Acta Neurobiol. Exp. 1994, 54 (Suppl.): 83-137

ABSTRACTS

OPENING LECTURE

WAS THE THIRD EYE THE FIRST EYE?

David C. Klein
NIH, Bldg 49, Rm 5A38, 9000 Rockville Pike, Bethesda, MD 20892, USA
Not received

SESSION I: EYE/RETINA 1

THE CYTOARCHITECTURE OF THE DOPAMINE CELLS IN VERTEBRATE RETINA

Jeanine Nguyen-Legros and **Axelle Simon**
Laboratoire de Neurocytologie Oculaire, I.N.S.E.R.M., 15, rue de l'Ecole de Medecine, 75270 Paris, France

Descriptions of the retinal dopaminergic system are remarkably uniform across species; it consists of intrinsic retinal neurones that are amacrine cells, mostly located at the inner margin of the inner nuclear layer and sending processes to the outmost sublamina of the inner plexiform layer.

Although the dopamine neurones are currently called "amacrine cells" they have been demonstrated to be polyaxonal rather than axonless neurones (1). Moreover, some of them, which send an axon to the outer plexiform layer, are called "interplexiform cells" but belong to the same cell class. Whether the same action of dopamine on horizontal and photoreceptor cells is dependent upon interplexiform cells to deliver dopamine more closely to its targets remains an open question, because interplexiform cells make very few synapses. Although it is currently thought that dopamine can reach its receptors by diffusion over distance, the structure-function relationships concerning interplexiform cells remain poorly understood. They were first described as existing only in fish, but the continuous improvement of immunocytochemical techniques led to their demonstration in a growing number of species. It can be hoped that they will be observed to be a common feature of the vertebrate retinal dopaminergic system.

Another problem of this system is the existence of two cell types, 1CA- and 2CA-cells, synthesizing either high or low quantities of dopamine, respectively (2). At the present time, the 2CA-cells appear especially developed in primates while they are not observed in cold blooded vertebrates. These 2CA-cells exhibit morphologies and, probably, functions quite different from those of the 1CA-cells, because their processes innervate the middle sublamina of the inner plexiform layer, and because interplexiform cells are not observed among them. They are especially sensitive to degeneration in pathologies of aging.

Finally, an additional problem concerning dopamine cells was raised by the discovery that in birds most of them are actually ganglion cells displaced to the amacrine cell layer (3). Since some tyrosine hydroxylase-immunoreactive fibres are observed in the nerve fiber layer of primates, and since the first tyrosine hydroxylase positive cells appear as ganglion-like cells in the human embryo, it is possible that dopamine cells will be useful as a model to understand transdifferentiation from ganglion-like to amacrine or polyaxonal cells during development.

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THE DOPAMINERGIC SYSTEM IN THE VERTEBRATE RETINA: MORPHOLOGICAL DIFFERENTIATION OF TH-IR CELLS AND LOCALIZATION OF D1 AND D2 RECEPTORS

H.J. Wagner, U.D. Behrens, E. Fröhlich and **K. Negishi**
Anatomisches Institut, Universität Tübingen, Österbergerstr. 3, 72074 Tübingen, Germany

All vertebrate retinae contain dopaminergic (DA) neurones. These are classified as amacrine cells (ACs) if their processes are restricted to the IPL, or as interplexiform cells (IPCs), if additional processes extend into the OPL. A comparison of the differentiation of DA neurones into ACs and IPCs across vertebrates does not yield a clear pattern: In amphibians, reptiles and birds, ACs are the only types of DAergic cells. On the other hand, in fishes and mammals, some species have been observed with ACs only, while others contain IPCs. In the present report we have investigated the morphology of the DAergic cells in 23 species of fishes (including elasmobranchs and teleosts) using light microscopic immunocytochemistry against tyrosine hydroxylase (TH). We find a close correlation between the presence of cones (ascertained by immunolabelling with antivisin) and the occurrence of IPCs, while pure-rod retinae contain ACs as the only type of DAergic cell.

In an attempt to study the retinal localization of DA receptors (DA-R) we used immunocytochemistry for D2-R, and binding of fluorescent antagonist for D1-R. The fluorescent-labelled D1-R antagonist SCH 23390 bound specifically to the outer and the inner plexiform layers. Especially in fishes, there was a higher labelling of the horizontal cells compared to the inner plexiform layer. For the D2-R localization, two different antisera were available: an antipeptide antiserum (courtesy of M. Ariano), and a polyclonal antiserum. These two antisera yielded essentially similar labelling patterns as regards the two synaptic layers. The Chemicon antiserum showed a distinct layering of the IPL as well as a patchy labelling on the plasma-membrane of subpopulations of ACs and ganglion cells. In the outer retina, the horizontal layer was D2-positive, also exhibiting a sublayering in some species. As for the photoreceptors, the anti-peptide antiserum stained both inner and outer segments, while the polyclonal antiserum, only the inner segments of cones and rods were labelled; in addition, the layer of synaptic terminals was fluorescent. The patterns of DA-R localization indicate that D2-Rs may act as autoreceptors in the horizontal layer while D1-R may serve a more traditional synaptic role. In the IPL, a possible colocalization, with a synergistic function of both receptor families, cannot be excluded. However, the observations obtained with the Chemicon antiserum suggest a role of D2-mediated signal transmission for specific subpopulations of amacrine and ganglion cells.

DOPAMINE RECEPTORS IN VERTEBRATE RETINA: CHARACTERIZATION AND SIGNAL TRANSDUCTION MECHANISMS

Michel Schorderet
Department of Pharmacology, University Medical Center, CH-1211, Geneva-4, Switzerland

In the early work of several teams, retinal dopamine (DA) receptors that were positively coupled to adenylate cyclase have been characterized by various dopaminomimetic agents including DA and shown to be blocked by typical neuroleptics (reviewed in 1). Early binding studies have also confirmed the existence of retinal DA receptors of D-1 type (1). Further binding studies have next demonstrated that the majority of mammalian species contain D-2 receptors as well (2). Autoradiographic studies performed with selective D-1 and D-2 ligands have localized either subtype in the inner and outer plexiform layers (2).

Retinal D-1 receptors are unequivocally coupled with a transduction mechanism involving G protein and concomitant increase in cAMP production (1). An agonist-induced desensitization of D-1 receptor has been achieved in experiments *in vitro* (3). An additional and independent coupling of D-1 receptors

with phospholipase C and concomitant formation of IP-3 has also been recently shown in chick retinal cell cultures (4). The transduction mechanisms that were implied for retinal D-2 receptors appear to be much more difficult to characterize (1). A negative coupling with adenylate cyclase activity has been found in lower vertebrates and some mammalian species including hen and rat (1). Alternative putative transduction mechanisms connected with D-2 receptors in other systems (e.g. opening and closing of K^+ and Ca^{++} channels, respectively) were not demonstrated in mammalian retinas. It is however conceivable that in physiological conditions the stimulation of retinal D-1 and/or D-2 receptors by DA would explain the role(s) of this transmitter in visual functions and/or melatonin biosynthesis (5-6). Interestingly, a time-dependent up-regulation of D-2 (as well as D-1) receptors in response to light deprivation has been found in rat retina (7).

The recent cloning, characterization and expression of additional subtypes of DA receptors (D-3, D-4 and D-5) that would be linked with specific functions in CNS (8) has also led to new investigation in retina. For example, it has been recently shown that mouse retinal photoreceptors posses D-4 receptors linked to the inhibition of adenylate cyclase (9). Furthermore, modulation of melatonin biosynthesis by DA may be achieved by the stimulation of D-4 subtype of receptors (10).

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MELATONIN BIOSYNTHESIS IN RETINAL PHOTORECEPTOR CELLS: MODULATION BY INTRINSIC AND EXTRINSIC SIGNALS

P. Michael Iuvone

Department of Pharmacology, Emory University School of Medicine, Atlanta, GA, USA

Melatonin biosynthesis in vertebrate retinal photoreceptors occurs as a diurnal or circadian rhythm, with the highest level of synthesis at night. Light exposure at night dramatically suppresses melatonin biosynthesis. The rhythmic synthesis of melatonin biosynthesis in retinal photoreceptors is regulated by parallel rhythms in the activities of tryptophan hydroxylase and serotonin N-acetyltransferase (NAT). Evidence will be reviewed for regulation of these enzymes by intrinsic mechanisms, which can be sustained in isolated photoreceptor cells, and extrinsic mechanisms, which involve retinal neuronal pathways and neurotransmitters. The intrinsic mechanism involves light-evoked changes in photoreceptor membrane potential with consequent changes in voltage-sensitive calcium channel activity and second messenger cascades. In addition, the intrinsic mechanism involves a circadian clock which appears to be contained within the photoreceptor cell. Hence, both circadian rhythms and light-evoked decreases in melatonin biosynthesis in photoreceptor cells are sustained in the absence of other neural inputs. The extrinsic mechanism involves light-evoked dopamine release and activation of dopamine receptors on photoreceptors cells. Dopamine inhibits the expression of NAT activity and appears to function as an amplifying signal for regulating melatonin biosynthesis under conditions of dim light. Adenosine is another potential extrinsic signal for regulating melatonin biosynthesis. Activation of A2a-like adenosine receptors on photoreceptor cells dramatically increases NAT activity, mimicking the effect of darkness. Adenosine receptor activation is

capable of stimulating NAT activity under photopic conditions to an extent that is comparable to that observed in darkness. Thus, the intrinsic mechanisms for regulating melatonin biosynthesis in retinal photoreceptors appear to be subject to both positive and negative control by neuromodulators.

CHARACTERISTICS OF THE DOPAMINE RECEPTOR REGULATING MELATONIN BIOSYNTHESIS IN VERTEBRATE RETINA

Jolanta B. Zawilska[1,2] and Jerzy Z. Nowak[1]

[1]Department of Biogenic Amines, Polish Academy of Science, 3 Tylna St., 90-950 Łódź and [2]Department of Pharmacodynamics, Medical University of Łódz, Łódz, Poland

Retinal dopamine (DA), originating from either a subset of amacrine cells or interplexiform cells (depending on the species), is a modulator of melatonin biosynthesis that takes place in photoreceptors. DA inhibits the nocturnal enhancement of the hormone formation, and in this respect it mimicks the effect of light on the melatonin generating system. The modulatory activity of both DA and light occurs at the level of serotonin acetylation, the reaction catalyzed by an inducible enzyme serotonin N-acetyltransferase (NAT). It is suggested that light-evoked suppression of NAT activity and melatonin content may be mediated, at least partially *via* DA, since light activates DA synthesis and release. Therefore, an important question is which type of DA receptors regulates NAT activity (and thus melatonin formation) in photoreceptors.

Earlier studies excluded a possible role of the D_1 DA receptor, since a D_1-selective agonist (SKF 38393) and an antagonist (SCH 23390) were inactive under different conditions. This suggested a role of a D_2 DA receptor (1,2). However, recently introduced molecular cloning techniques have led to the identification of novel subtypes of DA receptors, i.e., D_3 and D_4 - members of the D2 DA receptor family, and D_5 - a member of D1 DA receptor family. These findings set the stage for a re-evaluation of previous data concerning DA-melatonin interaction in the retina. Our recent experiments (3), carried out with DA itself, as well as with a number of DA agonists and antagonists acting on different DA receptor types (D_1, D_2, D_3, D_4, D_5) suggest that a D_4-like rather than a D_2-type receptor is responsible for the suppression of the stimulated (nocturnally or pharmacologically) NAT activity and melatonin content of the retina. The rank order of the agonists tested by us was: quinpirole > apomorphine = pergolide > LSD-25 > (±)-ADTN> bromocriptine = 7-OH-DPAT >> SKF 38393 (inactive). The quinpirole-evoked suppression of the night-driven NAT activity was antagonized by DA receptor blockers with the following order of potency: spiroperidol > YM-09151 (emonapride) > sulpiride > clozapine > (+)UH-232 > haloperidol > domperidone; butaclamol showed weak activity, whereas raclopride and remoxipride were practically inactive. In addition, sulpiride, which shows a pronounced stereoselectivity against D_2-type DA receptor ("S" form is much more active than "R" form), was practically equipotent in antagonizing the quinpirole-induced inhibition of NAT activity (3).

Exposure of chicks to light at night dramatically suppressed retinal NAT activity and melatonin levels, and this effect was antagonized by peripherial or intravitreal application of clozapine, spiroperidol and sulpiride. Furthermore, in chicks maintained in light (instead of being in darkness) the application of these neuroleptic drugs resulted in marked elevations of NAT activity and melatonin levels of the retina (4). Although quinpirole mimicked light in resetting the phase of a free-running rhythm of melatonin production and release *in vitro* in the retina of *Xenopus laevis* (5), it did not affect the circadian rhythm of NAT activity in retinas of living chicks. Thus, it is not certain whether the dopaminergic system is of major importance in a complex process of photoentrainment of the circadian oscillator generating the rhythm of melatonin biosynthesis in the retina of higher vertebrates.

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Supported by the KBN grant 0540/P2/93/04 to J.B.Z.

INCREASE IN MELATONIN AND DECREASE IN DOPAMINE RELEASE MAY PRECEDE RETINAL DEGENERATION IN RATS WITH HEREDITARY DEGENERATION

Hisako Ikeda, Marko Hawlina and **Mark Hankins**
Division of Physiology, UMDS, St. Thomas' Hospital, London, UK

The outer segment of both rod and cone types of retinal photoreceptors which contain photosensitive pigments are renewed continuously following a distinct circadian rhythm (1). The renewal process consists of phagocytosis of shedded old outer-segment discs by the retinal pigment epithelium (RPE) and formation of new discs at the photoreceptor inner segment. The circadian rhythm of the renewal process is known to be regulated by retinal melatonin and dopamine in reciprocal fashion (2). Failure in either process of the phagocytosis or the formation of new discs leads to retinal degeneration. This phenomenon is already demonstrated in the Royal College of Surgeons (RCS) rats, a well studied animal model of hereditary retinal degeneration (3). We examined the relationship between retinal melatonin, dopamine and functional integrity of the photoreceptor and the RPE in heterozygous control and dystrophic retinae of the RCS rats at different ages.

Our electroretinographic (ERG) studies (4) showed that the circadian rhythm of the photoreceptor renewal process taking place at the rod photoreceptor/RPE interface is manifested by diurnal variation in the amplitude of the c-wave of the ERG. The c-wave is the light-evoked trans-pigment epithelium potential, whose generation depends upon the anatomical and functional integrity of the RPE and the photoreceptor. The ERG c-wave was lowest at 1 - 1.5 hours after expected light "on" time, when the rod disc-shedding and the RPE phagocytosis are known to be at the peak. Furthermore, this diurnal rhythmicity of the ERG c-wave shown in control retinas was greatly reduced in the retinae of dystrophic RCS rats at the period preceding retinal degeneration (4).

A radioimmunoassay revealed that the lack of the ERG c-wave rhythmicity in the pre-degenerate retina of dystrophic RCS rats is associated with an abnormal increase in retinal melatonin level (4). Melatonin content was twice as high in the retinae of dystrophic rats compared with that in control retinae. In addition, a recent electrophysiological study on horizontal cells in isolated retinae (5) showed that when melatonin (0.5-1 μM) was perfused, cells in control retinae behaved similarly to those in the pre-degenerate retinae of dystrofic rats. In keeping with the fact that melatonin inhibits dopamine release (6), moreover, a significant reduction in endogenous dopamine release associated with the abnormal increase in melatonin was suggested in the retinae of dystrophic rats at the period preceding degeneration (5).

It appears, thus, that the reciprocal interaction of retinal melatonin and dopamine regulating the biological clock of the photoreceptor renewal process at the RPE/photoreceptor interface is essential in functional maturation and maintenance of normal retina.

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This work has been carried out at the Vision Research Unit of Sherrington School, The Rayne Institute, St. Thomas' Hospital.

SESSION I: EYE/RETINA 2

DOPAMINE-STIMULATED POTASSIUM EFFLUX IN THE CHICK RETINA

Jarmo T. Laitinen
Department of Physiology, University of Kuopio, POB 1627, FIN-70211 Kuopio, Finland

Potassium efflux (assessed as $^{86}Rb^+$ efflux) was studied in retinal suspensions of posthatched chicken. Dopamine (DA) increased K^+ efflux rate by 1.5-fold. This effect was dose-dependent (EC_{50} 22 μM), was mimicked by the D1-selective agonist SKF-38393 and reversed by the D1-selective antagonist SCH 23390 suggesting the involvement of D1-receptors. The DA-elicited K^+ efflux seems to be independent of adenylyl cyclase (AC) activation, as the membrane permeable cAMP analogs (dibutyryl cAMP and 8-bromo-cAMP) exhibited no potency in the efflux assay. Moreover, DA failed to affect cAMP levels in assay conditions where forskolin produced a massive increase in retinal cAMP levels. Analogs of cGMP had no effect on K^+ efflux suggesting that the nitric oxide-cGMP pathway might not be involved in the DA action. The protein kinase C (PKC) activator 4β-phorbol-12-myristate-13-acetate (4β-PMA) stimulated retinal K^+ efflux in a dose-dependent manner (EC_{50} 4nM). This effect was mimicked by 4β-phorbol-12,13-didecanoate but not by the inactive isomer 4-PMA suggesting possible involvement of PKC. When added together, DA and 4β-PMA stimulated K^+ efflux in an additive manner indicating that DA and 4β-PMA do not share a common mechanism of action. Moreover, DA failed to stimulate phosphoinositide hydrolysis, a commonly utilized pathway to generate diacylglycerol, an endogenous activator of PKC. These data suggest that the DA-elicited K^+ efflux was not mediated by the second messenger pathways generally linked to DA action.

In the efflux assay, increase in extracellular K^+ may result from enhanced K^+ efflux mechanisms and/or suppressed Na/K-ATPase activity. Therefore the effects of DA on the ion pump were also investigated. It was found that DA inhibited retinal Na/K-ATPase (assessed as ouabain-sensitive $^{86}Rb^+$ influx). However, the DA-elicited K^+ efflux was largely independent of this inhibition, as DA was able to stimulate K^+ efflux in the presence of ouabain (1 mM).

As melatonin receptors are enriched in the inner plexiform layer of chick retina and melatonin is known to interact with DA in the retina, the effects of this indole on retinal K^+ efflux were studied. However, melatonin had no effect on basal or DA- or 4β-PMA-stimulated K^+ efflux.

To summarize, these studies provide evidence that DA stimulates K^+ efflux in the chick retina *via* D1-receptors. This action seems to be independent of the second messengers typically linked to DA action. Melatonin does not seem to modulate basal or the DA-elicited K^+ efflux.

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LOCALIZATION OF THE D1-DOPAMINE RECEPTOR IN VERTEBRATE RETINAE

U.D. Behrens and **H.J. Wagner**
Anatomisches Institut, Universität Tübingen, Österbergerstr. 3, 72074 Tübingen, Germany

Dopamine is a retinal neurotransmitter synthesized in interplexiform (fish, mammals) and amacrine cells (amphibians, reptiles, birds and also mammals). It is released upon light stimulation and diffuses widely throughout the retina. Dopamine induces light adaptive changes in the outer retina of lower vertebrates (retinomotor movements of photoreceptor inner and outer segments and spinule formation of teleost cone-horizontal cell dendrites), and reduces the size of receptive fields by uncoupling horizontal cell gap junctions in all vertebrate species. The action of dopamine is mediated by two receptor families: D1 and D2. In order to understand dopamine receptor mediated effects, it is important to know the location

of the two receptor subtypes. We used the fluorescent receptor antagonist Bodipy-SCH 23390 (Molecular Probes) to study the D1-dopamine-receptor localization in the retinae of fish, amphibians, turtles, chick, rat and rabbit. Unfixed cryosections were incubated with 100 nM of the fluorescent D1-antagonist for 30-60 min at 4°C. The highest intensity of fluorescence was found in fish retinae at the level of the outer plexiform layer (OPL), whereas a less bright label was associated with the inner plexiform layer (IPL). Both plexiform layers were also stained in the retinae of the other vertebrates. In none of the species, binding was observed in the nuclear layers (ONL, INL, GCL). In control sections, an excess of unlabelled SCH 23390 removed the fluorescent signal indicating that the pattern of labelling was specific. In conclusion, the staining pattern of the fluorescent D1-receptor antagonist shows a preferential localization of the D1-receptor within the synaptic layers. The association of D1 receptors with the horizontal cell layer is in a good agreement with physiological and pharmacological experiments on dopamine mediated light adaptive effects.

CHARACTERISATION OF MELATONIN BINDING SITES IN THE QUAIL EYE

K. James[1], D.J. Skene[1], B. Stankov[2] and J. Arendt[1]

[1] School of Biological Sciences, University of Surrey, Guildford, UK, [2] Department of Pharmacology, University of Milan, Milan, Italy

The presence of melatonin (N-acetyl-5-methoxytryptamine) has been reported in the retina of many vertebrates including several avian species. Various local actions regarding retinal physiology have been demonstrated (e.g., effects on photoreceptor disc shedding, photomechanical movements and neurotransmitter systems). Within the eye of the quail there is reported to be a biological clock controlling the production of melatonin (1). The significance of this circadian release of retinal melatonin in terms of local actions and effects on the whole-body circadian system is, as yet, undefined. Thus there is much interest in the mechanism of action of retinal melatonin.

In these studies the existence of retinal melatonin binding sites in the adult quail was investigated. Tissues were removed from male quails maintained under a constant light routine and neural retina and choroid-retinal pigment epithelium (C-RPE) separated by dissection. A previously established rapid filtration radioreceptor assay utilising the iodinated ligand 2-[^{125}I]iodomelatonin was employed (2). Crude membrane preparations of neural retina showed specific binding over the radioligand concentration range 11-580 pM. Scatchard analysis revealed a high affinity, low capacity binding site (K_d 51.6± 2.5 pM (se) and B_{max} = 11.8± 0.3 fmoles/mg protein (se)). Linearity of the Scatchard plot and Hill coefficient approaching unity implied a single class of binding site. Choroid-retinal pigment epithelium membranes were also investigated in terms of their ability to bind 2-[^{125}I]iodomelatonin. Saturable, high affinity, low capacity binding, of similar order to that of the neural retina, was demonstrated.

Further analysis of the neural retina showed that association of the radioligand was rapid, binding equilibrium being achieved within 40 min at 28°C; association rate constant = 7.85 x $10^8 M^{-1} min^{-1}$. Binding was found to be reversible by addition of 1 μM cold melatonin; dissociation rate constant = 8.48 x 10^{-3} min^{-1}. Competition studies for inhibition of 2-[^{125}I]iodomelatonin binding showed the order of potencies to be 2-bromomelatonin = 2-iodomelatonin > melatonin = 6-chloromelatonin > 6-hydroxymelatonin >> N-acetylserotonin > 5-methoxytryptophol >> 5-methoxytryptamine > 5-hydroxytryptamine. Studies with the guanine nucleotides GTP and GDP showed a dose-dependent inhibition of 2-[^{125}I]iodomelatonin binding implying a potential signal transduction mechanism involving coupling of the binding site with a guanine nucleotide regulatory protein. There are therefore distinct similarities between this retinal receptor and those previously identified in other species.

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CHARACTERIZATION OF TRYPTOPHAN HYDROXYLASE IN THE RETINA OF THE GREEN FROG, *RANA PEREZI*

A.I. Valenciano, A.L. Alonso-Gómez, M. Alonso-Bedate and **M.J. Delgado**
Departamento Biología Animal II (Fisiología Animal), Facultad de Biología Universidad Complutense, Madrid 28040, Spain

Recent studies indicate that serotonin N-acetyltransferase (E.C.2.3.1.87) is not the only enzyme involved in the regulation of melatonin (MEL) biosynthesis in the retina. Tryptophan hydroxylase (TPH, E.C.1.14.16.4), the initial and rate-limiting enzyme in the serotonin biosynthetic pathway has been suggested as one of the most probable candidate involved in ocular MEL regulation. In the present study we have characterized the TPH activity *in vitro* from the retina of the green frog, *Rana perezi*. The enzymatic activity was estimated by measuring the 5-hydroxytryptophan (5-OHTrp) formed from L-tryptophan (L-Trp) by the action of the enzyme. Neural retina (THP activity in the choroid-pigmented epithelium complex was not detected) was sonicated in 50 mM potassium phosphate pH 6.6 and the homogenate was centrifuged (39.000 g, 20 min., 4°C). Supernatant samples were incubated at 25°C for 30 min., the reaction was stopped by addition of 100 µl cold perchloric acid and centrifuged (15.000 g, 1 min). 5-OHTrp was analyzed in 25 µl aliquots of supernatant fraction by high performance liquid chromatography (HPLC) with coulometric detection. Blank values were routinely obtained by performing the reaction in the absence of L-Trp in order to subtract the endogenous 5-OHTrp content in the neural retina from that obtained as product of enzymatic assay. TPH activity was located in the supernatant fraction (126 %) versus the precipitate fraction (6%) (100 %= total activity in crude homogenate). When retinas were sonicated in the presence of Triton X-100 (0.2 %), TPH activity exhibited a 40 % increase. In the analysis of the contribution of the various reaction components, we have found that:

(a) in absence of catalase (which acts as a peroxide scavenger) TPH activity showed a 50% reduction, the optimal concentration of catalase being 10 µg per tube.

(b) The addition of a reductor agent, such as dithiothreitol (DTT) at doses lower than 2 mM significantly protected the enzyme, but at higher concentration (50 mM) DTT exerted an inhibitory effect on TPH activity.

(c) The inhibition of aromatic L-amino acid decarboxylase with *m*-hydroxybenzylhydrazine does not affect the TPH activity. The time course of the tryptophan hydroxylation reaction conserved the linearity with time up to 60 min. at 5-35°C range. The enzyme showed a thermal instability, at room temperature (25°C) the activity was reduced to 17% for a time longer than 30 min. The enzymatic activity was preserved for 150 min at 5°C. Ocular TPH activity exhibited a marked response to buffer pH, with 6.6 as the optimal pH for this enzyme. The reaction linearity was highly conserved within the retinal fraction range tested (0.016-0.500). In relation to kinetic characterization of the enzyme, the K_M of TPH for the co-substrate, 6-methyltetrahydropterine (6-MPH_4) at 1 mM L-Trp, was 116.23±17.05µM, and the V_{max} was 525.± 0.23 nmol/h/mg protein. The K_M and V_{max} estimations for the L-Trp (at 1 mM 6-MPH_4) were 82.09±12.02 µM and 5.69±0.18 nmol/h/mg protein, respectively.

SEROTONIN AND MELATONIN RECEPTORS IN RETINAL PIGMENT EPITHELIUM

Neville N. Osborne and **Mark Nash**
Nuffield Laboratory of Ophthalmology, Oxford University, Walton Street, Oxford OX2 6AW, UK

Our studies have shown that cultured human and rat retinal pigment epithelial (RPE) cells contain serotonin receptors of the 5-HT_{1A} and 5-HT_2 type respectively, while melatonin receptors are present on both human and rat RPE.

Addition of serotonin to cultures of rat RPE cells is associated with phosphoinositide turnover and mobilization of intracellular calcium. At 10 µM serotonin induced a 2.5 fold increase in ^{3}H-inositol phos-

phates (more than 75% in the form of ^{3}H-inositol-1-phosphate) accumulation within 30 min in cells pre-incubated in ^{3}H-myo-inositol and exposed to 5 mM lithium chloride. The EC_{50} value of serotonin was approximately 0.9 μM and the saturation concentration was 100 μM. Serotonin analogues like tryptamine, 5-methoxytryptamine, α-methyl-serotonin and the 5-HT_2 agonist quipazine and DOI all stimulated inositol phosphates accumulation to some degree. Carbachol, noradrenaline, isoproterenol, dopamine, tryptophan, 5-hydroxytryptamine, 8-OH-DPAT, 5-methyl-serotonin and NECA were inactive. The serotonin-induced response was blocked most effectively by ketanserin and methysergide but not by 5-HT_3 and 5-HT_1 antagonists. Cultures loaded with FURA-2 showed a transient increase in calcium concentration in most cells on exposure to serotonin. The change in intracellular concentration was independent of external calcium and was attenuated by ketanserin but not by the 5-HT_3 antagonist, granistron.

In contrast, cultured human RPE showed no phosphoinositide turnover on exposure to serotonin. However, serotonin reduced basal levels and more emphatically the forskolin (5 μM)-elevated levels of cAMP. The reduction in elevated cAMP levels was dose-dependent with an EC_{50} value of around 1 nM and a maximum reduction of about 50%. The effect was mimicked by a variety of 5-HT_1-agonists including the specific 5-HT_{1A} agonist 8-OH-DPAT with EC_{50}s in the nanomolar range. The serotonin effect is significantly inhibited by the 5-HT_1 antagonists spiroxamine, spiperone and propranolol but not by ketanserin or methysergide.

Melatonin also caused a decrease in forskolin-elevated cAMP levels in both cultured human and rat RPE cells. However, in these cases the melatonin effect was insensitive to the presence of propranolol, showing that melatonin's action is not via serotonin receptors. Proof of this has been demonstrated by further comparison of the serotonin and melatonin influence on cAMP metabolism.

The combined findings show the existence of serotonin and melatonin receptors within RPE cells, but the type of serotonin receptors on human and rat cells differs.

DIURNAL RHYTHMS IN THE CHICKEN EYE AND THEIR RELATION TO MYOPIA DEVELOPMENT

Frank Schaeffel

University Eye Hospital, Department of Pathophysiology of Vision Neuroophthalmology, Division of Experimental Ophthalmology, 11 Röntgenweg St., 72076 Tübingen, Germany

The fine-tuning of axial length and focal length in the growing eye is under visual control. Because refractive errors are frequent among humans, considerable efforts are currently being made to understand the action of the responsible growth-controlling feedback loops. The chicken, although not closely related to humans, is an important model to study emmetropization because it is readily available and has excellent optics and fast ocular growth. Axial myopia can be induced in a few days by moderate retinal image degeneration induced by translucent eye occluders ("deprivation myopia"), or by treatment with negative spectacle lenses. Hyperopia can be induced by positive spectacle lenses. Deprivation myopia can develop only if there is an undisturbed dark period; it does not develop in continuous light or after retinal dopamine (DA) levels have been lowered by neurotoxins. In chickens, continuous light *per se* results in corneal flattening and severe hyperopia and a brake-down of diurnal growth rhythms. Deprivation-induced axial elongation is also suppressed. During deprivation myopia development, both diurnal dopamine rhythms in the eye and diurnal growth rhythms are lost. The result is different for lens-induced refractive errors: they develop both in continuous light (although superimposed to a more hyperopic baseline) and after re-

tinal DA levels have been lowered by intravitreal 6-OHDA application. Reserpine, which depletes both dopamine and serotonin stores also blocks myopia induced by negative lenses.

The complex pattern of visually-induced ocular growth changes shows that (1) deprivation myopia development is linked to diurnal rhythms in the eye, (2) spectacle lens-induced ocular growth changes are more resistant to disturbances of diurnal rhythms, and (3) positive lens-induced hyperopia (which has been shown to result initially from rapid choroidal thickening) occurs even after retinal dopamine and serotonin have been lowered by reserpine down to 20% of the initial value.

Supported by the German Research Council (SFB 307, TP A7).

DEPRIVATION MYOPIA AND ITS RELATION TO THE RETINAL DOPAMINE/MELATONIN SYSTEM IN CHICKENS

Michael Hoffmann and **Frank Schaeffel**

University Eye Hospital, Department of Pathophysiology of Vision and Neuroophthalmology, Division of Experimental Ophthalmology, 11 Röntgenweg St., 72076 Tübingen, Germany

Moderate amounts of retinal image degradation by translucent eye occluders produce exaggerated axial eye growth and myopia in young chickens ("deprivation myopia"). The visually-induced change in axial eye growth implicates dopaminergic mechanisms since dopamine (DA) agonists and neurotoxins 6-OHDA or reserpine suppress myopia development very efficiently, and DA-antagonists (sulpiride) enhance it. Myopia development is linked to disturbances in diurnal growth rhythms: deprived eyes grow both during the day and night whereas eyes with normal visual experience grow only during the day (1). It has been shown that diurnal retinal dopamine rhythms disappear during development of myopia (2, 3); retinal melatonin levels have not yet been studied. We have employed a melatonin radioimmunoassay (DRG, Marburg, Germany) and we have obtained the following results: (1) Diurnal melatonin rhythms were not changed during development of deprivation myopia (normal vision: day 20.829.45 pg/mg protein, night: 110.50±21.92, $P<0.001$; deprived: day 15.00±6.8, night 101.5±20.32, $P<0.001$). Also during blockade of myopia development by an intravitreal 6-OHDA injection, retinal melatonin levels remained unchanged (day, occluded: 20.53±6.35 *vs.* 17.43±6.64, NS) despite of severe reduction in retinal dopamine levels, as measured by HPLC-ED: 1.33±0.30 (vehicle injected) *vs.* 0.40±0.18 ng/mg protein (200 µg 6-OHDA intravitreal, $P<0.001$). Our results imply that: (1) deprivation myopia affects selectively dopamine rhythms but leaves melatonin levels untouched, (2) during deprivation myopia development, diurnal dopamine rhythms appear to be largely independent of retinal melatonin rhythms.

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Supported by the German Research Council (SFB 307, TP A7).

SEROTONIN AND MELATONIN RECEPTORS IN THE IRIS/CILIARY PROCESSES

Neville N. Osborne and **Glyn Chidlow**

Nuffield Laboratory of Ophthalmology, Oxford University, Walton Street, Oxford OX2 6AW, UK

Specific binding sites for ^{3}H-serotonin exist in the iris/ciliary body of the rabbit eye. The binding sites have been localized by autoradiography and shown to be associated with the ciliary processes. Biochemical characterization of these binding sites shows they are of a 5-HT$_{1A}$-type, primarily because the 5-HT$_{1A}$ ligand, 8-OH-DPAT, has an affinity for the ^{3}H-serotonin binding sites. Moreover, these binding sites are not displaced by melatonin, an analogue of serotonin. Evidence that these binding sites are receptors has come from physiological and pharmacological studies.

Serotonin influences adenylate cyclase metabolism in iris/ciliary processes by what appear to be 5-HT_{1A} receptors. Serotonin, the general 5-HT_1 agonist, 5-carboxamidotryptamine and in particular the 5-HT_{1A} agonist, 8-OH-DPAT, have no effect on basal cAMP levels but all reduce the forskolin-elevated cAMP levels in a dose-dependent manner. Spiperone and propranolol significantly antagonize these actions while ketanserin (5-$HT_{2A/2C}$ antagonist) and ICS 205930 (5-$HT_{3/4}$ blocker) were without influence.

Serotonin also stimulates phosphoinositide metabolism as well as the enzyme protein kinase C (PKC) in isolated iris/ciliary processes. These effects were elicited via a 5-$HT_{2A/2C}$-type receptor as they are blunted by the presence of ketanserin. Methysergide and mianserin also significantly attenuates the serotonin effect on phosphoinositide metabolism but the inhibition is never more than 50%. The serotonin influence on phosphoinositide metabolism is unaffected in iris/ciliary from animals which were denervated by superior ganglionectomy; however this was not the case for noradrenaline which also stimulates phosphoinositide metabolism but *via* α_1-type receptors.

Receptors to melatonin, a metabolite of serotonin, also exist in the iris/ciliary processes. Using the melatonin agonist iodomelatonin, ^{125}I-iodomelatonin binding sites have been shown to be associated with the rabbit iris/ciliary processes. The binding of the ligand was found to be saturable with a single population of binding sites. Importantly, serotonin and ketanserin had little affinity for the iodomelatonin binding sites compared with a variety of melatonin analogues. Melatonin also decreased the forskolin elevated cAMP levels but this process, unlike that of serotonin, was unaffected by ketanserin.

When serotonin (0.1-2%) is applied directly to the rabbit eye the intraocular pressure (IOP) is increased to reach maximal levels within 30-60 min. The degree of increase in the IOP is dependent on the concentration of serotonin applied. In contrast, when a 2% solution of melatonin is applied to the eye no change in the IOP is recorded.

The combined studies clearly show that melatonin and serotonin have independent functions in the iris/ciliary processes of the rabbit eye.

SESSION II: PINEAL GLAND: MAMMALS

NON-VISIBLE ELECTROMAGNETIC RADIATION AND PINEAL FUNCTION

Russel J. Reiter
Department of Cellular and Structural Biology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, 78284-7762 TX, USA

Classically, the pineal gland is known to be regulated by the visible portion of the electromagnetic spectrum, i.e., light (wavelengths of 400-760 nm). Within the visible spectrum, wavelengths in the blue/green range seem to be more suppressive to pineal melatonin production than are other wavelengths. These experiments, however, have been performed on only a few mammals and there may be as yet undiscovered species differences. Within the visible wavelength range there are widely different sensitivities between species in terms of the ability of light to suppress nocturnal melatonin levels. The most sensitive species studied to date is the nocturnal albino rat whereas diurnally active ground squirrels are relatively insensitive to light. In general, it has been shown that suppression of nighttime pineal melatonin production required a much lower intensity of light in nocturnal than in diurnal animals. The ranges of sensitivity between nocturnal and diurnal species can differ several thousand fold. In reference to the pineal gland, the discrimination of light sensitivity may occur at the level of the suprachiasmatic nuclei.

Red light, with wavelengths >600 nm, has typically been used as safe light in animals rooms because it was thought not to influence the circadian melatonin rhythm. Recent studies in the albino rat, however,

indicate that the pineal gland in this species responds to rather low intensity of red light. Thus pineal gland N-acetyltransferase (NAT) activity and pineal and serum levels of melatonin declined linearly in albino rats exposed to different intensities of red light (≥600 nm; low: 140 $\mu W/cm^2$; moderate 690 $\mu W/cm^2$, high: 1200 $\mu W/cm^2$) during the middle of the night. The high intensity red light was as effective as white light (780 $\mu W/cm^2$) in suppressing NAT activity and pineal and circulating melatonin. Red light inhibited nighttime NAT activity and suppressed nocturnal melatonin levels in both retinally degenerate and retinally normal rats. Pretreatment with the NMDA receptor antagonist MK-801 (10 mg/kg, i.p.) completely prevented the red light-induced inhibition of nighttime melatonin synthesis. $MgCl_2$ (300 mg/kg, i.p.) antagonized the effectd of low and moderate intensities of red light, but was ineffective when high red light intensity was used. Since retinally degenerate and retinally normal animals respond in the same way to both red light and pharmacological intervention with the NMDA receptor blocker MK-801, the findings indicate that the activation of central hypothalamic NMDA receptors mediate the photic inhibition of nocturnal melatonin synthesis in the pineal gland. The results also suggest, but do not prove, that the classical photoreceptors may not be involved in the red light suppression of pineal melatonin production.

Extremely low frequency (<100 Hz) electromagnetic fields have also been shown to reduce nocturnal pineal melatonin production in some species. Both pulsed static magnetic fields (MF) as well as 50-60 Hz MF exposure, either acute or chronic, reduces nocturnal melatonin production in rodents under some circumstances. The degree of inhibition of the biosynthetic activity of the pineal gland by MF exposure is typically of lower magnitude than that caused by light exposure. It has been speculated, although not proven, that the magnetoreceptor is within the eye, presumably in the retinas.

Work by the author was supported by NSF and by EPRI.

STUDIES ON RAT PINEAL α_1-ADRENERGIC RECEPTORS

David Sugden[1], Anthony K. Ho[2] and David C. Klein[3]
[1]Division King's College London, Campden Hill Road, London W8 7AH, UK,
[2]Department of Physiology, University of Alberta, Edmonton, Canada T6G 2H7;
[3]Section on Neuroendocrinology, LDN, NINCHD, NIH, Bethesda, MD 20892, USA

The primary regulator of nocturnal melatonin synthesis is noradrenaline (NA), released at night from the sympathetic neurones which innervate the gland. *In vitro* experiments using primary cultures of rat pinealocytes have shown that NA acts *via* both β- and α_1-adrenoceptors to induce serotonin N-acetyltransferase (NAT) activity, the rate-limiting enzyme in melatonin synthesis (1). *In vivo* experiments confirm a role for an α_1-adrenergic mechanism in regulating the physiological nocturnal elevation of melatonin synthesis. Pharmacological, and more recently, molecular cloning studies have established the heterogeneity of α_1-adrenoceptors; four α_1-adrenoceptor subtypes can be distinguished (2). Radioligand binding studies using the non-subtype selective α_1-antagonist [125I]-HEAT and subtype selective competitive antagonists have shown that the rat pineal α_1-adrenoceptor is predominantly the α_{1B}-subtype. Studies on second messenger responses in dissociated pinealocytes have shown that the increase in cyclic AMP synthesis following NA treatment has a major α_{1B}-component, although, in addition, there is evidence for a role of another α_1-subtype. The NA-induced elevation in $[Ca^{2+}]_i$ in pinealocytes appears to be mediated through an α_{1B}-adrenoceptor subtype. *In situ* and Northern blot analysis indicates that α_{1B}-adrenoceptor mRNA is abundant in rat pineal. These data suggest that pinealocyte α_{1B} -adrenoceptors play an important role in regulating nocturnal melatonin synthesis.

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CHOLINERGIC SIGNALING IN THE RAT PINEAL GLAND

Jarmo T. Laitinen and **Tarja Kokkola**
Department of Physiology, University of Kuopio, POB 1627, FIN-70211 Kuopio, Finland

Recent studies suggest that acetylcholine (ACh) may play some role in pineal physiology. Radioligand binding studies have revealed the presence of muscarinic ACh binding sites in the pineal gland of several mammalian species, including the rat (1). We have been interested in the possible functional role of these binding sites. In previous work, we have found that cholinergic stimulation of the rat pineal gland *in vitro* activates the phosphoinositide (PI) signaling pathway through a muscarinic ACh receptor (mAChR) with pharmacological characteristics of type M1 (relative potency of muscarinic antagonist in blocking the cholinergic response: atropine>pirenzepine>>galamine)(2). The pineal PI response to the cholinergic agonist carbachol (CCh) was not altered after removal of symphathetic innervation to the pineal gland *via* superior cervical ganglia, a manipulation that readily sensitized PI signaling through the adrenoceptors (2). *In vitro*, pineal muscarinic PI response was desensitized following 2 h treatment with CCh; this homologous desensitization was not mimicked by forskolin or phorbol esters suggesting that protein kinases A and C were not involved (3). On the other hand, sensitization of the PI signaling was seen in rats with advancing age (3). A possible physiological role of these receptors may be modulation of melatonin synthesis/release as cholinergic stimulation has been found to modestly increase melatonin release from pineal slices (4) or glands in organ culture (3).

More recent work using primary cultures of rat pinealocytes have revealed that CCh stimulated melatonin production in pinealocytes obtained from 18-w-old rats but not from 4-w-old rats. However, the response to CCh (1 mM) was only 8-fold as compared to 300-fold increases in melatonin production obtained with 10 μM norepinephrine (NE). For comparison, pinealocytes from 4-w-old rats produced 25-80-fold melatonin in response to 10 μM NE. How CCh elicits an increase in melatonin production is not yet known. No changes in pineal cAMP levels have been detected following stimulation with CCh. Further, CCh or the nitric oxide (NO)-donor sodium nitroprusside (SNP) have no effect on NE or isoproterenol stimulated melatonin production suggesting that CCh does not work through the NO-cGMP pathway. Moreover, muscarinic agonists do not seem to affect pineal cGMP levels (3, 5).

In conclusion, experimental data from this and other laboratories suggest that the pineal muscarinic binding sites may represent functional mAChRs that are linked to the PI signaling pathway. These mAChRs seem to modulate melatonin production *in vitro*. Whether this is also the case *in vivo* remains to be shown.

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PRESENCE OF NEUROPEPTIDERGIC CONTAINING PINEALOCYTES IN THE MAMMALIAN PINEAL GLAND: INDICATIONS FOR A PARACRINE REGULATION

M. Møller[1], A. Coto-Montes[2], S. Webb[3], E. Mato[3], P. Pevet[4], M. Masson-Pevet[4] and **J.D. Mikkelsen[1]**
[1]Institute of Medical Anatomy, University of Copenhagen, Denmark; [2]Department of Anatomy, Oviedo, Spain; [3]Department of Endocrinology, Hospital de la Santa Creu i Sant Pau, Autonomous University, Barcelona, Spain; [4]URA-CRNS 1332, Neurobiologie des fonctions rhythmiques et saisonnieres, Universite Louis Pasteur, Strasbourg, France

The mammalian pineal gland is innervated by a number of neuropeptide containing nerve fibres. Thus, neuropeptide Y (NPY) is colocalized with norepinephrine in the sympathetic fibres and in fibres origin-

ating in the brain (central fibres). Nerve fibres containing vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI) are located in non-sympathetic fibres with origin probably in the parasympathetic pterygopalatine ganglia. Further, pinealopetal nerve fibres containing substance P, calcitonin gene related peptide, opioidergic peptide, somatostatin, and vasopressin have been demonstrated.

However, in some species, it has now been possible to show that certain pinealocytes also contain neuropeptides. For example, in the European hamster, multipolar cells have been shown in the pineal gland which are immunoreactive to leu-enkephalin and met-enkephalin. By use of immunocytochemistry it could be verified, that these cells were pinealocytes and not neurones. The processes of the peptidergic pinealocytes made synaptic contacts with other pinealocytes indicating a neuropeptidergic regulation of the non-immunoreactive pinealocytes. It must be emphasized that such opioidergic pinealocytes have to date only been found in the European hamster.

In the male, adult Wistar rat few somatostatin containing pinealopetal nerve fibres are present. In addition, somatostatinergic neuronal-like, often bipolar cells, have been found. The presence of somatostatin synthesizing cells has been supported by *in situ* hybridization studies, using ^{35}S-labelled cDNA oligonucleotides. A strong hybridization signal was observed in few pinealocytes, verifying the presence of mRNA encoding somatostatin. It has up to now not been possible to study these cells on the electron microscopical level.

Therefore, the pinealocytes of the mammalian pineal gland are not, as previously believed, of only one cell type. A subpopulation of peptidergic pinealocytes are present, the function of which is a paracrine regulation of other pinealocytes.

NATRIURETIC PEPTIDES AND THEIR RECEPTORS IN THE MAMMALIAN PINEAL: IDENTIFICATION OF NEW REGULATORS OF THE CYCLIC GMP PATHWAY

J. Olcese[1], E. Maronde[1], C. Schmidt[1], D. Muller[1] and R. Middendorff[2]
[1]Institute for Hormone and Fertility Research, and [2]Institute for Anatomy, University of Hamburg, Hamburg, Germany

Recent studies involving nitric oxide, a direct activator of soluble guanylyl cyclases, have drawn attention to the regulation of cGMP in the mammalian pineal gland. We have recently identified both type A and B natriuretic peptide receptors in the rat and bovine pineal gland. These receptors possess intrinsic guanylyl cyclase activity and generate cGMP when activated by their natural ligands, which are atrial natriuretic peptide (ANP) or B-type natriuretic peptide (BNP) in the case of the type A receptor (GC-A), and the C-type natriuretic peptide (CNP) in the case of the type B receptor (GC-B). Using reverse transcriptase/polymerase chain reaction with subsequent DNA blot hybridization, transcripts of both the GC-A and GC-B forms of natriuretic peptide receptor were found. Photoaffinity cross-linking with iodo-ANP demonstrated the presence of the GC-A receptor (130 kDa). The addition of natriuretic peptides (10-1000 nM) to monolayer cultures of rat or bovine pinealocytes resulted in significant dose-dependent elevations of cGMP accumulation. More recently, we have obtained evidence for the presence of pineal cells that are immunopositive for ANP and CNP. These results reveal a new complexity in the pineal cGMP-generating system, i.e. the heretofore unrecognized involvement of membrane guanylyl cyclases, which are activated by natriuretic peptides to generate cGMP. Furthermore, it would appear that at least one source of these peptides is from cells in the pineal gland itself.

Supported by a grant to J.O. from the Deutsche Forschungsgemeinschaft #01 45/4-1.

CHARACTERISATION AND PHOTONEURAL REGULATION OF RAT PINEAL NITRIC OXIDE SYNTHASE

N.C. Schaad[1], J. Vanecek[2] and P.E. Schultz[1]

[1]Division of Clinical Pharmacology, Department of Psychiatry of the University of Geneva, 125 Chêne-Bourg, Switzerland; [2]Institute of Physiology, Czech Academy of Sciences, 1083 Videñská, 14220 Prague, Czech Republic

The rat pineal gland responds to norepinephrine (NE) with a 100-fold increase in cGMP formation. Neither the function nor the molecular mechanism controlling its formation are fully understood. It is known, however, that the NO generator sodium nitroprussiate stimulates guanylate cyclase activity in pineal membrane and the cytosolic preparations, and cGMP formation in intact pineal tissue. Accordingly, it appears that NO might be involved in the NE stimulation of pineal cGMP formation. To pursue this, we have determined if the pineal gland contains the enzyme which generates NO from arginine, NO synthase (NOS). A modification of a published method was used (PNAS, 83: 9030-33). Pineal NOS activity was about 40% of that in the cerebellum, 30% higher than in the hippocampus and 80% higher than in the cerebral cortex. Enzyme activity was cytosolic (95% of the total activity), Ca^{++}-dependent (EC_{50}=140 nM), and inhibited by trifluoroperazine. These results indicate that a constitutive, Ca^{++}/calmodulin-dependent form of NOS is present in the rat pineal gland. Pineal $[Ca^{++}]_i$ is regulated *via* an α_1-adrenergic mechanism, which points to the probability that NE activation of the pineal gland elevates NOS activity trough elevation of $[Ca^{++}]_i$ and the resulting increase in $[NO]_i$ plays a role in the stimulation of cGMP formation.

Developmental studies indicate that NOS activity is present before the cGMP response to NE treatment is first detected and circadian studies indicate that it does not change significantly on a 24-hour basis in the adult. The factors regulating the expression of the constitutive form of nitric oxide synthase (cNOS) in the central nervous system remain largely unknown. We report here a photoneural regulation of nitric oxide synthase (NOS) activity in the rat pineal gland. In the absence of the adrenergic stimulation following constant light exposure (LL) or superior cervical ganglionectomy, pineal NOS is markedly reduced. A maximal drop is measured after 8 days in LL. When rats are housed back in normal light:dark conditions (LD 12:12), pineal NOS activity returns to normal after 4 days. A partial decrease in pineal NOS activity is also observed when rats are placed for 8 days in LL 18:6 or shorter dark phases indicating that pineal NOS activity reflects the length of the dark phase. Because it its known that NE is released at night from the nerve endings in the pineal gland and this release is blocked by exposure to light, our data suggest that NOS is controlled by adrenergic mechanisms. Our observation may also explain the lack of cyclic GMP response to NE observed in animals housed in constant light.

ADRENOCEPTOR STIMULATION INDUCES NITRIC OXIDE FORMATION IN RAT PINEALOCYTES

R. Spessert, G. Hill, E. Layes and L. Vollrath

Department of Anatomy, Johannes Gutenberg University, 55099 Mainz, Germany

It is well established that in rat pinealocytes cGMP formation is under adrenergic control. For the full stimulation of cGMP formation by norepinephrine to occur, the activation of both α_1- and β-adrenoceptors is required. β-Adrenergic stimulation alone produces only a small increase whereas β-stimulation combined with α_1-stimulation results in an amplified increase in cGMP formation, α_1-adrenergic stimulation alone being without effect. The effect of α_1-adrenergic receptor stimulation on cGMP formation depends on Ca^{2+} influx, calmodulin and protein kinase C. In various neuronal tissues receptor-induced cGMP formation is mediated by the messenger molecule nitric oxide (NO). It is synthesized by the cata-

lytic action of nitric oxide synthase (NOS) and strongly activates the enzyme cytosolic guanylyl cyclase (cGC) resulting in an increase in cGMP formation.

In the present study we investigate a putative role of NO in adrenergic regulation of cGMP synthesis. Firstly, the occurrence of NOS-immunoreactivity in a subpopulation of pinealocytes supports our idea that NO may play a part in pineal cGMP synthesis. As shown by the inhibitory action of NOS inhibitors and cGC inhibitors we found that cGMP stimulation requires both NO synthesis and NO-dependent activation of cGMP formation. Using an experimental system which enables us to determine NO synthesis and NO-dependent activation of cGMP synthesis separately we elaborated that adrenoceptor stimulation induces NO formation. The effect on NO formation is suggested to involve α_1-adrenergic-stimulated Ca^{2+} and calmodulin which are known to activate NOS. Apart from the increase in NO, we found that NO-activated cGMP formation itself is not adrenergically regulated. However, it turned out to be strongly Ca^{2+}-sensitive suggesting that this step in cGMP transduction may be controlled by a non-adrenergic, Ca^{2+}-dependent mechanism.

In conclusion our findings show that adrenergic regulation of pineal cGMP formation is mediated by changes in NO formation. More generally, our findings show that, besides other types of receptor, adrenoceptors also regulate NO formation.

SESSION III: PINEAL GLAND: NON-MAMMALIAN SPECIES

PHOTORESPONSE AND MELATONIN REGULATION OF PINEAL PHOTORECEPTORS IN TELEOSTS

Hilmar Meissl and **Julian Yañez**
Max-Planck-Institute for Physiology and Clinical Research, W.G. Kerckhoff-Institute, 1 Parkstrasse, 61231 Bad Nauheim, Germany

The role of the pineal organ in physiological functions which are related to the circadian and circannual control of endocrine activity seems to be partly mediated by the photoperiodic dependent secretion of the pineal hormone melatonin with its elevated nocturnal and low daytime levels. In the pineal organ of the trout, *Oncorynchus mykiss,* melatonin production appears to be directly controlled by changes in the light environment and does not maintain a circadian pattern of melatonin secretion in the absence of external cues, i.e. the pineal does not contain an endogenous oscillator. From electrophysiological studies we have obtained numerous informations that the pineal is not only a simple light detector that acts as a kind of photometer, but that it is capable of processing the light information and to discriminate it from signals that have no meaning for its assumed photoperiodic function. This complex response pattern is possibly caused by an intrapineal interaction of second order neurones on photoreceptor activity. In order to elucidate the possible contribution of putative neurotransmitters and modulators on photoreceptor function, we have maintained trout pineal organs in whole organ culture under continuous superfusion conditions. Drugs were added directly to the superfusion medium and the release of the methoxyindoles melatonin, 5-methoxytryptophol, 5-methoxyindole acetic acid and 5-methoxytryptamine was directly measured in the superfusion medium by high pressure liquid chromatography (HPLC) with electrochemical detection. Melatonin synthesis under organ culture conditions is mainly regulated by light and dependent on irradiance and wavelength of the light stimulus. The response pattern is similar to the electrophysiologically observed photoresponses with the exception that the melatonin response seems to have a several log units lower sensitivity than the electrical response. Blocking synaptic transmission in the pineal by replacement

of the perfusion medium with a medium containing low calcium (0.13 mM), high magnesium (6.08 mM) concentrations blocked the light response of second order neurones completely and reduced melatonin release by 35% under scotopic and up to 66% under mesopic (6.5×10^{11} photons/cm^2/s at 520 nm) conditions. We assume that this reduction in melatonin output is caused by the removal of a neuronal feedback of second-order neurones onto photoreceptor cells in addition to the proposed role of calcium ions on melatonin synthesis . However, melatonin synthesis and release is also modulated by other chemical factors. Drugs acting on the $GABA_A$/benzodiazepine receptor complex are able to modify the daytime production of the pineal hormone. Melatonin formation was considerably increased after application of diazepam, a full agonist of the benzodiazepine receptor in a dose-related and reversible manner with strongest effects under mesopic conditions, when melatonin secretion is usually low. The site of action of diazepam is probably directly onto photoreceptor cells, because the effects survive blocking synaptic transmission. Furthermore, preliminary experiments suggest that melatonin may inhibit its own production by a kind of paracrine mechanism. After addition of exogenous melatonin to the organ culture in concentrations that are released by the gland at the same time interval, melatonin release decreased significantly. These data indicate that melatonin production is, in addition to its major control by the environmental light, regulated by internal factors.

THE ROLE OF ION FLUX IN MAINTAING DARKNESS-ASSOCIATED MELATONIN SYNTHESIS IN TROUT PINEAL ORGANS

William A. Gern and **Paul J. Gasser**
Department of Zoology and Physiology, University of Wyoming, Laramie, 82071 Wyoming, USA

Rainbow trout pineal organs do not contain an endogenous clock regulating melatonin synthesis; regulation is gained solely through light. Differential melatonin synthesis in response to light color and intensity has been documented (2, 3). Because of this photoresponsivity and lack of an endogenous clock, the rainbow trout pineal organ has been linked to an endocrine photometer (2). The base state for photoreceptor cells is encountered in darkness. Light initiates a series of biochemical events driving the cell from its base state. Meissl and coworkers (3) have shown that trout pineal photoreceptor cells display resting membrane potentials in darkness and hyperpolarize in response to light. We hypothesized that elevated melatonin synthesis is an integral part of the dark-state physiology of trout pineal photoreceptor cells and that light acts to decrease melatonin synthesis (2). This hypothesis implies that, rather than having darkness create "peaks" of melatonin synthesis from baseline, light carves "valleys" into the melatonin plateau.

We have examined various steps in the photoreceptor pathway to determine how light acts to inhibit melatonin synthesis in rainbow trout pineal organs maintained in whole organ culture. In a series of experiments we have been unable to measure a pertussis toxin-induced effect on melatonin synthesis, implying that melatonin synthesis is not directly regulated by the G-protein transduction. Similar results have been obtained by Falcón et al. (1). Manipulations of intracellular Ca^{2+} altered melatonin synthesis. Trout pineals incubated in Ca^{2+}-free medium in the dark, decreased melatonin synthesis to about 10% of control dark values but responded to light by further decreasing melatonin synthesis to baseline. Mn^{2+} (given as $MnCl_2$) decreased melatonin synthesis. Nitrendipine, an antagonist of "L"-type, voltage-sensitive calcium channels, decreased darkness-associated melatonin synthesis (4). Trout pineals treated with nitrendipine displayed a 70% decrease in darkness-associated melatonin synthesis, with light bringing melatonin synthesis to baseline. Cyclic-AMP overrode the nitrendipine blockade of melatonin synthesis, implying that cAMP acted downstream of Ca^{2+}. The "L"-type channel agonist, Bay K 8644, increased melatonin synthesis during darkness. Finally, treatment with tolbutamide, a K^+-channel antagonist, significantly increased trout pineal melatonin synthesis in both light and darkness.

We interpret these results the following way: 1) melatonin synthesis required Ca^{2+} influx through the "L"-type voltage sensitive calcium channel and this Ca^{2+} is involved in activating a cAMP system that increases N-acetyltransferase activity and melatonin synthesis; 2) light acts to increase the pineal photoreceptor cell membrane potential thus reducing Ca^{2+} influx; 3) tolbutamide blocks K^+ outflow thus reducing the photoreceptor cell membrane potential (compared to control pineals) allowing more voltage sensitive Ca^{2+} channels to remain open in both the light and dark states resulting in relative elevations in melatonin synthesis in both states. These results are compatible with the hypothesis that melatonin synthesis is an integral component of the dark state pineal photoreceptor cell physiology and that light inhibits melatonin synthesis by first altering the membrane potential which subsequently alters Ca^{2+} entry. This scheme is similar to the scheme proposed by Żurawska and Nowak (5) for regulation of retinal melatonin synthesis.

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IONIC MECHANISMS IN THE CHICK PINEAL GLAND: VOLTAGE- AND CGMP-ACTIVATED IONIC CHANNELS AND REGULATION OF INTRACELLULAR FREE CALCIUM

Stuart E. Dryer
Florida State University, Tallahassee, 32306-4075 FL, USA

Chick pineal cells contain intrinsic light-sensitive circadian oscillators that persist in dissociated cell culture. We have used patch-clamp and fura-2 imaging techniques to describe ionic mechanisms in acutely-isolated chick pineal cells. Chick pineal cells express voltage-activated Na^+ and Ca^{2+} channels, as well as several types of K^+ channels similar to those of excitable secretory cells. However, we have not observed these cells to fire action potentials, either spontaneously or in response to current injection, and the physiological significance of the Na^+ channels is unknown.

The amplitude and characteristics of Na^+ and Ca^{2+} currents are similar in nearly every pineal cell, but there is considerable variability in the amplitude and characteristics of K^+ currents in different pineal cells. Extrinsic or intrinsic modulation of K^+ currents could provide a powerful mechanism to regulate Ca^{2+} influx. With inside-out patches, we have detected two distinct populations of cyclic GMP-activated cationic channels, strikingly similar to those observed in vertebrate rods. These channels are permeable to Ca^{2+}, are not activated by physiological concentrations of cyclic AMP, and are not potentiated or inhibited by micromolar concentrations of Ca^{2+} applied to the cytoplasmic face of the patch membrane. But as with retinal photoreceptors, these channels are partially blocked by physiological concentrations of intracellular Mg^{2+}. These channels, along with rhodopsin, transducin, and arrestin, may serve to mediate the acute inhibition of melatonin secretion caused by light. It is unlikely that cyclic GMP-activated channels are involved in light-induced resetting of the circadian pacemaker. As expected, membrane depolarization evoked large increases in intracellular free Ca^{2+} as measured with fura-2. In addition, about 20% of chick pineal cells exhibited spontaneous Ca^{2+} oscillations. Application of thapsigargin caused increases in intracellular free Ca^{2+} due to mobilization of intracellular Ca^{2+} stores. Moreover, agents such as VIP, forskolin, 8-Br-cyclic AMP, and IBMX, which increase intracellular cyclic AMP levels, caused increases in intracellular free Ca^{2+}. These results suggest new mechanisms for the regulation of melatonin synthesis by extrinsic hormones and they raise the possibility of novel output pathways for the intrinsic circadian oscillator.

Supported by AFOSR Grant F-49620.

NEUROPHYSIOLOGICAL AND IMMUNOCYTOCHEMICAL RELATIONSHIP OF PHOTORECEPTORS AND NEURONES IN PHOTOSENSORY PINEAL ORGANS

Y. Morita, S. Tamotsu and **K. Uchida**
1st Department of Physiology, Hamamatsu University School of Medicine, Hamamatsu 431-31, Japan

Text of the abstract received late, printed at pages 136-137.

DOES HISTAMINE STIMULATE CYCLIC AMP FORMATION IN THE CHICK PINEAL GLAND *VIA* A NOVEL (NON-H_1, NON-H_2, NON-H_3) HISTAMINE RECEPTOR TYPE?

Jerzy Z. Nowak and **Barbara Sęk**
Department of Biogenic Amines, Polish Academy of Sciences, 3 Tylna St., 90-950 Łódź, Poland

Histamine is an established neuromodulator that is involved in the regulation of many diverse activities of the brain, including the neuroendocrine system. Although most "histamine" findings come from experiments carried out on different mammals, some also show the potency of the amine in non-mammalian species. Histamine was shown to be a stimulator of cAMP accumulation in pieces of chick brain and retina (1,2). Recently, we have observed (3,4) that histamine action in intact chick pineal glands was stronger than that in the cerebral cortex of chick, guinea pig or rabbit. Therefore, the aim of our work was to thoroughly characterize the effect of histamine on cAMP generation in the chick pineal, and to see whether there is a relation between this histamine action and melatonin biosynthesis.

Histamine (0.1-1000 μM) dose-dependently and potently activated production of pineal cAMP, the effect being significantly enhanced in the presence of forskolin or 3-isobutyl-1-methylxanthine (IBMX). The histamine effect was mimicked by several H_1- and H_2-receptor agonists (in decreasing order of potency): 4-methylhistamine (4MeHA), 2-methylhistamine (2MeHA), amthamine (AMTH), 2-thiazolylethylamine (2TEA); Rα-methylhistamine (RαMeHA; a selective H_3-receptor agonist) and dimaprit (an H_2-receptor agonist) were poorely active, while *tele*-methylhistamine (*t*-MeHA; main histamine metabolite), imidazole and L-histidine were inactive. The stimulatory effect of histamine on cAMP synthesis was not affected by mepyramine (H_1-blocker) and thioperamide (H_3-blocker), while it was antagonized by H_2-blockers: tiotidine > oxmetidine >> aminopotentidine (APT) = cimetidine = ranitidine (RAN). However, a more precise analysis of the antagonistic effect of APT and RAN (vs. histamine) showed a non-competitive mode of action of both drugs, and the order of potency of the studied antagonists (vs. histamine) does not fit the profile typical for H_2-receptor blocklade.

On the basis of the obtained results (3-5) we conclude that the pharmacology of the histamine effect on the cAMP generating system in the chick pineal does not indicate a role of any known histamine receptor class (H_1, H_2, H_3; according to mammalian criteria). However, based on the "antagonist" data, there may be some similarity of the chick pineal receptor to the mammalian H_2-type receptor. However, our latest data from the duck pineal suggest a possibility that the bird gland possesses a novel (avian-specific?) histamine receptor subtype. Preliminary findings suggest that stimulation of this histamine receptor leads to activation of melatonin biosynthesis in the chick pineal gland.

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Supported by the KBN grant 0366/P2/94/06.

EFFECTS OF PHOSPHODIESTERASE INHIBITORS AND FORSKOLIN ON IONIC CHANNEL ACTIVITY IN ACUTELY DISSOCIATED CHICK PINEAL CELLS

Theresa D'Souza and **Stuart E. Dryer**
Florida State University, Tallahassee, 32306-4075 FL, USA

Chick pineal cells express a light-sensitive circadian oscillator that persists when cells are maintained in dissociated cell culture. Light has two effects on chick pineal cells: an acute inhibition of melatonin synthesis, and phase-dependent resetting of the intrinsic circadian oscillator. These effects of light appear to utilize different phototransduction cascades. We have recently described cyclic GMP-activated cationic channels in chick pineal cells similar to those described previously in vertebrate rods (1,2). These channels are activated by physiological concentrations of cyclic GMP, but not cyclic AMP. It is possible that these channels are involved in the light-induced inhibition of melatonin synthesis, but it is not known if they change their gating in response to light. However, if phototransduction cascades similar to those of retina were present in chick pineal cells, then treatments that increase the intracellular concentration of cyclic GMP should increase the activity of these channels. This was examined using the cell-attached configuration of the patch-clamp recording technique. Channel activity was examined using pipette salines free of divalent cations to facilitate detection of cyclic GMP-activated channels. Cyclic nucleotide phosphodiesterases were inhibited by application of IBMX (100 μM) or papaverine (50 μM). These treatments, which increase intracellular concentrations of both cyclic GMP and cyclic AMP, caused a large and consistent increase in the activity of cationic channels in cell-attached patches. These channels exhibited marked "flicker" behaviour similar to that of cyclic GMP-activated channels recorded in the presence of millimolar Mg^{2+}. These channels had a unitary slope conductance of less than 15 pS, and reversed when the potential applied to the recording pipette was approximately +30 to +40 mV (corresponding to a cell reversal potential of approximately 0 mV). Application of 10 μM forskolin, which increases intracellular concentrations of cyclic AMP, did not cause activation of cationic channels. These results suggest a role for cyclic GMP hydrolysis in the control of low-conductance cationic channels in the plasma membrane of chick pineal cells, and they are consistent with the predictions of phototransduction cascades previously described in the vertebrate retina.

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Supported by AFOSR F-49620.

INTRINSIC CATECHOLAMINES MODULATE NEURONAL SIGNALS IN THE PHOTOSENSITIVE PINEAL ORGAN OF THE RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

R. Brandstätter, M. Zaunretter, E. Fait and **A. Hermann**
Department of Animal Physiology, Institute of Zoology, University of Salzburg, 34 Hellbrunnerstrasse, A-5020 Salzburg, Austria

Catecholamines play a major role in retinal signal modulation in all vertebrate classes. To investigate if this is also the case in the photosensitive pineal organ of teleosts, we performed electrophysiological, biochemical and immunocytohistochemical studies of the trout pineal. To reveal if catecholamines are synthesized within the pineal gland, we examined pineal catecholamines (organ content and release) and tyrosine hydroxylase (TH) activity by HPLC-ECD. HPLC-measurements of homogenized organs revealed the presence of L-DOPA, dopamine, its main metabolite DOPAC, norepinephrine, and epinephrine within the pineal parenchyma. Both, TH activity and dopamine release from isolated superfused pineals were significantly elevated during darkness. Immunocytochemistry revealed TH-immunoreactivity throughout

the pineal parenchyma, demonstrating TH-containing somata and fibres. Furthermore, both D_1- and D_2-dopamine receptors were labelled in the pineal parenchyma with fluorescent receptor probes.

To tackle the question if catecholamines are involved in intrapineal signal transduction, we examined the effects of exogenously applied dopaminergic and norepinephrinergic drugs on extracellularly recorded ganglion cell activity in isolated superfused organs. A total number of 99 cells were investigated during this study. Exogenous dopamine significantly increased the discharge activity of 75% of pineal neurones, 82% of the investigated cells showed a significant elevation of their discharge rate after application of exogenous norepinephrine. All effects were dose-dependent and reversible.

We used exogenously applied receptor antagonists to reveal the functional significance of endogenous catecholamines during light- and dark-adaptation. SCH-23390, a D_1-dopamine receptor antagonist, reduced the firing rate of about 50% of the cells tested, whereas in other 50% of cells the firing rate was increased. Eticlopride, a D_2-receptor antagonist was without effect (50%) or reduced the firing of the pineal neurones (50%). These data indicate that endogenous dopamine acts via both D_1 and D_2 receptors. Propranolol, a specific β-adrenergic antagonist significantly reduced the firing rate of all neurones tested. Low concentrations of all three catecholamine receptor antagonists ($<10^{-5}$M) revealed significantly stronger effects during dark adaptation indicating that endogenous catecholamines are particularly related to the dark signal. However, higher concentrations also affected light induced ganglion cell activity.

In our study we provide evidence for the intrapineal origin of catecholamines and their functional significance in the modulation of neuronal signals. Furthermore, we propose a preliminary model for the involvement of intrapineal catecholamines in the regulation and/or modulation of nycthemeral neuronal signals of the teleostean pineal organ.

Supported by the Austrian "Fonds zur Förderung der wissenschaftlichen Forschng (P9343BIO)".

SESSION IV: RETINA-PINEAL-BRAIN FUNCTIONAL RELATIONSHIPS

MELATONIN RECEPTORS AND THE MECHANISM OF SIGNAL TRANSDUCTION

Jiri Vanecek

Institute of Physiology, 1083 Videňská St., 14220 Prague, Czech Republic

Effects of the pineal hormone melatonin on seasonal and circadian rhythms are well known, but the mechanism of its action is not well understood. The effects of melatonin are mediated by specific high affinity melatonin receptors. The distribution of the receptors have been shown by *in vitro* autoradiography using ^{125}I-iodomelatonin as ligand (1). In rats, the receptors have been detected in pars tuberalis and distalis (AP) of anterior pituitary, in suprachiasmatic nuclei and in area postrema. In AP, the concentration of the receptors is the highest from all brain structures immediately after birth, but then gradually decreases and it is barely detectable at 30 days of life. In adulthood, the highest concentration of melatonin receptors is seen in pars tuberalis.

Melatonin receptors are located on plasma membranes. Therefore, the hormone may act on cellular metabolism through intracellular second messengers. Melatonin has been shown to decrease the concentration of several intracellular messengers and all these effects appear to be mediated by an inhibitory G-protein, according to the results of studies with pertussis toxin, which antagonizes the effects of melatonin (2,3). In immature rat AP cultured *in vitro*, melatonin inhibits basal and GnRH-stimulated cAMP accumulation (4). Melatonin also inhibits GnRH-stimulation of cGMP accumulation, of diacylglycerol formation and of arachidonic acid release from the gland. Recently we have found that melatonin inhibits GnRH-induced increase of intracellular calcium ($[Ca^{2+}]_i$) in neonatal rat pituitary cells (3). The effect of melatonin on $[Ca^{2+}]_i$ is due to inhibition of Ca^{2+} influx through voltage-sensitive channels. The mech-

anism of this effect may involve membrane potential, because melatonin hyperpolarizes plasma membrane of neonatal rat pituitary cells, and hyperpolarization blocks Ca^{2+}-influx through voltage sensitive channels.

Melatonin inhibits GnRH-induced LH and FSH release from neonatal rat gonadotrophs both *in vivo* and *in vitro* (5). The inhibitory effect of melatonin gradually disappears during postnatal development and is absent after 3 weeks of age, which closely correlates with decrease of melatonin receptors in rat pituitary during postnatal development. The inhibitory effect of melatonin on LH-release seems to be transduced by the inhibition of the GnRH-induced calcium increase. The same mechanism of action may be employed in other cells bearing the melatonin receptors.

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G-PROTEIN MEDIATED MODULATION BY MELATONIN OF CONSTITUTIVE SECRETION

Marina Bubis and **Nava Zisapel**
Department of Biochemistry, Tel Aviv University, Tel Aviv, Israel

The effects of melatonin on secretion of ^{35}S-methionine labeled proteins from cultured melanoma cells was investigated. At physiological concentrations (0.5-10 nM), melatonin inhibited the release early after plating or at low cell density, but facilitated the release later on, or at high cell density. To elucidate the involvement of G-proteins in these responses, the effects of melatonin on protein secretion from the cells were assessed in the absence and presence of guanosine 5'-O-(3-thiotriphosphate)(GTP S; which was introduced into the cells during the process of permeabilization and resealing with ATP), pertussis (PTX) and cholera (CTX) toxins. At low cell density, melatonin inhibited the release, but paradoxically enhanced it when GTP hydrolysis was blocked (by GTP S or CTX treatment). At high cell density, melatonin facilitated the release and so did GTP S. CTX treatment prevented the melatonin-mediated facilitation. Similar treatment of the cells with PTX, did not affect the melatonin-mediated inhibition or facilitation. The ability of melatonin to affect binding of GTP ^{35}S to melanoma proteins was examined. Melatonin significantly enhanced GTP ^{35}S binding to the melanoma cells proteins; this effect was more pronounced at the inhibition than facilitation phase. Pretreatment of the cells with CTX also increased GTP ^{35}S binding; the binding was further enhanced in the presence of melatonin. Photoaffinity labeling of GTP binding proteins in the cells in the absence and presence of melatonin indicated that melatonin enhanced the incorporation of the label into a protein with an apparent molecular weight of 45 kDa and decreased incorporation into a 40 kDa protein. These results indicate that the effects of melatonin on protein secretion are mediated by at least two G proteins, one of which belongs to the Gs class. The direction and magnitude of melatonin's effects are dynamic and determined by the prevailing activation of the G proteins involved, thus generating a see-saw type response.

IONOTROPIC GLUTAMATE RECEPTOR GENE EXPRESSION IN THE RAT BRAIN DURING DEVELOPMENT AND EFFECTS OF VISUAL DEPRIVATION

D.Z. Nowicka and **L. Kaczmarek**
Nencki Institute of Experimental Biology, 3 Pasteur St., 02-093 Warsaw, Poland

It is well established that ionotropic glutamate receptors (GluRs) play an important role in neural transmission and plasticity of visual pathways in the brain. However, it is much less clear whether expression of their genes can be modulated by sensory input. In order to approach this problem we have asked whether

kainate/AMPA (K/A) receptors' (GluR A-C or 1-3, flip and flop) as well as NMDA R1 (NR1) mRNAs are regulated by visual input in the developing rat cortex. In the first series of experiments, two-week old rat pups were visually deprived by surgical removal of the left eye. Then the animals were reared in the presence of visual input (natural day-night cycle) for two weeks. Next, *in situ* hybridizations to probes specifically recognizing flip and flop versions of K/A GluR A-C were performed. Computer-aided image analysis did not reveal any significant changes in the expression of any of the six mRNAs in the visual cortex. In the second series of experiments we attempted to investigate the influence of visual deprivation on the level of NR1 mRNA in visual structures such as lateral geniculate nucleus (LGN), superior colliculus (SC) and visual cortex area 17. In order to study the rat brain during development animals were sacrified on P7, P14, P19, P26 and P40. Additionally some animals were reared in darkness from P19 until P26 or P40. Again the level and distribution of NR1 mRNA were analyzed from autoradiograms with an aid of image analysis system. Our experiments revealed that hybridization pattern changes during development. In particular, we observed progressive increase in the level of NR1 in brain cortex. However, we did not notice any significant change in NR1 level in visual structures due to visual deprivation.

ELECTROPHYSIOLOGICAL CORRELATES OF VISUAL ATTENTION

Andrzej Wróbel
Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur St., 02-093 Warsaw, Poland

The idea that the rich cortico-thalamic projection controls the function of the lateral geniculate nucleus (LGN) was put forward long ago but still lacks good experimental support. The system is difficult to study because its activity is suppressed by anesthesia. We have therefore recorded the electrical activity from the primary visual cortex (VCx) and LGN in behaving and pretrigeminally transected cats (1,2). The animals were taught to attend to the location of disappearance of either visual or acoustic moving stimuli which signaled reward in the left or right foodwell. The frequency analysis of EEG activity recorded during this period revealed significant elevation of the 20 Hz band, at both VCx and LGN recording sites, only during visual trials that ended with the successful responses. This finding supported the notion that such a rhythmic activity was a neural correlate of attention processes. The specific 20 Hz activity was characterized by 0.1-1 s bursts of oscillations which tended to appear simultaneously in VCx and LGN. The amplitude and mean frequency of appearance of these bursts exceeded the relevant values observed during acoustic trials. The calculation of directed transfer function suggested that this activity was propagated by the descending visual pathway. Concomitant recording from acute preparations with pretrigeminal transections of the brainstem, with the typically desynchronized EEG, revealed decrease in both spontaneous and evoked neuronal activity of LGN neurones after reversible cool of the VCx (Waleszczyk, Bekisz and Wróbel, unpublished). This agrees well with our previous finding (3) that cortico-geniculate EPSPs in principal LGN cells are frequency potentiated and with repetitive cortical activation reaching 20 Hz, typically exceed the optic tract evoked EPSP. Thus, the cortico-thalamic projection can have a marked influence on the membrane potential of LGN cells. The set of behavioural and neuronal data strongly support the hypothesis that short synchronized bursts of 20 Hz frequency in the cortico-thalamic system might control the gain of geniculate relay during attentive perception.

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Supported by James S. McDonnell Foundation

SESSION V: RETINA-PINEAL RELATIONSHIPS

PHOTORECEPTOR-SPECIFIC PROTEINS IN THE MAMMALIAN PINEAL ORGAN: IMMUNOCYTOCHEMICAL DATA AND FUNCTIONAL CONSIDERATIONS

Ch. Schomerus, P. Ruth and **Horst W. Korf**
Center of Morphology, Section on Neurobiology, Johann Wolfgang Goethe University, 7 Theodor-Stern-Kai St., 60590 Frankfurt/Main, Germany

This contribution reviews immunocytochemical data on the distribution of photoreceptor-specific proteins (rod-opsin, S-antigen, recoverin, alpha-transducin, cyclic GMP-gated cation channel) in the pineal organ of various mammalian species. It also presents an *in vitro* model allowing combined analysis of functional parameters and immunocytochemical features of single pinealocytes. The immunocytochemical investigations have revealed conspicuous interspecific differences, but the following general conclusions can be drawn: Immunoreactions for S-antigen and recoverin labeled more pinealocytes than the rod-opsin immunoreaction. The intensity of the recoverin- and S-antigen immunoreactions varied from cell to cell. α-Transducin immunoreaction was absent from the pineal organ of all mammals investigated with the exception of the blind mole rat *Cryptomys damarensis.* Immunoreaction for the cyclic GMP-gated cation channel was undetectable in the pineal organ of all mammals investigated. These data show that only some components of the phototransduction cascade are expressed in the mammalian pineal. In regard to their function, it has been speculated that they become involved in the adrenergic transduction cascade which plays an important role in regulation of pineal functions in mammals and which resembles in several aspects the phototransduction cascade. To address the problem of a putative relationship between adrenergic transduction mechanisms and the expression of photoreceptor-specific proteins, we have started to analyze calcium responses of single rat pinealocytes to norepinephrine stimulation by means of the Fura-2 technique and to characterize these functionally identified cells further by means of immunocytochemical demonstration of S-antigen. These investigations showed that variation in S-antigen immunoreactivity is not correlated with differences in the rapid calcium response to stimulation with norepinephrine. It remains to be determined whether cells displaying different intensities of the S-antigen immunoreaction show different cyclic AMP responses to noradrenergic stimulation. Investigations along this line should help to clarify further whether there is indeed a relation between the expression of S-antigen and noradrenergic transduction mechanisms in the mammalian pineal organ.

5-METHOXYTRYPTOPHOL (ML) AND 5-METHOXYTRYPTAMINE (MT) IN THE RETINA AND PINEAL GLAND

D.J. Skene
School of Biological Sciences, University of Surrey, Guildford, UK

Apart from melatonin, two other 5-methoxyindoles (5-methoxytryptophol (ML) and 5-methoxytryptamine (MT)) are synthesised in the pineal gland and retina. Using a sensitive and specific radioimmunoassay (RIA) pineal and retinal ML has been measured in different species including man. Concentrations of ML varied depending upon the species, time of day, photoperiod, age, and season. Generally pineal ML levels showed a day/night variation with higher concentrations occurring during the day.

Using the Syrian hamster as a model, the role of ML and MT in photoperiodism has been studied. The daily rhythmic production of both pineal indoles was dependent upon the prevailing photoperiod, the duration of low night-time levels of ML (1) and MT (2) being proportional to the length of the dark phase. Whilst studies of the effect of ML on the gonadal axis are conflicting, MT (25 μg daily; 8 weeks) induced

gonadal atrophy in hamsters kept in long photoperiod (3). The site and mechanism of action of MT is not known but MT's agonist action on 5HT receptors ($5HT_{1A}$, $5HT_{1B}$, $5HT_4$) may be implicated.

The study of ML and MT in the retina is in its infancy. Both indoles have been detected in the retina of different species (review 4), and retinal synthesis of ML and MT from melatonin has been demonstrated in non-mammalian vertebrates (5). Using the frog retina as a model, day and night-time concentrations of ML and [^{125}I]ML binding have been determined and compared with melatonin content and [^{125}I]melatonin binding. Of the limited number of studies assessing the effects of exogenous ML and MT on retinal processes, a role for ML in rod disc shedding and phagocytic activity of pigment epithelial cells has been indicated. MT may function to modulate 5HT retinal processes. Until the necessary experiments are performed, a retinal action of these 5-methoxyindoles cannot be ruled out.

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COMPARISON OF MELATONIN SYNTHESIS IN THE RETINA AND PINEAL GLAND AT DIFFERENT TIMES OF THE YEAR

Stephan Steinlechner, Ilona Baumgartner, Gabriela Klante and **Russel J. Reiter**[1]
Institute of Zoology, Veterinary School at Hannover, 17 Bunteweg St., 30559 Hannover, Germany; [1]Department of Cellular and Structural Biology, UTHSC, San Antonio, 78284 TX, USA

It is generally assumed that melatonin synthesis in the retina follows the same metabolic pathway as has been shown for the pineal gland. In addition, regulation of retinal melatonin synthesis by light is thought to be equivalent to that seen in the pineal gland, i.e. light is inhibitory and hence melatonin is synthesized in the retina during the night only. However, several studies have reported elevated levels of melatonin during the daytime hours (1). In a preliminary study we measured daily profiles of N-acetyltransferase activity in the pineal gland and melatonin content in the retina of the same animals under natural photoperiodic conditions throughout the course of a year. These daily profiles turned out to be very different and no temporal correlation was detectable between melatonin synthesis in the pineal gland and retinal melatonin. Whereas pineal melatonin synthesis was always elevated during the scotophase irrespective of the time of year, peak levels of retinal melatonin were found at variable times of the day at different seasons. Retinal melatonin exhibited monophasic rhythms in April, June, August and September. A biphasic rhythm was measured in February and no rhythm at all in October. These results indicate that melatonin production in these two organs is regulated independently from each other. On the other hand, an increase in retinal melatonin was found eight weeks after pinealectomy (2). This led us to believe that a long term feedback regulation between the pineal gland and the retina exists which modulates retinal melatonin synthesis (3).

In order to further characterize the metabolic regulation of melatonin synthesis we developed a method using HPLC with electrodetection for determination of nine indole metabolites (tryptophan, 5-hydroxytryptophan, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, 5-hydroxytryptophol, N-acetyl-5-hydroxytryptamine, 5-methoxyindoleacetic acid, 5-methoxytryptophol and melatonin). Reliable measurements of all nine metabolites, however, were only possible in pineal glands. The methoxyindoles could not be measured in retina homogenates due to low levels of these metabolites combined with large amounts of interfering substances. Nevertheless, the results confirm our earlier findings, i.e. daily rhythms of indoles involved in melatonin synthesis are different in the retinae and pineal gland of the same animals. These

findings corroborate the results of Nowak (4) suggesting that the level of retinal melatonin is regulated and controlled locally within the retina itself. However, long term feedback mechanisms from other sources of melatonin are not excluded but rather likely.

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ARYLAMINE AND ARYLALKYLAMINE N-ACETYLTRANSFERASES IN RETINA, PINEAL GLAND, BRAIN AND LIVER OF CHICK: A COMPARATIVE STUDY

Jerzy Z. Nowak[1], Jolanta B. Zawilska[1,2], Anna Makowska[1] and **Agata Woldan[1,2]**
[1]Department of Biogenic Amines, Polish Academy of Sciences, 3 Tylna St., 90-950 Łódź, and [2]Department of Pharmacodynamics, Medical University of Łódź, Łódź, Poland

There are two classes of aromatic amine N-acetyltransferase (NAT); one class prefers arylalkylamines (e.g., serotonin, 5HT; tryptamine, T; β-phenylethylamine, PEA) and is referred to as arylalkylamine NAT (aaa-NAT); the other class of NAT demonstrates preferance towards arylamines (e.g., *p*-phenetidine, PHEN; procainamide, PRAM) and is referred to as arylamine NAT (aa-NAT). The 5HT-NAT (aaa-NAT) is a key regulatory enzyme in the biosynthesis of the hormone melatonin (MEL). Its properties have been described in detail for the pineal enzyme, whereas aa-NAT is involved in the metabolism of drugs and endogenous substances containing amine and hydrazine groups, and its activity is highest in the liver (detoxification processes). Although the presence of both aaa-NAT and aa-NAT have been described in several body tissues, the retina was only rarely studied in this respect.

In this study, we examined NAT activities in four tissues of the chick, i.e., the retina, pineal gland, brain and liver, using PHEN and PRAM (for aa-NAT) and T and PEA (for aaa-NAT). All substrates were used at 0.1-10,000 μM concentration; under some conditions the substrates were applied at 1 mM concentration. The tissues were taken from animals: (1) maintained under a 12:12 h light:dark illumination cycle and killed in the light or dark phase, (2) adapted to constant light or darkness, or (3) treated with various compounds (PDE inhibitors, cycloheximide, actinomycin D, dibutyryl-cAMP). Furthermore, the time course of inactivation (at 4°C) of aa-NAT and aaa-NAT was also investigated.

The obtained data indicate that the chick retina contains both aa-NAT and aaa-NAT. The two enzymes have distinct characteristics and the regulation of their activities is different. It seems that there are at least two forms of aa-NAT, and that, under specified conditions, the activity of one form may be regulated in an opposite manner to aaa-NAT activity. The retinal aa-NAT is similar to aa-NAT present in other tested tissues; however, aaa-NAT can be induced at night only in the retina and pineal gland.

Supported by the KBN grant 0540/P2/04 to J.B.Z.

EVIDENCES FOR ARYLAMINE N-ACETYLTRANSFERASE ACTIVITY IN THE FROG (*RANA PEREZI*) RETINA . DIFFERENTIAL CHARACTERISTICS AND REGULATION WITH RESPECT TO ARYLALKYLAMINE N-ACETYLTRANSFERASE

A.L. Alonso-Gómez, A.I. Valenciano, M. Alonso-Bedate and **M.J. Delgado**
Departamento Biología Animal II (Fisiología Animal), Facultad de Biología Universidad Complutense, Madrid 28040, Spain

The aim of the present study was the characterization of the arylamine N-acetyltransferase activity (A-NAT; E.C.2.3.1.5) from *Rana perezi* retina, and its comparison with the arylalkylamine N-acetyltransferase (AA-NAT; E.C.2.3.1.87). Enzymatic activities were determined by a radioenzymatic method (1) using p-phenetidine and tryptamine as A-NAT and AA-NAT specific substrates, respectively. A-NAT activity

is present in both, neural retina and choroid-pigmented epithelium complex, nevertheless the enzymatic activity expressed as nmol/h/mg prot was 10-fold higher in the neural retina. In contrast, AA-NAT activity was only detected in the neural retina. Subcellular localization showed that both enzymatic activities are in the supernatant fraction (39,000 g, 20 min). p-Phenetidine acetylation exhibited a significant linearity as a function of the neural retina amount in the assay (from 1/16 to 1 retina), and it was insensitive to phosphate buffer pH into the range 6.5-8.4. The A-NAT kinetic showed a hyperbolic shape for both cosubstrates, and the estimated kinetic constants were K_M=11.2 μM, V_{max}=0.49 nmol/h/mg prot for p-phenetidine (50 μM acetyl-CoA), and K_M=113.4 μM, V_{max}=3.1 nmol/h/mg prot for acetyl-CoA (5 mM p-phenetidine). p-Phenetidine and tryptamine did not compete for the catalytic sites, since both, p-phenetidine-dependent and tryptamine-dependent activities were additive when the substrates were added at the same time. Serotonin addition in the assay revealed important differences in the catalytic mechanism of both enzymes. For AA-NAT, serotonin acted as a strong mixed inhibitor, mainly competitive in nature (apparent K_M for tryptamine was increased). However, respect to A-NAT, serotonin acted as a weak mixed inhibitor, predominantly non-competitive (K_M was only slightly affected). Even though N-acetylserotonin was not quantified in the assay, it can be concluded that serotonin is a poor substrate for A-NAT attending to the low affinity (K_i) of the enzyme. Finally, regulation of both enzymatic activities by photoperiod and temperature is markedly different. Eyecups cultured for 12 hours at low temperature (5°C) showed significantly higher AA-NAT activity compared to the high temperature (25°C) cultured ones, while A-NAT was not affected by culture temperature. Moreover, AA-NAT activity showed a significant increase at subjective midnight with respect to midday, at both culture temperatures. However, A-NAT did not show this day/night oscillation. In conclusion, specific A-NAT characteristics, such as substrate specificity, reaction requirements, and regulation by temperature and light/dark cycles makes possible to discriminate this enzymatic activity from AA-NAT activity in frog retina. Retinal A-NAT is unable to acetylate arylalkylamines *in vitro*, and thus, probably this enzymatic activity does not contribute to *in vivo* melatonin synthesis.

1. Alonso-Gómez et al. (1992) J. Neurochem. 58: 587-592.

HYDROXYINDOLE-O-METHYLTRANSFERASE GENE EXPRESSION IN THE PINEAL GLAND AND RETINA

Pierre Voisin, Jerome Guerlotte, Marianne Bernard, Pierre Greve, Aline Grechez-Cassiau and **Jean-Pierre Collin**

Laboratoire Neuroendocrinologie Cellulaire, URA CNRS 1869, UFR Sciences, 40 Av. Recteur Pineau, 86022 Poitiers, France

Melatonin is an indolic hormone synthesized from serotonin in the pineal gland and to a lesser degree in the retina. This hormone is produced according to a daily rhythm and plays a role in synchronizing physiological functions with the photoperiod (seasonal breeding, circadian activity and retinal adaptation). The last step of the melatonin synthesis pathway is catalyzed by hydroxyindole-O-methyltransferase (HIOMT). An antibody directed against chicken HIOMT and cDNA encoding this enzyme have been produced in our laboratory and used to examine the effect of environmental lighting on HIOMT expression in the pineal gland and retina of chickens.

Long term effect of light: HIOMT activity increases 2-fold in the pineal gland of chickens kept in constant light for 2 weeks. Immunotitration and Western blot analysis revealed that this activity change reflects a 2-fold increase in HIOMT concentration. Furthermore, Northern blot analysis indicated that constant light causes a 2-fold increase in HIOMT mRNA levels, suggesting the regulation may be exerted at transcriptional level.

Day/night rhythm in the pineal gland: Although HIOMT activity and concentration remain practically unchanged through the day/night cycle, Northern blot analysis revealed that HIOMT mRNA con-

centration is 3- to 4-fold higher at midday than at midnight. The lack of rhythm in HIOMT activity might be due to a slow turnover of this protein. The day/night rhythm of HIOMT gene transcription was photosensitive at night but photorefractory during the day time, a result suggesting that it is controlled both by light and by a biological oscillator.

Day/night rhythm in the retina: A day/night rhythm of HIOMT mRNA concentration could be also observed in the retina. However, in contrast to the pineal, peak HIOMT mRNA levels occurred at midnight in the retina. As in the pineal gland, HIOMT gene transcription in the retina was photosensitive at night and photorefractory during the day time.

Characterization of the chicken HIOMT gene: Restriction mapping of chicken genomic DNA revealed that the HIOMT gene is about 20 kb-long and contains at least 8 introns. Screening a chicken genomic library with the HIOMT cDNA allowed us to isolate several fragments of the HIOMT gene and identify a clone that contains the 5'-flanking region. Partial sequencing of the clone revealed homologies with promotor and enhancer elements of other eukaryotic genes.

ICER IN RAT PINEAL GLAND AND RETINA: DIFFERENCES IN EXPRESSION AS A HINT FROM PHYLOGENY?

Jörg Stehle, Nick Foulkes[1], Paul Pevet[2] and Paolo Sassone-Corsi[1]

[1]Institute of Anatomy, University of Frankfurt, 7 Theodor-Stern-Kai St., 60590 Frankfurt, Germany; U184 and [2]CNRS URA 1332, University of Strasbourg, Strasbourg, France

The development and adaptive plasticity of biological systems depends on the regulated expression of specific genes. Within such regulative processes the activity modulation of transcription factors is pivotal. The transcription factor CREM plays an important role in the dynamics of (neuro)-endocrine processes (1-3). The mammalian pineal gland sets a paradigm for neuroendocrine transduction, with neural input regulating hormonal output. Dynamics in melatonin synthesis in rat pineal gland were recently shown to be linked to the expression of a transcription factor: a novel CREM isoform, ICER (inducible cAMP early regulator) was shown to have a nocturnally elevated expression with peak values in the second half of the dark period (3). ICER induction is caused by the release of noradrenaline from pinealopetal sympathetic nerve fibers and driven by the endogenous clock residing in the SCN. ICER encodes a potent repressor of cAMP-induced transcription (3). Using *in situ* hybridization, Northern blotting and RNase protection analysis we now investigated the developmental appearance and temporal regulation of ICER in the rat central nervous system. In the course of ontogenetic development ICER mRNA was not detectable in the central nervous system prenatally. The nocturnally elevated expression of ICER previously described in the pineal gland of adult rodents (3) was first observed in 8-day old animals. The developmental appearance of a day/night difference in pineal ICER expression coincides with maturation of the sympathetic innervation of the gland. There exists conclusive evidence for the phylogenetic origin of the mammalian pineal gland as a homologue of a directly photosensitive diencephalic structure in lower vertebrates, called the "third eye". Therefore, it is not surprizing that molecular biology and immunocytochemistry techniques have revealed a wealth of molecules including proteins of the phototransduction cascade that are expressed in both, the mammalian pineal gland and the retina (cf. Schomerus et al., this issue). It therefore seemed interesting to investigate ICER expression in rat retina. We observed a dramatic difference as compared to rat pineal gland, with ICER showing only very limited expression in rat retina, so far only detectable with RNA blots. Moreover, there is no daily fluctuation in ICER expression in the retina. In conclusion, our data support the fundamental role of the ICER within cAMP-induced transcriptional events in neuroendocrine tissues. Functional differences responsible for ICER pattern formation in rat retina and pineal gland need further explanation. It may be tempting to speculate that ICER is a requirement for fluctuations

in neuroendocrine activity: the presence of rhythmic neurohumoral coupling is linked to a fluctuating expression of ICER as in the case of rat pineal, absence eliminates the need of the repressing transcription factor ICER as in the case of rat retina.

1. Foulkes et al. (1992) Nature 355: 80.
2. Foulkes et al. (1993) Nature 362: 264.
3. Stehle et al. (1993) Nature 365: 314.

IMMUNOCYTOCHEMICAL DEMONSTRATION OF TAURINE IN THE RETINA AND PINEAL ORGAN OF THE PIGEON

M. Ueck, A. Hach and **N. Lake**
Institut fur Anatomie und Zytobiologie, Justus-Liebig-Universitat, 35392 Giessen, Germany

Taurine is a β-aminosulfonic acid discussed in the retina under different aspects. Taurine may be a neurotransmitter or neuromodulator or it may protect outer segments from exposure to toxic levels of light and chemicals. Taurine may be involved in the regulation of ion-flow and ATP-ase activity or in the elimination of substances which originate in photoreceptors during processing of light impulses.

On light- and electron microscopical levels the distribution pattern of taurine in the retina and pineal gland of the pigeon was demonstrated immunocytochemically and the results are discussed on the basis of the hypotheses mentioned.

SESSION VI: MELATONIN - LIGHT THERAPY - HUMANS

PINEAL MELATONIN REGULATION IN NORMAL HUMANS: ROLE OF OCULAR MECHANISMS

George C. Brainard, Frederick L. Ruberg, Felix M. Barker, John P. Hanifin and **Mark D. Rollag**
Department of Neurology, Jefferson Medical College, Philadelphia, 19107 PA;
Pennsylvania College of Optometry, Philadelphia, Pennsylvania, 1914; Department of Anatomy, Uniformed Services University of Health Sciences, Bethesda, 20814 MA, USA

It has been well established in both animals and humans that more light is needed to stimulate the circadian system than to stimulate the visual system. Specifically, in the case of melatonin regulation in humans, it is often stated that bright light of 2,500 lux or more is needed to acutely suppress melatonin or phase shift the melatonin rhythm. When exposure of the human eye is carefully controlled, however, illuminances as low as 5 lux of monochromatic green light (Brainard et al., Brain Res. 1988) or 100 lux of broadband white light (Gaddy et al., Endocrinology, 1993) can produce significant suppression of melatonin in normal human volunteers. To understand how such low levels of illumination can suppress melatonin, it is necessary to examine the ocular mechanisms which mediate this photic effect. Specifically, in humans, factors which can significantly alter the amount and spectral quality of light reaching the retina include: 1) gaze behavior relative to a light source, 2) the age of the ocular lens, and 3) pupillary dilation. For example, a study on postmortem lenses (n=288) showed that lenses of humans between 20 and 29 years of age transmit significantly more blue (440 nm) and green (540 nm) light than lenses of humans aged 50 to 59 years (P<0.0001 and P<0.03, respectively). Once a light stimulus reaches the retina, physiological processes within the retina and within the circadian system will determine the capacity of the stimulus to alter melatonin production. These include: 1) the sensitivity of the operative photopigments and photoreceptors, 2) the photoreceptor location in the retina, and 3) the ability of the circadian system to integrate photic stimuli spatially and temporally. A series of studies have been done which examine

how retinal and neural mechanisms contribute to melatonin regulation. For example, in a preliminary study of subjects with normal color vision (n=6) and color blind subjects (n=8), exposure to 200 lux of white light at night induced a significant suppression of plasma melatonin (P<0.02) in both groups. Further analysis indicated that there were no significant differences in the degree of melatonin suppression between the two groups (Ruberg et al., Soc. Neuroscience Abstracts, 1993). The results from that study suggest that a normal trichromatic photopic visual system is not necessary for the regulation of circulating melatonin by light. Given the increasing use of light to treat affective disorders and problems associated with circadian disruption, it is useful from both a scientific as well as a clinical perspective to elucidate the specific mechanisms in the eye which mediate the therapeutic and biological effects of light.

This work was supported by FDA Grant#785346, NASA Grant#NAGW1196, the National Electrical Manufacturer's Association (#LRI 89:DR:1), the Lighting Research Intsitute (#LRI 88:SP:LREF:6), USUHS Grant#R07049, and the Philadelphia Chapter of the Illuminating Engineering Society.

LIGHT AS A DRUG

Anna Wirz-Justice

Psychiatric University Clinic, 27 Wilhelm Klein-Strasse, CH-4025 Basel, Switzerland

In the last ten years there has been a revolution in concepts concerning the role of light in human neurobiology and pathophysiology. Retinal light input of sufficient intensity and duration, appropriately timed, can elicit phase shifts and changes in the amplitude of circadian rhythms. Light is considered to act directly on the circadian pacemaker and not on sleep regulatory processes. Therefore, the therapeutic application

	SAD (n=11)		
Parameter	Before light	After light	ANOVA
SIGH-SAD	21.4±7.6	4.7±3.3	***
VAS mood (mm)	38.8±6.7	50.0±11.2	**
VAS alertness (mm)	30.9±11.5	31.8±5.3	
VAS tension (mm)	49.5±10.2	48.3±8.6	
VAS hunger (mm)	37.8±19.6	37.8±17.4	
Time estimation (30")	34.0±9.00	32.7±3.9	
Performance (letter cancellation test)	173.1±38.0	194.6±41	***
Rectal temperature (^{o}C)	36.84±0.23	36.77±0.07	
Heart rate (beats/min)	66.8±8.4	64.1±7.6	** (n=10)
Resting metabolic rate (Kcal/day)	1292±185	1226±134	*
Respiratory Quotient	0.83±0.05	0.83±0.3	
Salivary melatonin (pg/ml)	10.4±4.1	10.4±4.2	
Salivary cortisol (nmol/l)	6.9±5.4	4.4±2.1	*
Urine volume (ml)	84.7±12.7	89.6±14.0	
Sleep efficiency (%)	88.6±4.0	91.3±4.7	**
REM Sleep (during first 352 min)	58.0±14.5	67.1±13.4	*
Delta (0.75-4.5 Hz) power density (V^2/Hz)	21.7±5.1	23.5±6.0	* (n=10)

treatment effect: *P≤0.05 ; **P≤0.01;***P≤0.001

of light is focussed mainly on the reentrainment of circadian rhythm-related sleep-wake disorders. However, as yet, the broadest consensus for the efficacy of light stems from studies of its antidepressant effect in seasonal affective disorder (SAD).

The direct effects of light have been little researched. Such effects can be considered as classically "pharmacological". Data from an ongoing study in women with SAD are presented to demonstrate the wide range of psychological, physiological, and hormonal variables that can be modified by light. We have used a 40-hour constant routine (CR) protocol to "unmask" circadian rhythms and to tease out direct effects of light from phase or amplitude modulation. The sleep EEG was recorded before and after each CR, and the CR was repeated after 5 days of light therapy (600 lux, 10-14 h). The single measures or weighed 24-hour mean values averaged over subjects (sd) are listed in a table to document the selectivity of this "drug" effect in SAD patients whose depressive mood improved after light treatment in winter.

Supported by Swiss National Science Foundation Grant No. 32-28741.90.

MELATONIN AND SLEEP DISTURBANCES IN THE ELDERLY: POSSIBLE CLINICAL APPLICATIONS

I. Haimov[1], M. Laudon[2], P. Lavie[1] and N. Zisapel[2]

[2]Tel Aviv University, Tel Aviv; [1]Technion, Haifa, Israel

Melatonin production and levels decrease with age in mammals. However, no disorder has so far been attributed to this decrease. We have investigated the possibility that poor sleep quality in old age is associated with alterations in the daily rhythm of melatonin. Urinary excretions of the major melatonin metabolite, 6-sulfatoxymelatonin, were compared in four groups:(a) Young healthy subjects (age 24±1.6);(b) Independently-living elderly (age 71.4±5.2);(c) Independently-living insomniacs (age 73.1±3.9); (d) Institutionalized insomniacs (age 82.1±8.8). Sleep-wake cycles were monitored for 7 consecutive days by wrist-worn actigraphs. The results indicated that 6-sulfatoxymelatonin excretion in independently living insomniacs was significantly lower and its peak delays, as compared to the age-matched controls. 6-Sulfatoxymelatonin excretion in institutionalized insomniacs was significantly lower than in independently-living insomniacs. No significant differences were found between 6-sulfatoxymelatonin excretion in independently-living elderly and young controls. These findings indicate association between insomnia and deficiency in nocturnal melatonin in old age.

To further elucidate this possibility, melatonin replacement therapy was initiated, using regular and sustained release melatonin formulation containing 2 mg melatonin. The results of this placebo-controlled, double-blind cross-over study indicated that melatonin administered daily in the evening (7 days), effectively reduced sleep latency. Long-term treatment (2 months) with the sustained-release formulation significantly improved sleep initiation and maintenance in the elderly insomniacs.

CIRCADIAN RHYTHM OF SERUM MELATONIN LEVEL IN HOSPITALIZED PATIENTS WITH DEMENTIA

K. Uchida, N. Okamoto[1], K. Ohara[1] and Y. Morita

I Department of Physiology and [1]Department of Psychiatry, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431-31, Japan

Demented patients often show some disorders of the daily rhythms, such as sleep-wake disturbances and nocturnal wandering. In this study, we observed daily changes in serum melatonin levels of patients with dementia of degenerative type by means of radioimmunoassay. Twelve patients were studied. Control groups consisting of 13 age-matched healthy people (control group 1) and 8 healthy young adults (control group 2) were also studied. Further, to evaluate the different circumstances between the patients and the

healthy volunteers, 7 patients hospitalized without dementia in the same clinic where the demented patients stayed were studied (control group 3). The age of the patients was also matched. Twenty seven control subjects exhibited a clear daily rhythm of melatonin, whereas one subject in control group 1 did not show the rhythm. Four persons among the 12 patients did not show the rhythm. In the 5 persons without the melatonin rhythm, 2 patients revealed clinical symptoms of the rhythm disorder. One of these 2 patients was severely demented and the response to stimuli was poor, and the other was in delirium. One patient with the melatonin rhythm showed clinical symptoms of delirium and a disturbed sleep-wake cycle. These results suggest that the possibility of disappearance of the melatonin rhythm is much higher in demented patients than in people without dementia. However, the disappearance of the rhythm does not always accompany a clinical symptom. The sensitivity of patients to Zeitgeber and the functional connection of the circadian system to the rest of the nervous system should be important for clinical symptoms. Daytime melatonin levels of the patients showing the rhythm were significantly higher than that of the control groups 1 and 2 though there was no difference between the patients and control group 3. Illuminance in the ward was under 1,000 lux even on a sunny day. The outside was over 20,000 lux and 5,000 lux on a cloudy day. Since the patients in this study stay in the ward for the whole day, they take less sunlight compared to people in normal daily life. The difference of the daytime melatonin levels between the patients and the healthy subjects in this study could be induced by difference in the amount of daylight. Demented patients tend to be exposed to bright light less than healthy people (1). The circadian system of the patients should thus be hard to entrain to the LD cycle. Phototherapy for the patients (especially the hospitalized patients) could be of benefit in preventing rhythm disorders.

1. Campbell S.S. et al. (1988) Physiol. Behav. 42: 141-144.

SESSION VII: CIRCADIAN PACEMAKER MECHANISMS

THE RETINO-SUPRACHIASMATIC-PARAVENTRICULA-PINEAL PATHWAY IN RODENTS: GATING THROUGH THE SUPRACHIASMATIC NUCLEUS

Jens D. Mikkelsen, Philip J. Larsen, Gerard Mick, Niels Vrang and **Morton Møller**
Institute of Medical Anatomy, University of Copenhagen, Blegdamsvej 3, 2200 Copenhagen, Denmark

The anatomical pathways linking the retina with the pineal gland have neuroanatomically not been fully elucidated. Lesions of the retinohypothalamic pathway, the suprachiasmatic nucleus (SCN), the paraventricular nucleus (PVN) result in abolishing of the regulation of pineal serotonin-N-acetyltransferase activity by light is therefore believed to interfere with the photoperiodic regulation. Therefore, the pathways linking the retina and the pineal gland must be channeled through the SCN and the PVN. The aim of this study was to define the localization and neurochemical phenotypes of SCN neurons in the rat and the Syrian hamster responsible for the photoperiodic regulation of the pineal gland.

In rodents, neuronal tract-tracing have shown that the ventrolateral part and a smaller dorsal subpart of the SCN are innervated directly from the retina. The same regions contain the cells in which light induces c-*fos*. The retinohypothalamic pathway has been shown to contain glutamate and substance P. Our first aim was to localize the target neurons of this projection by localizing glutamergic and substance Pergic receptors in the SCN by using *in situ* hybridization biochemistry and immunocytochemistry. *In situ* hybridization histochemistry revealed the presence of NMDA-R1 and NMDA-R2C mRNA subunits. Further, mRNA for the non-NMDA GluR5 and KA2 was highly expressed in the rat SCN, and finally, the metabotropic glutamate receptor mGlu1 and mGlu5 mRNA was identified in the SCN. SP-receptor-immunoreactivity was detected exclusively in an area covering the dorsal cap of the SCN and in the adjacent

subparaventricular area, and double - staining revealed that this area overlapped with the zone occupied with c-*fos* immunoreactive cells after light. Because the NMDA receptor blocker MK-801 injected into the SCN area can prevent the morning decline of pineal NAT activity, the NMDA receptor expressing neurons may be important in transmission of the photic input to the pineal gland. We found, however, expression of the heterooligomeric NMDA-R1/2C exclusively in the dorsal part of the SCN whereas the entire SCN contained the homooligomeric NMDA-R1 mRNA, indicating that the SCN can be divided into retinorecipient zones exhibiting distinct neurochemical receptor mRNAs.

We considered these two retinorecipient zones to be involved in different mechanism and the second aim was to determine the efferent outputs of the two zones with special reference to the innervation of the PVN. Restricted injections of the anterograde neuronal tracer Phaseolus vulgaris leucoagglutinin (PHA-L) was placed in the two SCN subregions. As opposed to injections involving the entire SCN, injections of PHA-L centered in the dorsomedial subdivision of the SCN resulted in a relatively larger number of PHA-L immunoreactive fibres in the parvocellular subdivision of the PVN whereas the terminal field in the subparaventricular area was less substantial. In agreement, injections of the tracer Cholera toxin *subunit B* in the medial part of the PVN, known to be the the main source of descending axons to the brain stem and with a direct central innervation to the deep pineal tissue, resulted in retrogradely labeled neurones mostly in the dorsal and medial portions of the SCN. These data imply a multi-synaptic route linking the retina and the pineal gland that involves a substance P and/or glutamatergic retinal input to the SCN, and which is transmitted out of the SCN *via* neurones in the dorsal SCN to the PVN. More studies are now underway to define the neurochemical messengers in these neurones.

CIRCADIAN ORGANIZATION IN JAPANESE QUAIL

H. Underwood

Department of Zoology, North Carolina State University, Raleigh, 27695 NC, USA

Both the pineal organ and the eyes of quail synthesize melatonin rhythmically and both organs secrete melatonin into the blood. The eyes contribute about one-third of the melatonin found in the blood and the pineal contributes the remaining two-thirds. It is possible to entrain the melatonin rhythm in one eye 180° (12 h) out of phase with the melatonin rhythm expressed by the other eye by exposing each eye to a LD cycle which is 12 h out of phase. The clock driving the ocular melatonin rhythm (OMR) is located within the eye itself because cutting the optic nerves does not prevent the entraining effect of light on the OMR. The eyes do not appear to be coupled one to another because (1) patching one eye and illuminating the bird causes an acute suppression of melatonin levels only in the illuminated eye and (2) patching one eye and exposing the bird to a shift in the phase of the LD cycle causes the OMR to shift in the illuminated eye, but not in the patched eye. Light can also entrain the pineal melatonin rhythm *in vivo* either directly or *via* the retina.

The eyes play a major role within the quail's circadian system because removal of the eyes causes the activity and body temperature rhythms to decay into arrhythmicity in continuous darkness (DD). The eyes appear to have both a neural and a hormonal input to the central circadian system (suprachiasmatic nuclei?) because severing the optic nerves, but leaving the eyes *in situ*, does not abolish rhythmicity in most birds, but complete eye removal abolishes rhythmicity in all birds. The hormonal output from the eye may be melatonin because exogenous administration of melatonin to sham-operated or pinealectomized quail causes a lengthening in the period of the free running body temperature rhythm as well as a decrease in scotophase body temperature levels. Because a biological clock exists in the quail's eye it is proposed that the (multiple) oscillators located in the suprachiasmatic nuclei require a periodic input from this ocular clock to retain rhythmicity and/or coupling. The circadian system driving the activity and body tempera-

ture rhythms is functionally organized as a 2-oscillator system. In some cases the body temperature rhythm, or the activity rhythm, can dissociate into two different circadian components. Dissociation of the body temperature rhythm into two circadian components has been observed in normal birds, pinealectomized birds, and birds subjected to optic nerve section. In contrast to the major effects of blinding, removal of the pineal has little effect on the activity or body temperature rhythms in DD. The loss of two-thirds of the circulating melatonin levels due to pinealectomy cannot disrupt the circadian system if the "eye-SCN" axis is intact.

CIRCADIAN MODULATION OF RETINAL FUNCTION IN THE JAPANESE QUAIL

Robert B. Barlow
Institute for Sensory Research, Syracuse University, Syracuse, NY 13244, USA

Retinal sensitivity exhibits a circadian rhythm in the Japanese quail. When the animal is maintained under conditions of constant darkness (DD), retinal sensitivity as measured by the ERG b-wave is high and rod dominated at night and low and cone dominated during the day.

We hypothesize that dopamine and melatonin mediate the circadian rod-cone shifts. During the day, dopamine antagonists (haloperidol, sulpiride) injected into the vitreous shift the retina from cone to rod dominance, and at night a dopamine agonist (quinpirole) has the opposite effect. Thus dopamine appears to mediate cone dominance during the day when retinal dopamine synthesis is high as indicated in DD by DOPA levels following inhibition of DOPA decarboxylase activity *in situ*. We suggest that melatonin mediates rod dominance at night when retinal melatonin synthesis is high as indicated by tryptophan hydroxylase activity and melatonin levels measured in DD. Light adaptation can disrupt normal rod dominance at night suggesting that light and dopamine can independently modulate the circadian rhythms in retinal sensitivity.

Supported by NFS-BNS 9309539 and NIH EY 00667.

CIRCADIAN CLOCKS IN VISION: EYE AND BRAIN

Maureen K. Powers
Department of Psychology and Vision Research Center, Vanderbilt University, Nashville, 37240 TN, USA

Visual sensitivity changes with time of day. Humans are more sensitive to photons during the night than during the day, and the rhythm in fluctuation of sensitivity matches that of body temperature in humans (Bassi and Powers 1984). In poikilotherms (fish, for example) these same fluctuations also occur: that is, animals are more sensitive at night than during the day, even though their daily body temperature does not fluctuate. We have investigated the parameters of visual rhythms in fish, in an effort to determine, first, what their characteristics are, and, second, where the clock(s) that time the rhythms might be located. We find that rod disc shedding is diurnal but not circadian; visual sensitivity is circadian and can be shifted according to the light cycle; electroretinographic (ERG) sensitivity is circadian; retinomotor movements are diurnal but not circadian. Our analysis shows that the circadian change in ERG is due to some factors within the eye itself, for when we cut the optic nerve (the only connection between the eye and the brain), the circadian rhythm in sensitivity remains intact. We suggest that visual sensitivity in vertebrates is regulated by circadian clocks located in the eye(s); however, it is also likely that clocks located in the brain play a role as well.

Supported by NIH grants EY 08256 and EY 08126.

ROLE OF EXTRARETINAL PHOTORECEPTION IN INSECT CIRCADIAN RHYTHMS

Bronislaw Cymborowski
Department of Invertebrate Physiology, Warsaw University, 93 Żwirki and Wigury St., 02-089 Warsaw, Poland

In some dipteran insects (e.g., *Musca domestica, Calliphora vicina*) complete bilateral lobectomy failed to interrupt the circadian rhythm of locomotor activity or its entrainment to a light-dark cycle. Therefore, it was postulated that the relevant pacemakers are not located within the optic lobes and that the photoreceptors for entrainment may lie in the brain. An immunocytochemical study using S-antigen (arrestin) antibody, which can be considered a marker for retinal and extraretinal photoreceptors in both vertebrate and invertebrate species, revealed many groups of neurones bilaterally distributed in various parts of the blow fly (*Calliphora vicina*) brain. Furthermore, the injection of S-antigen antibody into the brain of this insect interferes with the phototransduction cascade and partially blocks both entrainment and the effects of constant light on the circadian rhythm of locomotor activity in the adult blow fly. Presented data strongly suggest that the brain of some dipteran insects might be a site for both the clock and the extraocular photoreceptors.

POSTERS

EYE-PINEAL RELATIONSHIPS AND ANTIDEPRESSANT EFFECT OF IMIPRAMINE

E.B. Arushanian and **K.B. Ovanesov**
Department of Pharmacology, Medical Insitute, Stavropol, Russia

Experiments were performed on blinded (B), pinealectomized (Px), sham-pinealectomized (SPx) and B-Px rats in order to determine the effects of these procedures on forced swimming. We have previously observed that forced swimming has a rhythmic structure characterized by periodic alterations of active swimming and immobility cycles lasting several seconds. After bilateral ocular enucleation more periods of immobility without shifts in the time-course of swimming were observed. Interestingly, under these conditions an attenuated antidepressant effect of imipramine with a concomitant increase in a rhythmological index of depression occured. The antidepressant action of imipramine found in Px animals was significantly weaker than that measured in the SPx group. Paradoxically, the antidepressant effect of imipramine in the B-Px rats was not altered. It is suggested that a disorganization of relationships between the eyes, pineal gland and suprachiasmatic nuclei could account for the observed changes in the antidepressant action of imipramine.

NOT ZODIAC SIGN, BUT SEASONAL TIME-DEPENDENT LIGHT EXPOSURE INFLUENCES THE PSYCHOSOMATIC FEATURES OF A NEW BORN CHILD

A. Brodziak, W. Romanowski, D. Kaszuba, R. Braczkowski and **B. Zubelewicz**
5th Department of Internal Medicine, Silesian University School of Medicine, Bytom, Poland

The are numerous reports showing that the pineal gland and its main hormone, melatonin, may influence the secretion of other hormones. It is suggested that the pineal gland of a pregnant woman may effect the function of the endocrine system of the fetus and, additionally, it may have an essential influence on psychosomatic features of a new born child. Our observations indicate that: 1. mortality from cancer and cardiac disorders is significantly greater in patients born under some specified "Zodiac Signs" than under other signs. 2. patients who were born during a specific season of the year are predisposed to cancer or cardiovascular disorders. We would like to hypothesize that a seasonal time of light exposure has a important influence on incidence of cancer and cardiovascular diseases.

EFFECTS OF THE PINEAL GLAND AND DIFFERENT LIGHT CONDITIONS ON ^{125}I PRL DISTRIBUTION IN SELECTED ORGANS, TISSUES AND BLOOD IN FEMALE RATS

Barbara Buntner and **Irena Caus**
I Department of Pathophysiology, Silesian Academy of Medicine, 2 Traugutt Pl., 41-800 Zabrze, Poland

It is widely recognized that the activity of living organisms is adapted to well defined light rhythms. Any change in the rhythm caused by prolonged exposure to darkness or light significantly effects neurohormonal function, especially the function of the pineal gland. However, there remains to be elucidated the broad spectrum of prolactin activity, and particularly the effect of light cycles on prolactin (PLR) distribution and the role of the pineal gland. Our study examined the effects of altered light cycle on ^{125}I PRL distribution in selected organs, tissues and blood in female rats after sham pinealectomy (SPx) and pinealectomy (Px). Experiments were performed on 240 female Wistar rats, mean body weight 150±10 g. The animals underwent a 2-week adaptation at 21-22°C, 80-85% humidity and artificial lighting (glow - lamps 360 Lx). Both the experimental and control groups (120 animals each) were divided into three subgroups of 40 rats and kept under different light cycles. After two weeks the rats were administered 10 ng of ^{125}I PRL intraperitoneally, and decapitated at 15, 60, 120 and 240 minutes. The liver, kidneys, adrenals, mammary glands, ovaries, heart, lungs, brain, muscular and fatty tissues, bones and the blood were weighted and placed in scintillation vials. The results were measured in impulses per minute and then converted into number of pg/g tissue. Two-week exposure of mature female Wistar rats to altered light cycles (long and short days) reduced ^{125}I PRL uptake by the examined organs, tissues and blood. Irrespective of the light condition, pinealectomy reduced bioavailability of PRL for organs, tissues and blood examined.

DOPAMINE-DEPENDENT CYCLIC AMP-GENERATING SYSTEM IN CHICK RETINA AND ITS RELATION TO MELATONIN BIOSYNTHESIS

Teresa Derbiszewska[1], Jolanta B. Zawilska[1,2] and **Jerzy Z. Nowak**
[1]Department of Biogenic Amines, Polish Academy of Sciences, 3 Tylna St., 90-950 Łódź;
[2]Department of Pharmacodynamics, Medical University of Łódź, Łódź, Poland

Dopamine (DA) is an established retinal neuromodulator and/or neurotransmitter. It is synthesized and released by either a subclass of amacrine cells or interplexiform cells, depending on the species (1). Biological actions of DA in the CNS are mediated *via* several dopamine receptors, named D_1, D_2, D_3, D_4 and D_5. However, it is not certain at the moment whether all these receptor types exist in the retina (2). D_1 and D_5 receptor types belong to the D1 family of DA receptors (they are usually positively coupled with adenylyl cyclase), while D_2, D_3 and D_4 receptor types represent the D2 family of DA receptors (they are unlinked or negatively linked to adenylyl cyclase).

One of the effects of DA in the vertebrate retina is to modulate retinal melatonin biosynthesis, which takes place in photoreceptors. In this respect, DA mimicks the effect of light and it suppresses the nocturnal activity of a crucial regulatory enzyme in melatonin biosynthesis, i.e. serotonin N-acetyltransferase (NAT), thus leading to decreases in melatonin content. It was suggested that the receptor mediating this DA action represents a D_2-type receptor that is negatively coupled with adenylyl cyclase (3,4). However, our recent findings suggest that the receptor under consideration may be a D_4-type receptor (5,6). Since the induction of NAT activity is a cAMP-dependent process (requiring protein synthesis) we asked whether the suppressive effect of DA and quinpirole (a predominant D_4 receptor agonist) on stimulated NAT activity is related to these drugs' inhibitory action on cAMP formation in the chick retina.

Firstly, we studied the response of adenylyl cyclase (AC) to 100 μM DA, 1 μM forskolin, and combination of both compounds in homogenates of retinas taken from 1-, 7-day and 1- and 3-month old chicks. We found that the basal activity of AC was highest in retinas of posthatched birds and then progressively declined. Forskolin was always more potent than DA; however, the effect of DA was potentiated by forskolin. The DA effect (alone or in the presence of forskolin) was antagonized by 3 μM SCH 23390 (a blocker of D_1 and D_5 receptors) and unaffected by 50 μM sulpiride (an unselective blocker of D2 family of DA receptors). Quinpirole (0.001-10 μM) had no effect on either basal or forskolin-stimulated AC activity of the chick retina.

Further, we investigated cAMP formation in intact retinas (eye-cup preparations; prelabelled with ^{3}H-adenine) that, prior to experimentation, were kept in darkness for 1 h. In such preparations, DA (300 μM) and 7-OH-DPAT (10 μM; a selective D_3-DA receptor agonist) did not modify cAMP accumulation in the presence of forskolin and 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor). However, quinpirole (0.01-10 μM) significantly suppressed (in a dose-dependent manner) the enhancement of cAMP accumulation evoked by forskolin and IBMX. This effect of quinpirole was reversed by clozapine (0.1-1 μM; an atypical neuroleptic with high affinity for the D_4-type receptor), sulpiride and spiroperidol (blockers of the D2 family of DA receptors).

In conclusion, our data show the presence of a DA-sensitive cAMP generating system in the chick retina. However, the responsiveness of this system (in *in vitro* assays) seems to be dependent on experimental conditions. Although quinpirole was highly potent in suppressing stimulated (nocturnal or post-forskolin) NAT activity in living chicks, it showed no activity on basal or forskolin-stimulated AC. Yet, it decreased forskolin-stimulated cAMP production in intact retinas, suggesting that the cAMP response may be a secondary event, not directly related to quinpirole-D_4-type receptor interaction. Thus, the precise mechanisms underlying the modulatory action of DA on cAMP- and melatonin-generating systems require further study to be clarified.

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Supported by the KBN grant 0540/P2/93/04 to J.B.Z.

IMMUNOHISTOCHEMICAL LOCALIZATION OF VASOPRESSIN IN THE RAT RETINA AND HARDERIAN GLAND

Yasmina Djeridane

Institute of Biology of Sciences and Technology, U.S.T.H.B., Algiers, Algeria

The presence of vasopressin in the retina and Harderian gland of the Wistar rat was examined immunohistochemically. Immunostaining was accomplished with the peroxidase-antiperoxidase method. In the retina, vasopressin is restricted to the ganglion cell layer, whereas in the Harderian gland, the excretory duct cells are the main source of vasopressin immunoreactivity. The finding of immunostaining for vasopressin in the retina and Harderian gland of rat is in agreement with radioimmunoassay data (1), which demonstrated that the transsection of the optic nerve decreases retinal vasopressin content. These data suggest that vasopressin is not synthesized in retina, but, instead, it is transported to retina by neuronal fibres and subsequently accumulated within ganglion cells. Indeed, the centrifugal innervation of the rat retina has previously been suggested (2). The physiological role of vasopressin in retina is at present unknown. It is suggested that this peptide may be involved in the regulation of intraocular pressure and/or transmission of photic information.

In the Harderian gland, 45% of the total cell population displayed vasopressin immunoreactivity. The histological and ultrastructural examination of the rat excretory duct revealed the presence of two categories of serous cells in this tissue (3). However, with the aid of light microscopy, it was impossible to ascertain which type of these cells exhibits vasopressin immunoreactivity. As the Harderian gland contains a photosensitive pigment (porphyrins), it has been proposed to play a role of an extraretinal photoreceptor organ that receives photic information from the environment and transmits it to pineal gland (4). Interestingly, vasopressin has also been detected in the rat pineal gland by radioimmunoassay (1) and immunohistochemical techniques (5). Thus the presence of vasopressin in the Harderian gland is not surprising, because of the functional similarities between these two tissues. Interestingly, the Harderian gland has been proposed as a link in the retinal-pineal axis. Such a link could use a humoral agent, possibly vasopressin.

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GONADOTROPIN RELEASING HORMONE (GnRH) CONTENT IN MEDIAN EMINENCE AFTER SUPERIOR CERVICAL GANGLIONECTOMY IN OVARIECTOMIZED AND ESTROGEN TREATED FEMALE RATS

B. Dziedzic, A. Walczewska and **W.Z. Traczyk**
Department of Physiology, Insitute of Physiology and Biochemistry, Medical University of Łódź, 90-131 Łódź, Poland

The aim of the present study was to examine if superior cervical ganglionectomy (SCGx) modified gonadotropin releasing hormone (GnRH) content in median eminence (ME) of female rats in different hormonal states. Experiments were carried out 12 h before sacrifice on female rats subjected to SCGx or sham operated. The animals were decapitated and their MEs were removed. GnRH content in ME was measured by RIA in the following groups: (1) cyclic female rats in diestrous state, (2) rats that were ovariectomized (OVX) 2 weeks earlier, and (3) OVX female rats implanted subcutaneously with pellets filled with 17β-estradiol (E_2) which was absorbed within 2 weeks.

SCGx performed 12 h earlier resulted in a decrease of GnRH content in ME of cyclic female rats (335.9±77.4 pg GnRH/mg tissue*, $P<0.05$) as compared with sham operated control (696.9±145.6 pg GnRH/mg tissue). OVX caused a decrease of GnRH content in ME (259.4±33.7 pg GnRH/mg tissue, $P<0.05$). A greater decrease of GnRH content was found in OVX rats after SCGx (84.4±23.5 pg GnRH/mg tissue, $P<0.05$). The difference in GnRH content in ME between E_2 treated OVX rats subjected to SCGx (289.1±71.2 pg GnRH/mg tissue) and E_2 treated OVX rats subjected to sham operation (396.4±57.1 pg GnRH/mg tissue) was not statistically significant.

It is concluded that SCGx has some transient influence on the GnRH storage and release from the median eminence of rats in different hormonal states. (*mean ±SE)

The study was supported by a grant from the Medical University of Łódź.

CHOLINERGIC MODULATION OF NEURONAL SIGNALS IN THE PHOTOSENSITIVE PINEAL ORGAN OF THE RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

E. Fait, R. Brandstätter and **A. Hermann**
Department of Animal Physiology, Institute of Zoology, University of Salzburg, 34 Hellbrunnestrasse, A-5020, Salzburg, Austria

The functional role of putative intrapineal neurotransmitters in light- and dark-adaptive processes, which are the basis for nycthemeral rhythms, is still elusive. Among others, acetylcholine (ACh) is a can-

didate as an intrapineal neurotransmitter. Acetylcholinesterase (1) and choline-acetyltransferase (2) were found to be present in the pineal organ of teleosts. Furthermore, both nicotinic and muscarinic receptors were found to be present in the trout pineal organ (3).

To further elucidate the functional significance of acetylcholine (ACh) in the pineal organ of rainbow trout, we performed extracellular recordings from neurones in isolated superfused pineal organs. Application of exogenous ACh (n=54) resulted in a dose-dependent reversible excitation of 95% of the neurons tested. The relative sensitivity of pineal neurones to ACh increased during stepwise light adaptation, resulting in a significant reduction of light sensitivity. Effects of exogenous ACh could be blocked by the specific muscarinic antagonist atropine sulphate but were not affected by the nicotinic antagonist hexamethonium chloride.

Application of specific nicotinic or muscarinic receptor agonists (n=13) revealed that both nicotine tartate (specific nicotinic agonist) and DL-muscarine chloride (specific muscarinic agonist) mimicked the effects of exogenous ACh, which is in accordance with an autoradiographic demonstration of both receptor types in the trout pineal (3). We used specific nicotinic (hexamethonium chloride) and muscarinic (atropine sulphate) ACh-receptor antagonists to reveal the functional significance of endogenous ACh. Whereas hexamethonium chloride showed only slight or no effects, atropine significantly reduced the discharge rate of all neurones during light- and dark-adaptation, which resulted in an increase of relative light sensitivity.

These data indicate that the excitatory effect of exogenous ACh is mediated *via* both nicotinic and muscarinic receptors, whereas endogenous ACh seems to act *via* muscarinic receptors. To our knowledge, this is the first demonstration of a cholinergic modulation of pineal neuronal signals in a teleost. Our data further suggest that intrinsic neurotransmitters may be involved in the regulation of rhythmic pineal signals by modulating light- or dark-adaptive mechanisms. ACh, which may be released from intrapineal neurones, influences the activity of pineal projection neurones, which transmit photoperiodically induced signals to integrative diencephalic and mesencephalic brain regions (4).

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Supported by the Austrian "Fonds zur Förderung der wissenschaftlichen Forschung (P9343BIO)".

CIRCADIAN RHYTHM OF TSH SECRETION AFTER MELATONIN ADMINISTRATION IN MORPHINE DEPENDENCE AND WITHDRAWAL SYNDROME CAUSED BY NALOXONE IN RATS

J. Górski, D. Kajdaniuk, J. Głogowska-Szeląg, M. Nowak, B. Buntner and **B. Jarząb**
I Department of Pathophysiology and Medical Analytics, Silesian University School of Medicine, 2 Traugutt Pl., 41-800 Zabrze, Poland

It is generally known that there are changes in hormone secretion during administration of morphine or other opioids. We examined the level of TSH and its changes in the circadian rhythm after melatonin administration in long lasting morphine dependence and withdrawal syndrome. The study was done using 3-weeks old male Wistar rats. Blood was taken every 4 h. The TSH concentration in blood was examined with RIA method and the circadian rhythm of its secretion was presented with cosinor method. The results were analyzed using t-tests. Results: (1) Melatonin administration has no influence on TSH secretion in morphine dependence. (2) The level of TSH secretion in the afternoon and at darkness in the withdrawal syndrome is decreased in comparison to the control group. There was no such influence in the morning. This may be connected with circadian changes in the sensitivity of the melatonin receptors.

MELATONIN INFLUENCE ON CORTICOSTERONE SECRETION IN MORPHINE DEPENDENCE AND IN WITHDRAWAL SYNDROME CAUSED BY NALOXONE IN RATS

J. Górski, D. Kajdaniuk, Z. Ostrowska, M. Nowak, J. Głogowska-Szeląg and **B. Buntner**
Department of Pathophysiology and Medical Analytics, Silesian Academy of Medicine, 2 Traugutt Pl., 41-800 Zabrze, Poland

It is generally known that there are changes in hormone secretion during administration of morphine. We examined the level of corticosterone and its changes after melatonin administration in long lasting morphine dependence and withdrawal syndrome caused by naloxone. Blood was taken three times a day. Corticosterone levels were examined using RIA method. The results were analyzed statistically using t-tests. Results: 1. Corticosterone secretion in morphine dependence was increased after melatonin administration. 2. Melatonin administration had no influence on corticosterone secretion in the withdrawal syndrome caused by naloxone.

EFFECTS OF MELATONIN AND DIAZEPAM ON NEURONES OF THE SUPRACHIASMATIC NUCLEI (SCN) IN THE RAT IN RELATION TO CIRCADIAN PHASE

E. Grossmann and **H. Meissl**
Max-Planck-Institut für Physiologische und Klinische Forschung, W.G. Kerckhoff-Institut, 1 Parkstrasse, D-61231 Bad Nauheim, Germany

The mammalian circadian system is dominated by a neuronal pacemaker, the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. The SCN is capable of endogenous rhythm generation with a periodicity of around 24 h. The clock mechanism is reset by different external (e.g., light environment) and internal (e.g. neurotransmitters) stimuli and seems to undergo circadian changes in its sensitivity to resetting stimuli (1).

Melatonin as well as diazepam seem to induce phase shifts of the SCN rhythm and it was suspected that they exert their effects through a combined mechanism (2) or an interaction of two different mechanisms (3). In the present study we have investigated the role of GABA, diazepam, a full agonist of the $GABA_A$/benzodiazepine receptor, and of the pineal hormone melatonin on neuronal activity of the rat SCN. The psychopharmacological effects of melatonin are similar to transquilizing drugs such as the benzodiazepines and, therefore, we studied the influence of brief applications of both drugs on neuronal excitability of the SCN. The electrical activity of SCN neurones was recorded extracellularly from rat brain slices of 500 µm. The slices were perfused at 37°C with glucose/bicarbonate-supplemented salt solution (NaCl, 124mM; KCl, 5mM; KH_2PO_4, 1.25mM; $MgSO_4$, 1.3mM; $CaCl_2$, 1.1mM) saturated with 95% O_2, 5% CO_2. Drugs were directly added to the perfusion medium for 5 min at different times of the day and night. The effects of the drugs were assessed by recording the change of the spontaneous discharge rate of the neurones. The electrical activity of individual neurones was monitored during periods of 60 min up to 300 min. Drug-induced phase-shifts in the rhythm of neuronal electrical activity were observed in these short recording periods. Application of GABA ($5x10^{-5}$ M), diazepam (10^{-4} M) and melatonin (10^{-6} M) for 5 min resulted in a complex response pattern consisting of a short initial excitation followed by a long lasting inhibition. Sometimes we observed a biphasic response with an initial inhibition followed by an excitation. Using the duration and the strength of the inhibitory response as criterion, the neurones seem to be more sensitive to the drugs during subjective day. The action of all drugs was longer at night and the strength of the inhibitory response was enlarged after melatonin and diazepam application. These data indicate that GABA, diazepam and melatonin can directly affect SCN neurones with a similar response profile.

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CALCIUM DECREASES NITRIC OXIDE-ACTIVATED cGMP ACCUMULATION IN RAT PINEALOCYTES

Gabriele Hill, Rainer Spessert and **Lutz Vollrath**
Department of Anatomy, Johanness Gutenberg-University, 55099 Mainz, Germany

Recent studies have indicated that in rat pinealocytes β-adrenergic stimulation of cyclic nucleotide formation requires activation of neuronal nitric oxide synthase (nNOS) and NO-dependent activation of cytosolic guanylyl cyclase (cGC). β-Adrenergic stimulation of cyclic guanosine monophosphate (cGMP) formation is potentiated by α_1-adrenergic-induced influx of Ca^{2+}. In this study we focussed on the regulatory role of α_1-adrenoceptor stimulation and Ca^{2+} on NO-stimulated cGC activity. It was found that α_1-adrenergic stimulation did not affect NO-activated cGC. Therefore, α_1-mechanisms act prior to cGC. In view of the ineffectiveness of α_1-mechanisms on NO-stimulated cGC and considering that α_1-mechanisms require Ca^{2+} influx, we were surprised to find that Ca^{2+} strongly depresses NO-stimulated cGC activity. Therefore, it appears that NO-dependent stimulation of cGC occurs in a cell/compartment not under adrenergic regulation.

LIGHT-INDUCED SUPPRESSION OF NOCTURNAL SEROTONIN N-ACETYLTRANSFERASE ACTIVITY IN CHICK PINEAL AND RETINA: A WAVELENGTH COMPARISON

Adam Jarmak[2], Jolanta B. Zawilska[1], Grzegorz Owczarek[3] and **Jerzy Z. Nowak[1]**
[1]Department of Biogenic Amines, Polish Academy of Sciences; [2]Department of Ophthalmology, Military Medical University of Łódź and [3]Institute of Physics, Technical University of Łódź , Łódź, Poland

Chick retina and pineal gland synthesize melatonin in a light-dependent circadian rhythm, with peak values in the dark phase (1). The nocturnal increase in melatonin level and activity of serotonin N-acetyltransferase (NAT, a penultimate and key regulatory enzyme in melatonin biosynthetic pathway) is suppressed by light. In experiments performed on rats and hamsters it has been demonstrated that this inhibitory effect of light depends on the wavelength of light, with green light having the strongest effect (2,3). As the sensitivity of the melatonin-generating system to white light exhibits pronounced variations among species, some species-dependent differences in response of melatonin production to monochromatic light seem likely to occur. The aim of our study was to examine effects of monochromatic light (blue, green and red) on nighttime NAT activity of chick pineal gland and retina.

Experiments were performed on male chicks (3-4 weeks old) raised under a 12 h light: 12 h dark illumination schedule. At the beginning of the third h of the dark phase the animals were exposed to monochromatic light (with wavelengths: blue - 440 nm, green - 550, and red - 600 nm; the light intensity was 17-22 lux) for 5, 10, 30 or 60 min, and then sacrificed. In another set of experiments birds were exposed to a 5-min pulse of light, and sacrificed 5, 15, 30, 60 or 120 min later. Pineal glands and retinas were isolated and used for determination of NAT activity. NAT of the pineal gland was more sensitive to the inhibitory effect of light than the enzyme of retinal origin. Although in both tissues the light-induced suppression of the nocturnal NAT activity was dependent on the wavelength of light (blue ≥ green > red), the observed differencies were evidently less pronounced than those reported for rodents (2,3). Furthermore, in contrast

to rat (3), following the 5-min light pulse NAT activity of chick pineal and retina declined maximally by 30-55% (15 min) and returned to control values 1 hr after the end of the pulse. It is suggested that the sensitivity of an individual animal species to the suppressive effect of monochromatic light on melatonin biosynthesis may be dependent on types of photoreceptor cells involved and a complex interplay among them.

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Supported by the KBN grant 0540/P2/93/04 to J.B.Z.

THE EFFECT OF SHORT PHOTOPERIOD ON OXYTOCIN RELEASE IN MALE SYRIAN HAMSTER

M. Juszczak, R. Steger, L. Debeljuk, C. Fadden and **A. Bartke**
Department of Physiology, Southern Illinois University School of Medicine, Carbondale, 62901-6512 Illinois, USA

Oxytocin release in the rat can be altered by pinealectomy or melatonin treatment (1). It is also controlled by noradrenergic and dopaminergic mechanisms (2). Pineal function and melatonin synthesis depends on light:dark cycle being increased during dark and decreased during light phase of the light:dark cycle. Experimental manipulation of photoperiod may produce long-term shifts in pineal activity as well as pineal-mediated changes in neuroendocrine function and reproductive competence in Syrian hamsters (3). Short photoperiod-induced suppression of gonadal activity in the hamster is accompanied by a reduction in noradrenaline (NE) and dopamine (DA) turnover in the median eminence (4). The purpose of this study was therefore to investigate the effects of various light conditions on plasma and neurohypophyseal (NH) oxytocin content as well as hypothalamic (Hth) and NH neurotransmitter turnover in the male Syrian hamster.

Adult male Syrian hamsters were kept under long photoperiod (16 h light and 8 h dark) or transferred to short photoperiod (6 h light and 18 h dark) for 1, 4 or 10 weeks. Oxytocin content in plasma and NH was measured by RIA and neurotransmitter turnover was calculated from HPLC measurements of the depletion of NE and DA in the Hth and NH 60 minutes after α-MPT administration (α-MPT, ip 250 mg/kg).

Neurohypophyseal oxytocin content was significantly elevated both in saline- and α-MPT-treated animals after 10 weeks of exposure to the short photoperiod. In saline-treated animals kept under the short photoperiod for 1 and 4 weeks, oxytocin content in the NH was not changed, when compared to animals kept under the long photoperiod. Injection of α-MPT did not modify the NH oxytocin storage but a significant increase of oxytocin in the blood was observed in all α-MPT-injected animals, when compared to animals injected with saline. After one week of exposure to the short photoperiod, DA turnover in the NH was sharply elevated whereas NE and DA turnover in the Hth was significantly diminished. Hypothalamic NE and DA turnover remained depressed in all short photoperiod-treated animals.

The present results suggest that in the Syrian hamster the response of oxytocinergic neurones to a short photoperiod may be mediated at the hypothalamic level, at least in part, by dopaminergic and noradrenergic mechanisms. At the level of the neural lobe, some other mechanisms are involved in the pineal-neurohypophyseal interrelationship.

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RELATIONSHIP BETWEEN CORTICOTROPIN-CORTISOL AXIS FUNCTION AND MELATONIN LEVEL DURING 24-H PERIOD. LONG-TERM EFFECT OF PREDNISONE TREATMENT

Beata Kos-Kudła, Zofia Ostrowska, Bogdan Marek, Krystyna Żwirska-Korczała, Marek Kudła and **Barbara Buntner**

I Department of Pathophysiology and Clinical Biochemistry, Silesian Academy of Medicine, 2 Traugutt Pl., 41-800 Zabrze, Poland

There is no conclusive evidence of an interaction between the pineal and adrenal glands but administration of dexamethasone or hydrocortisone is known to decrease serum melatonin level in humans and animals. In the present study a relation between 24-h levels of melatonin, ACTH and cortisol was determined in 37 asthmatic patients (19 women and 18 men) aged 35 to 50 years (mean 39.0), who had been treated with prednisone in daily doses of 8 mg for seven days. The control group consisted of 21 asthmatic patients not treated with prednisone (10 women and 11 men) and 40 healthy volunteers (20 women and 20 men) aged 29 to 50 years (mean 35.6). All subjects were hospitalized during the study period and their daily activities, times of meals and nocturnal rest were synchronized. Blood samples were collected at 3-h intervals over a 24-h period, starting at 08.00 h. Melatonin, ACTH and cortisol were measured using RIA methods and the urinary 17 OHCS excretion using Porter-Silber method. Student's t-test and the cosinor method were used for statistical analysis of unpaired data and circadian rhythms, respectively. All subjects showed circadian rhythms of melatonin, ACTH and cortisol. Prednisone therapy did not change the characteristic pattern of melatonin, ACTH and cortisol rhythms but it reduced significantly their mean 24-h serum levels when compared to both control group values. In healthy subjects, the correlation between melatonin and ACTH and cortisol was found to be negative, while after prednisone therapy the correlation was positive. In this group urinary 17 OHCS excretion was significantly lower in comparison with control values. Changes in melatonin diurnal fluctuations can be connected with alterations in the activity of hypophyseal-adrenal axis.

MELATONIN - ARGININE VASOTOCIN - RELATIONSHIPS - A MODEL

Ewa Kulczykowska

Marine Biology Center, Polish Academy of Sciences, 5 Św. Wojciech St., 81-347 Gdynia, Poland

In teleosts, the pineal photoreceptor cells are the major place of photoperiod-dependent melatonin synthesis and are involved in the control of rhythmic adaptations to daily and seasonal cycles. Arginine vasotocin (AVT) is produced according to osmotic stimuli in the hypothalamic neurosecretory neurones concentrated in the preoptic nucleus in teleost fish and is released in the neurohypophysis. AVT plays a role in osmoregulation, cardiovascular activity, endocrine secretion and reproductive processes. AVT is thought to be a neurotransmitter and/or neuromodulator in the central nervous system in fish. Since both AVT and melatonin participate in systems controlling the physiological adaptation of fish to daily and seasonal environmental changes (e.g., light, temperature, salinity), the interactions between these hormones are considered. In this study a model of melatonin-AVT relationships in fish is proposed. Melatonin synthetized in pineal during darkness may inhibit the vasotocin synthesis in hypothalamic neurones. Low levels of melatonin during the light is insufficient to inhibit the vasotocin synthesis in hypothalamic neurones and therefore the AVT- neuron's activity may be high. On the other hand, the AVT synthetized in hypothalamus may inhibit the synthesis of melatonin in the pineal. Since melatonin synthesis and secretion is directly controlled by light, melatonin may regulate AVT levels, with negative feedback from AVT. The rhythmic secretion of melatonin may be crucial to keep up a rhythmic activity in the "SCN" region in fish in the absence of a discrete circadian oscillator. The melatonin-AVT interaction may be of a great

importance especially for migrating fish, in which the adaptation mechanism to both light and salinity play a key role. It is interesting that the migratory form of the salmonid fish (smolt) still living in fresh water is perfectly preadapted to sea water and is able to migrate without transitory osmotic disequilibruim. It is possible that the smolt pineal converting photic information into hormonal signals, changes directly or indirectly the synthetic activity of AVT neurones in the hypothalamus and thus adjusts the osmoregulatory mechanisms in fish to salinity changes.

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DEMONSTRATION OF NITRIC OXIDE SYNTHASE IN THE RAT PINEAL GLAND

Elisabeth Layers, Rainer Spessert and **Lutz Vollrath**
Department of Anatomy, Johannes Gutenberg-University, 55099 Mainz, Germany

Nitric oxide (NO) formed by the neuronal form of nitric oxide synthase (NOS) is an activator of the cGMP forming enzyme - soluble guanylyl cyclase (sGC). NOS additionally shows NADPH-diaphorase (NADPH-d) activity that can be demonstrated histochemically. The aim of the present investigation was to demonstrate the presence of NOS in the rat pineal organ. NADPH-d reaction revealed dark blue NADPH-d positive cells (NADPH-d+) in between unstained NADPH-d negative cells. The presence of NOS in rat pineal was shown by western blot immunohistochemical staining using a polyclonal antibody raised against rat cerebellar NOS. Further, NOS has been demonstrated immunohistochemically. Pinealocytes showing immunoreactivity (IR) were found lying together in small groups. Strongest IR was to be seen in pinealocyte cell bodies, whereas no stained cell processes were observed. Our findings agree with previous biochemical investigations which suggest a role for NOS in rat pineal cyclic GMP signal transduction. Further investigations are required to understand the significance of NOS localization concerning cGMP regulation.

EFFECTS OF α-METHYL-DL-p-TYROSINE AND CLORGYLINE ON PINEAL MORPHOLOGY AND FUNCTION IN PIG. I. PLASMA MELATONIN PROFILES OVER 110 HOURS IN CONTROL AND EXPERIMENTAL ANIMALS

Bogdan Lewczuk, Barbara Przybylska and **Zygmunt Wyrzykowski**
Department of Histology and Embryology, Olsztyn University of Agriculture and Technology, Olsztyn, Poland

The number of studies regarding melatonin secretion in the domestic pig is very limited. The results obtained so far show that plasma melatonin patterns in the pig present clear difference from the other examined mammalian species which secrete melatonin in a circadian fashion. Night - day differences in the concentration of melatonin in pig plasma were observed only when the lengths of the light and the dark periods were equal or when the light intensity during day was high. No nocturnal rise of plasma melatonin was observed in either long or short photoperiods. It is well known that in mammals melatonin secretion is under sympathetic control. The aim of the present study was to determine if the drugs which influence the level of norepinephrine modulate the plasma melatonin patterns in prepubertal gilts housed in photoperiod 12L:12D.

Fifteen pigs, aged 984 days at the beginning of the experiment were used in the study. The animals were housed under a 12L:12D photoperiod (250 lux of light during the day and less then 3 lux of dim red light during the night). After two weeks of the 12L:12D photoperiod, venous cannulae were inserted. Four

days after cannulation the pigs of the first group were treated with α-methyl-DL-p-tyrosine methyl ester hydrochloride (3g, twice daily per animal) - an inhibitor of tyrosine hydroxylase. The pigs of the second group were treated with clorgyline (60 mg, twice daily per animal) - an inhibitor of monoaminooxidase. Both drugs were dissolved in 20 ml saline and infused *via* cannulae for 20 min starting one hour after the beginning of both the light and dark phases. The animals of the third group functioned as the control group and were infused with saline. All animals received eight infusions over four consecutive days. Blood samples were taken every 1 or or 2 h over a period of 110 h, starting 23 h before the first infusion. Plasma concentrations of melatonin were measured by direct RIA (Fraser, 1983) using Stockgrand antiserum (G/S/704-6483) and H^3-melatonin (specific activity 86 Ci/mmol). Several procedures were performed to validate this method for the assay of pig plasma.

In control individuals considerable differences in concentration of melatonin in plasma were observed during the sampling period. The changes in the level of melatonin were usually connected with shifts of light-dark phases of the circadian rhythm, but they weren't regular or rhythmical. After the administration of α-methyl-DL-p-tyrosine a decrease in melatonin concentration was observed after the third infusion in four of five examined pigs. The level of plasma melatonin was lower during both phases of the circadian rhythm, but the nocturnal rises of melatonin were clearly visible. Clorgyline (a potent stimulator of melatonin secretion in other examined species) had no marked effect on plasma melatonin in pig.

Supported by grant KBN 5 5925 92 03.

THERAPEUTIC USE OF THE PINEAL HORMONE MELATONIN IN HUMAN NEOPLASMS: UPDATE RESULTS

P. Lissoni, S. Barni, G. Tancini, A. Ardizzoia, F. Brivio, R. Braczkowski[1], B. Zubelewicz[1] and W. Romanowski[1]

Division of Oncological Radiotherapy, S. Gerardo Hospital, Monza, Milan, Italy;
[1] V Deparment of Internal Medicine Silesian University School of Medicine, Bytom, Poland

Despite the well documented antitumor properties of the pineal gland and its most investigated hormone melatonin (MLT), the clinical use of pineal substance(s) in the therapy of human cancers is still in its early stages. Some years ago, the antitumor activity of MLT was known only from simple experimental evidence, but at present it has been demonstrated that MLT may counteract tumor growth through at least 4 distinct mechanisms, including stimulation of host anticancer immunobiological response, a possible direct cytostatic action, an inhibition of tumor growth factor production, and a differentiating action on cancer cells. On this basis, over the last 9 years we have carried out several clinical studies in oncologic patients with MLT alone or in association with antitumor cytokines. At present more than 300 patients have been treated with MLT according to different medical protocols. MLT was always given orally during the dark period of the day, because of its greater biological activity in this period. No MLT-related toxicity was found; on the contrary, it may result in some subjective benefits, including relaxation, relief of asthenia, pain and anorexia, and an increase in pleasure. MLT has been given as a palliative therapy in metastatic solid tumor patients, for whom no effective standard therapy was available, in patients treated with radical surgery as an adjuvant endocrine therapy, and in association with cytokines, mainly IL-2 and TNF, and as a neuroimmunotherapy of human cancer. The clinical results obtained up to now with MLT in cancer patients may be summarised as follows: (1) MLT alone may improve the quality of life and prolong the survival time, mainly in nonsmall cell lung cancer, and in metastatic cancer patients, who progressed in response to chemotherapy. (2) MLT may prolong the free-from progression period in cancer patients surgically treated for gastric cancer, non-small cell lung cancer, and perhaps node recurrence due to malignant melanoma. (3) MLT alone may prolong the survival time and improve the quality of live by antagonizing

steroid-induced side-effects in patients with brain metastases due to solid tumors. (4) MLT alone may increase blood cells number, prolong the survival time and counteract the evolution into nonlymphoid leukaemia in cancer patients with myelodysplastic syndrome secondary to cancer chemotherapy. (5) MLT may amplify the biological and antitumor efficacy of IL-2, with the possibility to achieve objective tumor regressions in most solid tumor histotypes, whereas it is extremely rare to obtain tumor regression with MLT alone. For some tumor histotypes, such as non-small cell lung cancer, hepatocarcinoma and pancreatic adenocarcinoma, the efficacy of neuroimmunotherapy with low dose of IL-2 plus MLT seems to be superior with respect to conventional chemotherapy. (6) MLT seems to be useful in reducing the toxicity induced by TNF immunotherapy.

In conclusion, according to our clinical experience, MLT has to be considered as an essential drug in the curative or palliative therapy of human neoplastic diseases.

RHYTHM OF MELATONIN SECRETION AND THE ENDOGENOUS OPIOID ACTIVITY DURING THE REPRODUCTIVE CYCLE IN SHEEP

T. Misztal, K. Romanowicz and **B. Barcikowski**
The Kielanowski Institute of Animal Physiology and Nutrition, 05-110 Jabłonna n/Warsaw, Poland

Sheep are considered to be short day breeders. It is believed that cyclic secretion of the reproductive hormones throughout the year is modulated by melatonin (MLT). Endogenous opioid peptides (EOP), particularly beta-endorphin (B-END), play an inhibitory role in the control of LH secretion, apparently acting within the hypothalamus, to inhibit the pulsatile release of LHRH. In 4 adult Polish Lowland ewes, the seasonal changes in rhythm of MLT secretion and EOP activity were determined. Blood was sampled hourly during a period of 24 h, 9 times during a year, at days of different daylength. Plasma concentration of MLT and B-END were measured by RIA. The concentrations of MLT from the 7-h periods, from 9.00pm to 3.00am, were used as an index of the mean nightly concentration of the hormone, to determine the seasonal rhythm of secretion.

During the period of short days, high concentrations of MLT in blood plasma were observed: Oct. 248.3+36.6(SD) pg/ml; Dec. 210.5+24.6 pg/ml; Feb. 261.0+50.1pg/ml; Mar/Apr. 246.7+48.5 pg/ml. Lower concentrations of MLT were noted during long day: May 151.3+24.0 pg/ml; June 195.9+41.1 pg/ml; the begining of July 125.4+11.6 pg/ml; the end of July 142.7+19.7 pg/ml; Sept. 180.5+27.5 pg/ml. Concentrations of beta-endorphin oscillated between levels of 13.1+6.4 - 27.5+17.9(SD) pg/ml from Feb. to Sept. A clear rise of beta-endorphin occured in Oct. (107.0+62.4 pg/ml) during the period of sexual activity of sheep.

These data indicate an evident relation between MLT and beta-endorphin secretion. Increasing concentration of MLT during the shorter days stimulates EOP activity within the hypothalamus, what may be a part of the mechanism by which MLT affects the reproductive cycle in sheep.

DAY/NIGHT MELATONIN PROFILE IN WOMEN WITH ACROMEGALY BEFORE AND AFTER TRANSSPHENOIDAL SURGERY

Zofia Ostrowska, Krystyna Żwirska-Korczała, Elżbieta Świętochowska, Beata Kos-Kudła and **Barbara Buntner**
I Department of Pathophysiology and Clinical Biochemistry, Silesian Academy of Medicine, 2 Traugutt Pl., 41-800 Zabrze, Poland

Some endocrine disorders may or may not stimulate the pineal gland to produce and secrete melatonin. Some investigators have recently found changes in melatonin blood rhythms and/or mean 24-h concen-

tration of this hormone in hypothalamo-pituitary-gonadal, or thyroid disorders. Others observed lack of melatonin rhythm in Cushing's disease or acromegaly. Piovesan et al. (1) believed the suppression of melatonin rhythm to be due to an increase in daytime levels of this hormone in untreated acromegalic men. Such an abnormality may be related to a derangement of neurotransmitter tone linked to acromegaly or a direct effect of growth hormone hypersecretion. The aim of the present study was to evaluate the circadian variations of melatonin and to find a possible relationship between melatonin (MEL) and growth hormone (GH), prolactin (PRL) and insulin-like growth factor (IGF-I) in acromegalic women before and after adenectomy. The study group consisted of 15 women aged 30 to 45 years (mean 38). The controls were 18 healthy women aged 28 to 48 years (mean 36). Both groups were studied after an overnight fast. Blood samples were taken at 3-h intervals over a 24-h span (starting at 8.00 h) for determination of GH (OPiDI, Świerk, Poland), PRL (IRMA, Finland), IGF-I (MEDGENIX, Belgium), MEL (DRG, USA). Circadian rhythms were evaluated using cosinor method. The circadian rhythm of MEL was not disturbed before surgery, but the mean 24-h MEL levels and the amplitude values were lower in subjects with higher concentrations of GH and IGF-I. After surgery, the serum MEL levels normalized parallel to decreasing concentrations of GH and IGF-I. On the other hand, there was no correlation between the nighttime serum values of the hormones studied.

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CHRONIC MODULATORY EFFECT OF MELATONIN ON RAT THYROTROPIN (TSH), TRIIODOTHYRONINE (T_3), THYROXINE (T_4), TESTOSTERONE (T) AND COTRICOSTERONE (B) DURING THE DAY/NIGHT CYCLE

Zofia Ostrowska, Krystyna Żwirska-Korczała, Bogdan Marek, Beata Kos-Kudła, Elżbieta Świętochowska and **Barbara Buntner**

I Department of Pathophysiology and Clinical Biochemistry, Silesian Academy of Medicine, 2 Traugutt Pl., 41-800 Zabrze, Poland

Melatonin, a pineal hormone, plays a neuromodulatory role in animals. An interaction between pineal gland melatonin and the pituitary-thyroid, -adrenal and -gonadal axis has been reported in some species. The effects of pinealectomy and prolonged melatonin administration (100 μg/100 g b.w., i.p. -twice daily over a one month period) on thyrotropin (TSH), thyroid hormones (T_3, T_4), testosterone (T) and corticosterone (B) secretion in sexually mature rats were examined (RIA method) at 3-h intervals under a 12:12 h light-dark cycle. The results were analysed using Student's t-test and the cosinor method. Clear circadian rhythms of TSH, T_3, T and B were detected in control rats. Pinealectomy abolished the rhythmical character of B secretion and disturbed the 24-h levels of T_3, T_4 and T, but did not change the TSH circadian rhythm. In addition, the mean 24-h levels of TSH, T_3, T_4 and T were increased, whereas B level was decreased. The circadian fluctuations of TSH, thyroid hormones and T were altered after chronic melatonin administration, with mesor and amplitude reduction. Daytime concentrations of TSH, T_3, T_4 and T showed a greater decrease in sham-operated rats. During the dark phase, the inhibitory effect of melatonin was prevalent in pinealectomized rats, and stimulation was observed in sham-operated rats. Chronic melatonin administration inhibits rhythmical T secretion and stimulates both daytime and nocturnal corticosterone secretion.

EFFECT OF DARKNESS ON CIRCADIAN RHYTHM IN THE ACTIVITY OF MIXED FUNCTION OXIDASES SYSTEM IN RATS

Andrzej Plewka and **Marcin Kamiński**

Department of Histology and Embryology, Silesian School of Medicine, Katowice-Ligota, Poland

Circadian changes in the hepatic enzymes involved in xenobiotic metabolism have important pharmacological and toxicological aspects. Understanding of these phenomena may provide data for optimaliz-

ation of drug application and dosage, especially for agents with low therapeutical index. Circadian changes in cytochrome P-450 and cytochrome b_5 content and activity of NADPH-cytochrome P-450 and NADH-cytochrome b_5 reductases have been studied in rat liver microsomes in autumn.

Male 6-month-old Wistar rats were obtained from the Central Experimental Animal House of the Silesian School of Medicine. The rats were kept in plastic cages, one rat per cage. The animals had free access to food and water. Fixed temperature (about 20° C), humidity (60%) and complete darkness were kept during the 2 months of the experiment. All animals were deprived of food for 12 h before decapitation. Liver samples were obtained during 48 h, and sampling was done every 4 h. The microsomal fraction of the liver was isolated according to Dallner. Purified microsomal fraction was suspended in Tris-HCl buffer (pH = 7.4) containing 0.25 M sucrose and 20% of glycerol. The enzymatic assays were done immediately. Cytochrome P-450 and cytochrome b_5 contents were measured according to the method of Estabrook and Werringloer. The activity of NADPH-cytochrome P-450 and NADH-cytochrome b_5 reductases were estimated by use of the spectrophotometric method of Hodges and Leonard. The protein content of the microsomal fraction was determined by the method of Lowry et al. with bovine albumin solution as a standard.

The results obtained indicate, that in the normal group of rats (light/dark changes 12:12; 6.00 - 18.00 light period) cytochrome P-450 content and NADPH-cytochrome P-450 reductase activity showed a 12 h rhythm with a maximum at 10.00 and 22.00. Cytochrome b_5 content and its reductase activity has a 24 h rhythm with a maximum at 14.00. Long lasting deficiency of light changed the circadian cytochrome P-450 rhythm to a 24 h one with a maximal value at 10.00. The mesor and the amplitude were also slightly decreased. The NADPH-cytochrome P-450 reductase showed a 24 h rhythm in the experimental conditions, with a maximal value at 10.00, but the mesor showed a two-fold increase. The darkness did not affect the cytochrome b_5 content. The rhythm of NADH-cytochrome b_5 reductase activity was significantly changed to a 24 h one with a maximal value at 6.00. The amplitude was also increased.

EFFECTS OF α-METHYL-DL-p-TYROSINE AND CLORGYLINE ON PINEAL MORPHOLOGY AND FUNCTION IN PIG. II. ULTRASTRUCTURE OF PINEALOCYTES

Barbara Przybylska, Bogdan Lewczuk and **Zygmunt Wyrzykowski**
Department of Histology and Embryology, Olsztyn University of Agriculture and Technology, Olsztyn, Poland

Previous studies in our laboratory demonstrated that cytoplasmic dense bodies are the specific and the most active structures of pinealocytes in the domestic pig. It has been found that their relative volume and inner structure changes in different physiological and experimental conditions and a relationship of these bodies to the secretory processes has been postulated. There is some evidence that drugs which influence the biosynthetic pathway of the indoloamines cause clear changes in the cytoplasmic dense bodies of pinealocytes. The present study was performed to examine if the drugs which influence the level of norepinephrine also effect the ultrastructure of pinealocytes, especially volume and structure of the cytoplasmic dense bodies.

Fifteen pigs, aged 98±4 days at the beginning of experiment, were used in the study. The animals were housed under a 12:12 LD photoperiod (250 lux of light during the light phase and less then 3 lux of dim red light during the dark phase). After two weeks of maintenance under the 12L:12D photoperiod venous cannulae were inserted. Four days after cannulation the pigs were treated as follows: 5 animals with α-methyl-DL-p-tyrosine methyl ester hydrochloride (MPT) - an inhibitor of tyrosine hydroxylase - 3g daily per animal; 5 animals with clorgyline - an inhibitor of monoaminoxidase - 60 mg daily per animal. Both drugs were dissolved in 20 ml of saline and infused via cannulae for 20 min starting one hour after the beginning of both light and dark phases. The last five animals functioned as controls and were given saline.

All animals received eight infusions during four consecutive days. The pigs were killed 4 h after the last infusion (between 00.30 and 01.30). The pineal glands were removed as quickly as possible, fixed and prepared for investigation of ultrastructure. The thin sections were examined in TEM Tesla BS 500. Point count analysis was employed to estimate the relative volume of various cell components.

The treatment with MPT and clorgyline caused clear changes both in the relative volume and the structure of cellular organelles. The administration of MPT resulted in an increase in relative volume of both forms of cytoplasmic dense bodies (multilamellar and membrane bounded with wide variation of internal structure) and decrease in numerical density of multivesicular bodies. The treatment with clorgyline caused a decrease in relative volume of cytoplasmic dense bodies and an increase in numerical density of granular vesicles. The present results strongly support the participation of dense bodies in secretory process of pinealocytes in the pig. The increase in dense bodies (particularly their multilamellar forms which are considered to be the inactive part of the system of cytoplasmic dense bodies), simultaneous with the decrease of plasma melatonin level (part I of the present study) point to observed changes as the signs of decreased activity.

Supported by grant KBN 5 5798 91 02.

THE COMPARATIVE ANALYSIS OF PINEAL ORGAN MORPHOLOGY AND CELLULAR COMPOSITION IN SEVERAL ECOLOGICAL GROUPS OF THE BLACK SEA FISHES

Victoria Radchenko and **Modest Aleyev**

Laboratory of Ecomorphology, Institute of Biology of Southern Seas, Ukraininian Academy of Sciences, 2 Nakhimov St., 335011 Sevastopol, Crimea, Ukraine

Fishes representing different ecological groups - benthos (B), nekton (N), etc. inhabit biotopes with very different light intensities. B species are illuminated from above it at all and their life cycle (especially the circadian component) greatly depends on correct measurement of daylength, while pelagic species usually have enough photoinformation from the environment. Between B and N many intermediate stages exist - benthonecton (BN), etc. This presentation describes an attempt to test a hypothesis that the pineal organ in fishes of these ecological groups differs distinctly in morphology and raties of various cell types (R). Using light microscopy we have studied the brain morphology in 18 species of ordinary Black Sea fishes. All B species (*Uranoscopus scaber*, etc.) had large, well-differentiated PO (with very long pineal stalk terminating above the bulbi olfactori) and R of neural:glial:photoreceptory cells was 3:5:10. N species (*Trachurus mediterraneus ponticus*, etc.) were characterized by short pineal stalks (in some species the pineal stalk was hidden between the lobi optici and corpora striata) and R was 3:5:7. In BN species (*Crenilabrus tinca*, etc.) the pineal stalk terminated mainly above the upper structure of the corpora striata and R was 3:5:8. This study revealed marked changes in relative numbers of various cell types in the pineal organ from benthic to nektonic fishes that reflects a conversion of the pineal organ from a photosensory structure to a secretory one.

EFFECT OF NOREPINEPHRINE ON THE SPONTANEOUS REGULAR AND RHYTHMIC ELECTRICAL ACTIVITY OF PINEALOCYTES *VIA* NITRIC OXIDE - AN *IN VITRO* STUDY

J. Schenda and **L. Vollrath**

Institute of Anatomy, University of Mainz, 55099 Mainz, Germany

Pinealocytes are known to express a circadian rhythm which can be observed in a change of spontaneous electrical activity *in vivo*. In addition short-term firing patterns were noted (Reuss 1987). In these experiments it could not be clarified whether the spontaneous activity and the rhythmic firing pattern are due to

the connection of the pineal to the CNS or to an intrinsic function of the organ. Moreover, nothing is known about the function and regulation of this spontaneous activity. The biochemical function of the pineal gland is regulated by norepinephrine (NE). Biochemical experiments have shown that adrenergic stimulation induces NO-mediated cGMP formation (Spessert et al. 1993).

The aim of the present study was to investigate the origin of the spontaneous activity and its modulation by NE in *in vitro* experiments. The pineals of adult Spargue-Dawley rats were quickly removed, fixed in a perfusion chamber and superfused by oxygenated artificial cerebrospinal fluid (ACSF). Extracellular recordings of multiple units were made with glass-covered platinum/iridium electrodes and a conventional electrophysiological setup. The spontaneous activity was recorded for 2 h under control conditions. Then the perfusion system was switched to ACSF plus norepinephrine (NE, 10^{-7} M), NE plus N-Methyl-L-Arginin (NMLA, 10^{-6} M, NE 10^{-7} M), NMLA alone (10^{-6} M) or sodium nitroprusside (SNP, 10^{-9} M). After 1-2 h the system was switched back to normal ACSF for 2 h. The spontaneous activity of the multiple units was lower than 10 impulses/s. Two groups of firing patterns could be distinguished : regularly firing (RE) and rhythmically firing (RH) cell clusters (duration 6.9±0.1 min or 12.6±0.2 min). NE reduced the frequency of both RE and RH cell clusters up to 85% of the control activity or had no effect (19% of the registrations). An increase of activity was never observed. Interestingly the rhythmic firing pattern of all RH cells was abolished. These depressing effects could be canceled by the addition of NMLA, an inhibitor of NO-synthase, to the NE-containing ACSF. NMLA itself had no effect on the firing rate. On the other hand SNP, an NO-analog had the same depressing effect on the frequency of RE and RH cell clusters and on the rhythmic firing pattern of all RH cells.

These results show that the spontaneous electrical activity of the pinealocytes appears to be an intrinsic function of the pineal which can be modulated by NE via NO as an intercellular messenger. A role of NO in adrenergic stimulation of cGMP has also been shown in biochemical investigations (Spessert et al. 1993).

MELATONIN BINDING SITES IN CHICKEN LYMPHOID GLANDS, GONADS AND CENTRAL NERVOUS SYSTEM

K. Skwarło-Sońta[1], E. Wolińska-Witort[2], T. Dziwiński[1], M. Snochowski[2] and J. Sotowska-Brochocka[1]

[1]Department of Vertebrate Animal Physiology, University of Warsaw, Warsaw; [2]Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jabłonna n/Warsaw, Poland

Previously we have shown that in chicken, contrary to mammals, melatonin did not exhibit any immunostimulatory and anti-glucocorticoid activity, mediated *via* an opiatergic mechanism. The aim of the present study was to demonstrate and characterize melatonin binding sites in the lymphoid gland taken from cockerels kept from hatching under controlled light conditions (L:D=12:12) and to compare them with those present in the central nervous system and gonads. Three-week-old birds were divided into two group, one of them was immunized ip with sheep red blood cells (SRBC), the second being intact controls. Chickens were sacrificed at the age of 11, 21, 24, and 28 days (the two last sacrifices were done 3 and 6 days after immunization, respectively), at mid-light period. There were 7-10 birds per group. The dissected organs were quickly weighted, frozen in liquid nitrogen and kept in -80°C until assayed, and next used for membrane preparations. Saturation and kinetic assays were performed using 2-[^{125}I]iodomelatonin of specific activity 2200 Ci/mmol, purchased from NEN, Dupont. Melatonin binding sites were measured in the following tissue membrane preparations: optic tectum (OPT), cortex (CTX), hypothalamus (HYP), gonads (TST), thymus (THY), spleen (SPL) and bursa of Fabricius (BF). It was found that examined membrane preparations contained high affinity, low capacity melatonin binding sites, and Scatchard analysis generated a linear plot indicating a single class of binding sites. The distribution of melatonin binding sites

in the examined structures of central nervous system was as follows: the highest density (measured as fmol/mg protein) was in OPT (13.87-21.77), lower in HYP (8.43-20.98) and CTX (6.86-14.93). These results are comparable with those found by other authors (1, 2). Much lower density of melatonin binding sites was found in chicken testes (0.12-2.12 fmol/mg protein). Of the lymphoid glands examined, the highest melatonin binding was in the bursa of Fabricius (0.23-3.20 fmol/mg protein), next in the spleen (0.23-1.24) and only traces in the thymus (0.05-0.25). The melatonin binding in chicken lymphoid glands is comparable with the resuls obtained by other authors in birds (3, 4), but is much lower than in rat thymus (5). It seems worthwhile to examine whether the lack of immunostimulatory activity of melatonin in chicken observed by us could be, at least partly, attributed to the lower number of melatonin binding sites in the avian than in mammalian thymi. There are no clear-cut statistically significant differencies during 4 weeks of postnatal development and there is no effect of immunization as well, therefore the research is continuing.

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TOXIC LIGHT LEVELS INDUCE PROGRAMMED CELL DEATH (APOPTOSIS) IN THE RAT RETINA

P.J. Szczęsny[1], M. Weller[2], A. Von Hochstetter[3] and Z. Zagórski[1]
[1]Department of Ophthalmology, Medical Academy, Lublin, Poland; [2]Internal Medicine and [3]Pathology, University Hospital, Zurich, Switzerland

Visible and ultraviolet (UV) radiation can induce ocular damage, especially in the lens and the retina (1). Reduced stratospheric ozone may result in increased outdoor exposures to UV during travel and leisure activities (2). Prolonged light exposures including light therapy increasingly used for treatment of winter depression and circadian rhythm disturbances caused by shift work or jet lag may also have cumulative deleterious effects (3).

The purpose of the study was to investigate the phototoxicity of diffuse white light on the retina in a rat, the time scale of retinal changes and the pattern of tissue responses to such injury.

Male albino rats (300 g) were dark-adapted for 36 h and sacrificed or exposed to 1,000 lux of diffuse white overhead light for 2 h, divided into 5 groups (n=5 per group) and killed at the end of light exposure (0) or 24, 48, 72 and 144 h thereafter. The eyes were processed for light and electron microscopy and for analysis of internucleosomal DNA cleavage by agarose gel electrophoresis.

Retinal degeneration was observed in lower temporal retina by 144 h after light exposure. At 0 h numerous photoreceptors were undergoing apoptosis, were shrunken and stained darkly, showed pyknosis of nuclei and condensation of cytoplasm. The onset of apoptosis in retinal pigment epithelium manifested by nuclear cupping and condensation was observed 24 h after light damage. At later stages (<24 h) numerous monocytes and macrophages, but no other inflammatory cells, were present within retina and choroid. Retinal recovery was observed in other retinal regions primarily characterised by vesicular alterations of rod outer segments. DNA cleavage, characteristic for apoptosis, was observed immediately after the end of light exposure (0 h) and was more pronounced at 24 h.

Apoptosis is a mode of cell death in addition to necrosis and occurs physiologically during development and tissue remodelling (4). It can also be induced by various deleterious conditions including electromagnetic radiation and light. This study demonstrates that apoptosis is an acute photoreceptor response to toxic light levels in the rat and followed by apoptotic changes in the retinal pigment epithelium resulting in chorioretinal scar formation within a week after the light injury.

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Supported by the Swiss National Science Foundation grant No 31.-30131.90.

MELATONIN RECEPTORS IN THE AVIAN BRAIN: A SPECIES COMPARISON

Jolanta B. Zawilska[1,2] and Jerzy Z. Nowak[1]
[1]Department of Biogenic Amines, Polish Academy of Sciences, 3 Tylna St., 90-950 Łódź and [2]Department of Pharmacodynamics, Medical University of Łódź, Łódź, Poland

In birds, the hormone melatonin, through an action in the brain appears to be involved in the regulation of the circadian timing system. The hormone exerts its actions *via* specific receptors.

Melatonin receptors were characterized in brains of four avian species (goose, duck, turkey and pigeon) by *in vitro* binding technique, using 2-[^{125}I]iodomelatonin as a labelled ligand. The experiments were performed in April-May on the animals living under natural environmental lighting conditions. The birds were killed in the middle of the day, under light conditions, and whole brains (minus cerebellum and pons-medulla) were removed. Binding assays were perfomed as described by Dubocovich and Takahashi (1) with some modifications. The specific binding of 2-[^{125}I]iodomelatonin to crude brain membrane preparations of the tested species was rapid, stable, saturable, reversible and of high affinity. Schatchard analysis revealed a single population of binding sites with an affinity constant (K_D; in nM) of 3.39 (goose), 2.7 (duck), 2.36 (pigeon and turkey) and a total number of binding sites (B_{max}; in fmol/mg protein) of 72.8 (goose), 159.1 (duck), 101.5 (pigeon) and 136.5 (turkey).

Competition studies (carried out with melatonin, MEL; N-acetyltryptamine, NAT; N-acetylserotonin, NAS; serotonin, 5-HT; and 5-methoxytryptamine, 5-MT) showed a monophasic reduction of 2-[^{125}I]iodomelatonin binding with a following order of potencies - goose: MEL > NAS > NAT 5-HT >> 5-MT; duck: MEL >> NAT > NAS = 5-HT > 5-MT; pigeon: MEL >> NAT > NAS = 5-HT >> 5-MT; turkey: MEL >> NAT = NAS = 5-HT >> 5-MT. Therefore, the pharmacological characteristic of melatonin receptors in brains of goose, duck, turkey and pigeon resembled that described for brains of chicken (2-4) and quail (5). On the other hand, the apparent densities of these receptor sites were about 2-3 times higher and their affinities were one order of magnitute lower than the values reported for chicken and quail brain membranes (2-5). The reason for these differencies is not known, but could be related to animals' living conditions (natural *vs.* laboratory environment) and species-related variations.

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Supported by the KBN grant 0540/P2/93/04 to J.B.Z.

RHYTHMIC MELATONIN SYNTHESIS IN THE CHICK EMBRYO PINEAL GLAND UNDER *IN VIVO* AND *IN VITRO* CONDITIONS

M. Zeman[1], D, Lamošova[1], M. Mackova[1] and E. Gwinner[2]
[1]Institute of Animal Biochemistry and Genetics, SASci, Ivanka pri Dunaji, Slovakia; [2]Max-Planck-Institut für Verhaltensphysiologie, D-82346 Andechs, Germany

Our previous studies showed that rhythmic melatonin synthesis occurred both in the pineal gland and eyes of chick embryos incubated in a light:dark (LD) cycle. The rhythm followed a typical pattern in both

these organs, concentrations were low in the daytime and high in the nighttime. Embryos incubated in constant darkness did not exhibit rhythmic melatonin production.

Results of recent experiments demonstrate that a LD cycle has a predominant effect on rhythm development. Different LD cycles (16:8, 12:12 and 6:18) were able to induce the melatonin rhythm expression in 19-day-old chick embryos. To demonstrate whether an endogenous component of the melatonin rhythm is developed at the end of the embryonic period, embryos were incubated in LD 12:12 for first 18 days of incubation and subsequently in constant darkness for next 48 hours. The rhythm in plasma melatonin concentrations induced by the LD cycle did not persist over the 48 h incubation period in constant darkness.

In vitro experiments demonstrate that pineal cells isolated from 19-day-old chick embryos and cultured under a LD cycle expressed rhythmic melatonin production. The shape of the rhythm was as expected. High concentrations were found during the nighttime and low during the daytime. This finding illustrates that chick pineal cells at the end of the embryonic development are photosensitive and they are able to synchronize their melatonin production to a prevailing LD cycle. The presence of a circadian component of the melatonin rhythm in 19-day-old embryos was tested by exposing synchronized pineal cells (5 days in LD 16:8) to constant darkness for 48 h. The melatonin rhythm was damped during a culture for two days in constant darkness. Both *in vivo* and *in vitro* experiments indicate that an endogenous component of the melatonin rhythm is not fully developed at the end of the embryonic period and it may develop perinatally in chicks.

The obtained results suggest that mechanisms underlying rhythmic melatonin production develop at a different rate during the ontogeny of the chicken. As a circadian component of the rhythm may develop during a discrete time interval, this period may be useful for analysing molecular mechanisms that develop at the same time. Hence, incorporation of developmental aspects into the classical model of chick pineal gland may offer further advantages for studies of the molecular basis of circadian oscillations in vertebrates.

LACK OF MELATONIN RHYTHM IN ACTIVE ACROMEGALY AFTER RADIOTHERAPY

Krystyna Żwirska-Korczała, Zofia Ostrowska, Beata Kos-Kudła, Barbara Buntner and **Marek Kudła**

I Department of Pathophysiology and Clinical Biochemistry, Silesian Academy of Medicine, 2 Traugutt Pl., 41-800 Zabrze, Poland

The relation between pineal and pituitary gland activities with regard to PRL, GH, ACTH and TSH is still controversial. Prolactin concentration returns to normal after oral administration of melatonin in some patients with idiopatic hyperprolactinaemia. Some investigators observed an increase in nocturnal secretion of PRL and GH after the administration of this indoleamine. Others noted an increas in PRL secretion after oral melatonin administration to healthy subjects. Still others reported no influence of exogenous melatonin on GH, PRL and TSH concentrations. It is generally accepted that the 24-h melatonin profile is not changed in pituitary adenomas. Piovesan et al. (1990) observed a suppression of melatonin circadian rhythm in acromegalic patients following its higher daytime secretion. The aim of this study was to evaluate the interrelation between the levels of growth hormone (GH), prolactin (PRL), insulin-like growth factor (IGF-I) and melatonin (MEL) determined at 3-h intervals over a 24-h period (8.00, 11.00. 14.00, 17.00, 20.00, 23.00, 2.00 and 5.00). The study group included 8 acromegalic patients 7 to 9 years after radiotherapy. Eleven healthy volunteers served as controls. The circadian hormonal rhythms and the interrelation between hormonal and IGF-I levels were evaluated using Halberg's cosinor method and correlation analysis, respectively. Our study showed a clear circadian rhythm of melatonin in healthy subjects with high

nocturnal and low daytime values. In men with active acromegaly we observed abolition of the melatonin rhythm, with significantly lower nocturnal values. No correlation was found between nocturnal melatonin levels and prolactin, growth hormone or insulin-like growth factor-I either in healthy or in acromegalic subjects. In conclusion, radiotherapy seems to be responsible for the lack of melatonin rhythm.

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NEUROPHYSIOLOGICAL AND IMMUNOCYTOCHEMICAL RELATIONSHIP OF PHOTORECEPTORS AND NEURONES IN PHOTOSENSORY PINEAL ORGANS

Y. Morita, S. Tamotsu and **K. Uchida**
1st Department of Physiology, Hamamatsu University School of Medicine, Hamamatsu 431-31, Japan

Morphological and functional relationship between nervous information processing and endocrine activity in the pineal organs of river lamprey, *Lamperta japonica,* was studied electrophysiologically, immunocytochemically and by means of HPLC.

Nervous information and responsible cells

Electrophysiological evidence:

Typical response patterns from pineal organs to photic stimuli consist mainly of two modes of electrical response, i.e. hyperpolarization of resting membrane potential recorded from pineal photoreceptors, and spike potentials accompanied by membrane potential changes (depolarization or hyperpolarization) from pineal neurones (ganglion cells). The peaks of spectral sensitivity curves obtained by these measurements are 380 nm and 525 nm (505 nm in larva) intracellularly recorded from photoreceptors, and 380 nm (inhibitory) as well as 540 nm (excitatory) for spike potentials from neurones.

Immunocytochemical evidence:

Both types of photoreceptors, rod-type and cone-type, are demonstrated in the pineal organ. Rod-type photoreceptor was identified by means of anti-rhodopsin antibody, and cone-type photoreceptor was shown by anti-visinin antibody and anti-iodopsin antibody. Visinin is Ca^{2+}-binding protein obtained from chick retinal cones, and iodopsin is visual pigment of red cone in chicken retina.

Photoreception and endocrine activity

It became evident that photoreceptors of lamprey pineals have two types if classified by serotonin immunoreactivity. Furthermore, in serotonin-positive photoreceptors, both types of rod-type and cone-type photoreceptors are identified. Rod-type photoreceptors are found often in the pineal end-vesicle, and cone-type photoreceptors are mainly demonstrated only in the periphery of the end-vesicle. Both rod-type and cone-type photoreceptors are observed at the atrium. Iodopsin immunoreactivity (I-IR) cells are found along Tractus pinealis, where no rhodopsin-immunoreactivity (R-IR) was observed.

In the pineal organ of lower vertebrates, 3 types of pineal cells have been classified, i.e. typical photoreceptors, modified photoreceptors, and pinealocytes *sensu stricto* (p.s.s.). Many p.s.s. are found around the Tractus pinealis in lamprey. Therefore, these p.s.s. could be assumed to have I-IR. It was suggested that p.s.s. may also be the site where the photic information could be converted to the endocrine activity, as well as in the modified photoreceptors.

Color discrimination and photoregeneration

Antagonistic effect of short and long wavelength light is remarkably demonstrated in the measurement by HPLC. Irradiation to orange light following cis-to-trans isomerization by blue light caused trans-to-cis isomerization. Pineal visual pigment photoregenerated. Such a process has never been observed so far in the vertebrate retina.

This antagonistic activity was confirmed also electrophysiologically. The inhibitory effect of UV light (380 nm) to spontaneous spike discharges from chromatic neurones is prompt and decreases in several seconds. However, simultaneous exposure to longer wavelength light (orange) holds the inhibitory effect of UV light. The mechanism of keeping sensitivity to UV light must contribute to the regulatory function of pineal organs according to seasonal changes.