that melatonin has no effect on Ca^{2+} oscillations evoked by intracellular injection of IP_3 (Zemková and Vaněček 2000, 2001) could indicate that melatonin inhibits the GnRH-stimulated signaling pathway upstream of IP_3 receptor activation. Melatonin has been shown to inhibit the GnRH-induced increase in DAG (Vaněček and Vollrath 1990), indicating that it may inhibit phospholipase C activity. However, this is an unlikely explanation, because potentiation of phospholipase C has been found in cells transiently expressing melatonin receptor (Godson and Reppert 1997, Brydon *et al.* 1999), as well as in neonatal gonadotrophs (see below). Because the majority of GnRH-stimulated DAG production comes from phospholipase D pathway (Zheng *et al.* 1994), it is likely that melatonin inhibits this pathway. An alternative explanation for the observed effects on GnRH-induced Ca^{2+} mobilization is the inhibition of adenylyl cyclase pathway. Cross-talk between the cAMP and phosphoinositide signaling pathways is well documented. For example, in hepatocytes the frequency of calcium oscillations triggered by hormones linked to IP_3 production is increased by activation of receptors positively coupled to adenylyl cyclase (Walaas *et al.* 1986, Furuichi *et al.* 1989, Mignery *et al.* 1990). Because both melatonin receptor subtypes expressed in neonatal gonadotrophs are negatively coupled to adenylyl cyclase, it is reasonable to speculate that sensitivity of IP_3 receptors for IP_3 is lowered in cells with activated melatonin receptors.

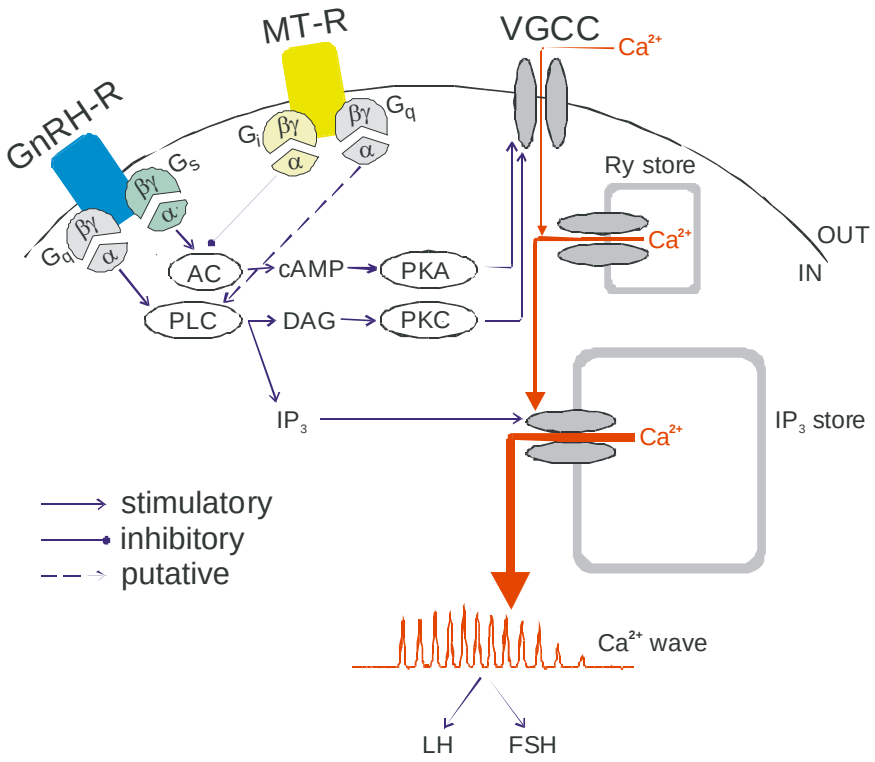

Fig. 5. Model of GnRH and melatonin action in neonatal gonadotrophs. Stimulation of gonadotropin-releasing hormone receptors (GnRH-R) causes activation of phospholipase C (PLC) and through G_q-dependent signaling pathway, leading to the production of diacylglycerol (DAG) and inositol (1,4,5)-triphosphate (IP_3). DAG together with Ca^{2+} stimulates protein kinase C (PKC). The cross-coupling of GnRH signaling pathway accounts for stimulation of adenylyl cyclase (AC), leading to increase in cAMP production and activation of protein kinase A (PKA). Both PKC and PKA are involved in control of voltage-gated calcium channels (VGCC). The influx of Ca^{2+} through VGCC triggers Ca^{2+} release from ryanodine (Ry)-sensitive calcium pool. Calcium and IP_3 coordinately regulate opening of IP_3 receptor-channels expressed in endoplasmic reticulum (IP_3 store), leading to a massive Ca^{2+} releases from this pool, which frequently occurs in an oscillatory manner. The rise in Ca^{2+} is sufficient to trigger release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Melatonin receptors (MT-R) signal through G_i pathway to inhibit AC. Melatonin also stimulates PLC, but only in high concentrations and $\beta\gamma$ dimer of G_i or α subunit of G_q accounts for stimulation of PLC. PKA phosphorylates IP_3 receptor-channel, an action that facilitates IP_3/Ca^{2+}/controlled calcium release. Such organization of calcium signaling provides an effective mechanism for amplification of signal from VGCC through Ry store to IP_3 store (indicated by thickness of arrows), whereas down-regulation of AC activity by MT-R provides an effective mechanism for inhibition of this cascade.

Melatonin exhibits two different effects on GnRH-induced $[Ca^{2+}]_i$ increases in neonatal rat gonadotropes, depending on its concentration (Zemková and Vaněček 2001). In the physiological concentration range, melatonin consistently inhibits Ca^{2+}-oscillations induced by lower GnRH concentrations (2 nM) or delays the responses, with half-maximal inhibition at about 30 pM. These values are in good agreement with the 60 pM dissociation constant of ^{125}I-melatonin binding found in binding studies on neonatal gonadotropes (Vaněček 1988a), as well as with the 20-40 pM K_d value reported for the recombinant MT_1 melatonin receptor (Reppert *et al.* 1994). At higher (nanomolar) concentrations, melatonin also enhanced GnRH-induced Ca^{2+}-dependent K^+ current, but only in about 10 % of the neonatal gonadotropes stimulated with 10 nM GnRH (Zemková and Vaněček 2000). Melatonin alone (in the absence of GnRH) was unable to trigger Ca^{2+} signals (Zemková and Vaněček 1997) or to change the pattern of calcium signals triggered by intracellular introduction of IP_3 (Zemková and Vaněček 2001). Thus, the potentiating effect of melatonin could be due to the cross-coupling of melatonin receptors to G_q signaling pathway, as demonstrated in cells transiently expressing the recombinant melatonin receptors (Brydon *et al.* 1999), or more probably by coupling of βγ-subunits of PTX-sensitive G_i protein to phospholipase C (Godson and Reppert 1997). In general, the physiological relevance of such coupling is low, because melatonin-induced $[Ca^{2+}]_i$ increase has only been observed in response to non-physiological concentrations of melatonin and in about 10 % of cultured ovine pars tuberalis cells expressing endogenous MT_1 receptor.

Why melatonin receptors disappear from pituitary?

It looks contradictory to have operative a very sophisticated mechanism for pulsatile GnRH release in hypothalamus and in the same time also to possess an effective system to block the action of GnRH in target pituitary cells as it was demonstrated in neonatal animals. However, such a dual control of gonadotroph function is physiologically justified. It provides an effective mechanism to down-regulate, but not to abolish, LH and FSH secretion. During the development, reproductive functions have to be arrested, but low levels of plasma gonadotropins are needed for keeping operative gonadal steroidogenesis, which is critical for numerous cellular pathways. Thus, GnRH release is required in prepubertal period, but its action should be attenuated in order to keep plasma gonadotropin levels below the threshold for initiation of peripubertal changes.

In general, rapid desensitization of GnRH receptors could provide the potential mechanism for down-regulation of gonadotropin secretion. However, that is not the case with mammalian GnRH receptor, this receptor lacks the C-terminal tail, which slows down the rate of receptor desensitization. In other scenario, the transient expression of high-density melatonin receptors in gonadotrophs appears to be critical to the fetal programming of the reproductive axis (Vaněček 1998), i.e. melatonin may affect the onset of puberty (Heideman *et al.* 2001) through its inhibitory effects on the pubertal activation of the reproductive functions (Ebling and Foster 1989). Furthermore, the developmental down regulation of melatonin receptors could serve to establish gradually normal action of GnRH on gonads and seasonal effects of melatonin on reproduction.

In contrast to the physiological relevance of transient expression of melatonin receptors in gonadotrophs, the mechanism by which the developmental down-regulation of melatonin receptor expression is mediated is not clear at the present time. During embryonic development, anterior pituitary cells express mRNAs for several hormones in various combinations. For example, in mice at embryonic day 16 plurihormonal population represents more than 60 % of cells, at postnatal day 1 it is approximately 35 %, and at postnatal day 38 it is only about 25 % (Seuntjens *et al.* 2002). Thus, it appears that developmental disappearance of melatonin receptors in anterior pituitary cells parallels the development loss of plurihormonal population of cells, suggesting that both phenomena are controlled by the same mechanism (Hazlerigg 2001).

Conclusions

Although the roles of melatonin in control of reproductive functions have been established a long time ago, still very little is known about the mechanism by which melatonin receptors contribute to this process. It is obvious that melatonin transduces information about daily and seasonal photoperiods on two effectors: pituitary cells and GnRH-secreting neurons. The direct action of melatonin in pituitary is a part of a more complex mechanism by which this messenger interferes with reproductive functions.

Our work in neonatal gonadotrophs revealed two important aspects of melatonin actions: a gradual loss of melatonin receptors and actions, which temporally coincides with the loss of multi-hormonal cells, and the coupling of GnRH receptors to ryanodine receptors, which also appears to be limited for prepubertal period. These observations raised the questions about the mechanism of gonadotroph differentiation and the physiological relevance of the delay in this process in order to provide an effective control of GnRH action by melatonin in neonatal cells. It also appears that the effect of melatonin on calcium signaling in neonatal gonadotrophs is mediated by both subtypes of high-affinity melatonin receptors, MT_1 and MT_2. Which subtype of melatonin receptor is responsible for the inhibitory effect of melatonin remains to be established.

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