THESIS

Marissiaux Jean-Baptiste
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University of Veterinary Medicine of Budapest

Department of Physiology and Biochemistry

Pineal gland & melatonin in ewes: a review in understanding and optimizing their reproductivity

By:
Marissiaux Jean-Baptiste

Supervisor:
Kiss Dávid Sándor, PhD

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Abstract

Melatonin is produced in the pinealocytes, granulosa cells, the oocytes, and the cumulus oophorus. The secretion of this hormone by the pineal gland is modulated by both intrinsic and extrinsic pathways, respectively centered on the pineal synaptic ribbons (SRs) and on environmental photic stimuli perception. As the cellular development of a mammalian embryo advances, the intrinsic pineal SRs behaving at first like direct photoreceptors progressively develop into cellular organelles enabling neighboring pineal cells – or pinealocytes – to modulate their functions in a paracrine manner. Aside from this intrinsic modulation, the pineal cells are under extrinsic control originating from the retinal photic stimuli perception, as well as being under the circadian clock's influence. The functions of melatonin – and its metabolites – on mammalian physiological and reproductive systems are numerous, including antioxidant properties, arousal control and sleep inducer. Moreover, maternal melatonin possibly promotes cellular development in the embryo, while entraining the circadian rhythmicity in the developing fetuses. The pineal melatonin secretion and mammalian reproductive systems appear to be under mutual feedback mechanisms. Besides the circulatory melatonin influencing the reproductive cycles in ewes, it also seems that reproductive hormones can in turn modulate the pinealocytes' melatonin secretion. For that reason, melatonin treatment along with superovulation protocols in ovine species in summer anestrus could, in the future, allow a better productivity and economic return in ovine farming throughout the world.

In order to achieve a general understanding of the pineal gland's modulation of melatonin production, we will initially discuss the intrinsic and extrinsic modulating pathways of pineal melatonin secretion. In a second time, we will encompass the functions of melatonin in regard to reproductive purposes in ewes and rams, beneficial to enhancing the reproductive farming protocols' efficacy. Furthermore, we will aim at laying grounds for future scientific research to clarify the role of SRs in pinealocytes in relation to the melatonin secretion in ewes, and to determine the potential effects of melatonin on estrus, follicular development, and early embryonal development in ewes.

Abbreviations

5HT	5-hydroxytryptamine, serotonin
AA-NAT	arylalkyl N-acetyl transferase
aCSF	artificial cerebrospinal fluid
AII	angiotensin II
AVP	arginine vasopressin
CALB	calbindin
CALR	calretinin
CAST	CAZ-associated structural protein
CAZ	cytomatrix at active zone
CtBP1	COOH-terminal binding protein 1
E1 & E2	estrone & estradiol
ELISA	enzyme-linked immunosorbent assay
ERα & β	estrogen receptor alpha & beta
ERC	ELKS-rab6-interacting protein CAST
ET	embryo transfer
FF	follicular fluid
FSH	follicle stimulating hormone
GABA	gamma (γ) aminobutyric acid
GAL	galanin
GnRH	gonadotropin-releasing hormone
GRP	gastrin releasing peptide
GSH	glutathione peroxidase
hCG	human choriogonadotropin
HIOMT	hydroxyindole-O-methyltransferase
IL	interleukins
IVF	in vitro fertilization
IVM	in vitro maturation
KIF3A	kinesin family member 3A
LD	long days
LPS	lipopolysaccharide
mENK	met-enkephalin
Munc13-1	mammalian ortholog uncoordinated-13 type 1
NA	norepinephrine, noradrenaline
NPY	neuropeptide Y
NT	neurotensin
PMSG	pregnant mare serum gonadotrophin
PVN	paraventricular nucleus
qPCR	quantitative polymerase chain reaction
RC	ribbon complex
RIA	radioimmunoassay
RIM1 & 2	regulating synaptic membrane exocytosis 1 & 2
ROS	reactive oxygen species
SCG	superior cervical ganglion
SCN	suprachiasmatic nucleus

SD	short days
SOD	superoxide dismutase
SR	synaptic ribbon
SV	synaptic vesicle
SV2	synaptic vesicle glycoprotein 2
TH	thyroid hormone
TNF	tumor necrosis factor
VIP	vasoactive intestinal peptide

Introduction

The pineal body, epiphysis cerebri, or conarium, is a small gland belonging to the endocrine system of the brain of most vertebrates. This gland is located between the two cerebral hemispheres in the region called the epithalamus, where the two halves of the thalamus join, outside of the blood-brain-barrier. The pineal gland develops as an evagination from a committed area of the neuroepithelium lining the roof of the 3rd cerebral ventricle prenatally, and its maturation continues postnatally in the first period of life, or neonatal period. The conarium is the only non-paired organ in the center of the skull (as opposed to the hypothalamus for example which is a paired one). In humans, it becomes calcified towards adulthood, and it is therefore an easily recognizable structure at the midline of the brain by radiologists.

Mammals dispose of various organs constituting the so-called "endocrine organs": testes, ovaries, pancreas, thyroid gland, parathyroid gland, hypothalamus, adrenal glands, pituitary gland, and pineal gland. Their role in the organism is mainly to produce and excrete a wide range of hormones, that will take part in the production, regulation, and stimulation (or inhibition) of various metabolic and biochemical reactions within the body. The pineal gland is responsible, inter alia, for the greater part of melatonin production. Blood melatonin levels have thoroughly been associated to the regulation of circadian rhythmicity. The circadian rhythm/cycle is the inner clock of an animal – usually of 24 hours – varying in direct response to environmental light stimulation. This cycle regulates the behavior (alertness, sleepiness…) and the physiology of an animal. The melatonin is a hormone responsible for several functions including antioxidant activity, decreasing body temperature and increasing sleep ability, controlling a mammal's arousal, sexual maturation and partaking in the regulation of seasonal reproductive cycles.

Histologically, the mammalian pineal gland is composed in minority of fibroblasts, and astrocytes (microglia), that play a role in pineal gland formation and homeostasis through the regulation of precursor cells, remodeling blood vessels and clipping sympathetic nerve fibers. Besides these cells, the pineal gland is mostly composed of pinealocytes (95%) that are responsible for the production and secretion of melatonin (Rodriguez et al., 2016).

Phylogenetically, the conarium belongs to a photoreceptor set of organs, meaning that the secretory functions from the cells of the pineal gland – or pinealocytes – are regulated through environmental light stimuli. In the epithalamus of some reptiles and amphibians for example, the

pineal body is linked to a photosensitive organ known as the parietal eye, or third eye. This latter structure plays a role – along with the pineal gland – in regulating the circadian rhythmicity and thermoregulation. As a photoreceptive organ, the conarium produces a varying amount of melatonin due to changes in length of daytime. These diurnal varying levels of melatonin directly affect the sexual cycle of seasonal breeders such as ewes and rams. Specifically, photoperiodic information is forwarded to the reproductive neuroendocrine system by a circadian secretion of melatonin from the pineal gland (Abecia et al., 2006).

Though it had previously been shown that pineal organs of some submammalian vertebrates – such as chelonians and birds – contained direct photoreceptive elements highly akin to that found in the retina, the purpose of a study carried out by Zimmerman in 1975 was to assess these possible similarities in higher vertebrates. It had been suggested and confirmed that the cells of the pineal body undergo a photoreceptor-like differentiation during a transient neonatal period partaking until 17 days of age in the rat (Zimmerman & Tso, 1975).

The pineal gland hormones production is regulated by both intrinsic and extrinsic pathways. The intrinsic pathway of the pineal gland is sparsely described and known in mammals, especially in ovine animal species. Until now, studies have described the intrinsic pathway components involving the action of so-called "synaptic ribbons". Ribbons are subcellular organelles mostly described in the photic mechanisms of the retina. Similar complexes in pinealocytes were observed and would serve as both a direct photoreceptive organ and a secretory organ within cells. They would ensure a rapid and sustained neurotransmitter release, with rod-like structures holding surrounding vesicles close to the active zone of the "pineal synapse" (Spiwoks-Becker et al., 2008). The role of these synaptic ribbons in the regulation of melatonin secretion is, however, not clarified yet and will be further debated in **chapter 1** of this thesis.

The extrinsic pathway however is well defined in mammals. The light stimuli perceived by the retina will convey a descending signal to the suprachiasmatic nucleus (SCN), which is the main "clock" or rhythm-generating system in mammals. From the SCN an information is transmitted towards the superior cervical ganglion (SCG). It is finally from the SCG that an ascending signal towards the pineal gland itself – via sympathetic pathways – will inhibit or stimulate the production of melatonin during daytime or nighttime respectively. The extrinsic neural pathway is later discussed into details in **chapter 2** of this thesis.

Moreover, pinealocytes seem to be influenceable by circulating estrogen (17-beta-estradiol, E2 and estrone, E1) levels. The pinealocytes nuclei presents both receptors alpha and beta for estrogen (ERα and ERβ), which could imply that the pineal gland is a direct target of estrogen (Hernández-Díaz et al., 2001; Semm et al., 1981). Thereby, the estrogen-regulated reproductive system would impact the regulatory role of the melatonin of pineal-origin on female reproductive and circadian cycles. Further evidence suggests that E2 also plays a role in the modulation of pineal sensitivity to adrenergic stimulation (Sànchez et al., 2004). The effects of the mammalian reproductive system – especially of sexual hormones like estrogen – on melatonin production, and the effects of melatonin on ovine reproductivity will be discussed in **chapter 3** of this thesis.

In the lack of targeted research about the relationship between synaptic ribbons and melatonin secretion in mammalian pinealocytes, as well as the specific effects of exogenous melatonin protocols on ovine reproduction, further experiments are necessary. These would be decisive to understand the influence of intrinsic regulations on melatonin secretion in pinealocytes and help us improve the productivity of ovine breeding programs. Possible experimental grounds will be discussed in **chapter 4** of this thesis.

<u>Chapter 1:</u> INTRINSIC pathway - Synaptic ribbons structure & morpho-functional development

A- Demonstration of a photoreceptor-like activity of pinealocytes in neonatal period and ribbons structures in pinealocytes of higher order mammals

Neonatal rats keep their eyelids closed during the neonatal period and are not opened until the age of 14-17 days. Bio-chemical responses were yet observed in the pineal body of neonatal rats, even after enucleation. (Zimmerman & Tso, 1975). The absence of such responses in adult rats suggested the presence of photoreceptors in the skull extraocularly, progressively lost throughout the animal's development into a more typical secretory function for a gland.

Accurately, the study showed a specialized differentiation of pinealocytes in neonates, distinct from that of an adult, first observed at 4 days and no longer apparent at 17 days of age. At day 1 we can observe dense clusters of randomly arranged parenchymal cells with peripheral regions infiltrated with connective tissue and vascular channels. In the first 4 days of life, a rat's pinealocytes already present well-formed "synaptic ribbons" surrounded by "synaptic vesicles".

Between 4 and 12 days of life, the pinealocytes are organized into cordlike formations surrounded by connective tissue and vascular matrices. The synaptic ribbons (SRs) are typically found at the nuclear pole of the pinealocytes within the cell body itself. These ribbons appear in groups of 2 to 6, oriented perpendicular to the plasma cell membrane, forming arciform densities. The SRs, though structurally similar, differ depending on their histological locations: in the retina these are located within the rod spherules or cone pedicles at the terminals of axons, while in the pinealocytes these ribbons are located within the cell bodies. At the opposite pole, the pineal cells developed elongated cell processes – which number peaks at 12 days of age – into luminal spaces and were attached to adjacent cells by structures resembling zonulae adherentes. Cilia from the tip of these processes were observed, extending into other adjacent cells' lumens. This polarization of the cell, along with the cilia formation which is typical of non-motile sensory epithelia – such as the retinal cells – are very similar characteristics to retinal photoreceptors. Vesicles present within the terminal expansion of a cilium appear like the "rudimentary outer segments" in the pineal organs of certain birds and reptiles (Figure 1, Figure 2). Even though no well-developed disk characteristic of outer segments of photoreceptors is observed in the rat, it has been shown that

immature photoreceptors with immature formed outer segments can elicit a response to light. During this transient neonatal period of mammalian life, we may suppose that a photoreceptor-like development occurs in the pineal gland. This transient period of pinealocytes may be the photoreceptive site explaining why enucleated rats still showed light-induced biochemical changes.

After 12 days of life, the cilia of pineal processes progressively disappear up to 17 days of life, while the SRs complexes are retained in the adult life. Thereby, the neonatal development in rats suggests that murine pinealocytes initially function like retinal photoreceptors, in order to compensate the yet underdeveloped neuronic pathways. The retained ribbon complexes later play a different role in the modulation of pineal functions, enabling a communication of signals among neighboring pinealocytes via neurotransmitters vesicular release. Even though mammalian pinealocytes are considered to have a strict endocrine secretory function, it also should be regarded as a "specialized neuron", or chemical synapse.

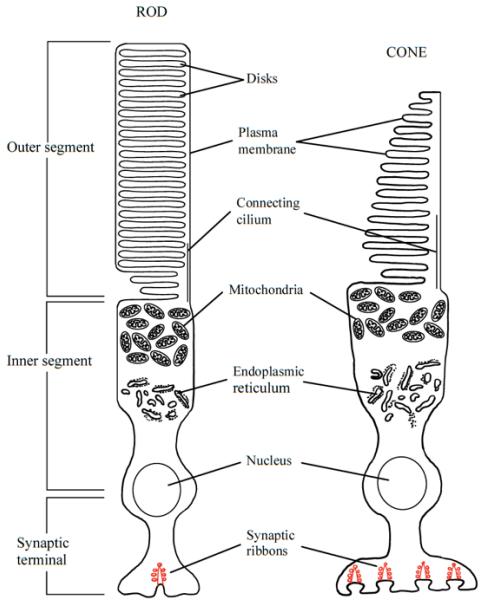

Figure 1: Vertebrate Rod and Cone schematics (adapted from Cote, 2006) - *Both cones and rods of the retina are photoreceptors. Both are constituted of the same bricks of elements: an outer segment perceptive to light stimuli, and a connecting cilium connecting the former to a second inner segment. This latter segment is far less sensitive to environmental photic stimulation, but its role predominates in modulating and centralizing the accumulating stimuli towards the photoreceptor cell's synaptic terminal, sending information through neurons into the central image-processing areas of the brain.*

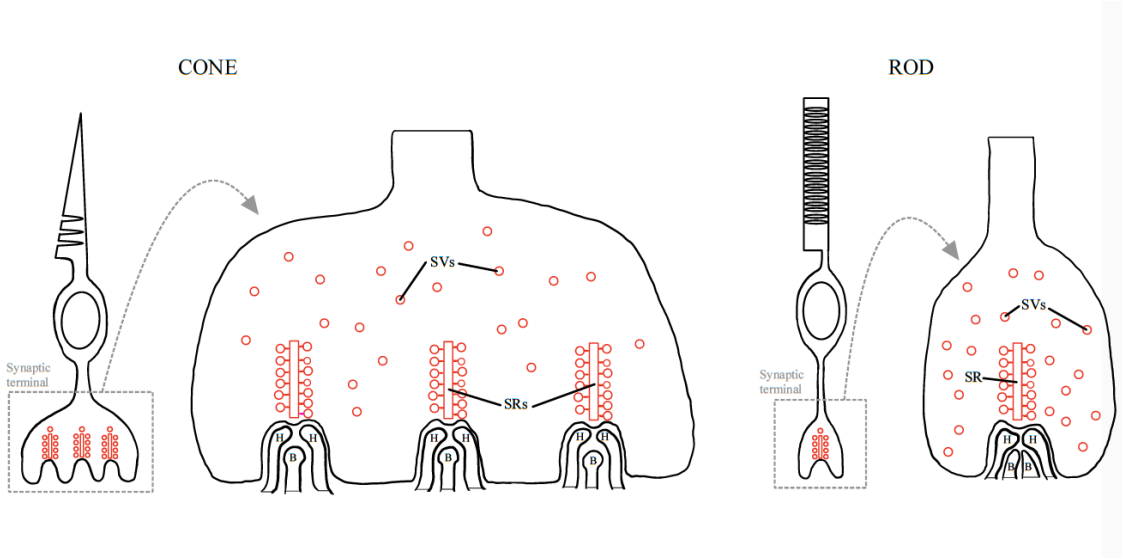

Figure 2: Retinal photoreceptor SRs (adapted from Schmitz et al., 2012) - *Synaptic terminals of photoreceptors (A-cone, B-rod), well-known structures harboring synaptic ribbons (SRs). The SRs of a retinal cone photoreceptor will present themselves in a pedicle conformation, while the SRs of a retinal rod are rather spherule shaped.*

SRs are electron-dense structures tethering synaptic vesicles (SVs) through tiny stalk-like materials. At first described as "vesicle-crowned rodlets" in the pineal (Zimmerman & Tso, 1975), the increasing resolution of electron microscopy over the past five decades enabled us to better observe these complexes. Oriented perpendicular to the plasma membrane, these rod-like organelles were observed to form groups of 2 to 6 ribbons in an arciform density formation at the presynaptic membrane. Many microtubules oriented parallel to the pinealocytes' long axis tethered a set of vesicles close to the "synaptic cleft". Each individual pineal SR is a flat plate-like structure, appearing just about 30-40 nm long, 200-400 nm high and 800 nm wide in murine pinealocytes (Voorn & Vogl, 2020). The arciform formation of pineal SRs clusters appears quite similar to the formations observed in the cone photoreceptor's terminal synapse termed as cone pedicles, rather than to the rod spherules.

SRs of the mammalian pinealocytes were at first proposed to be non-functional phylogenic relics of an embryonal or neonatal neurosensory organ. But evidence has since revealed that the mammalian pineal ribbons in adults are located near the cell membrane, in close topographical relationship to their neighboring pinealocytes, and undergoing diurnal morphological changes that resemble the typical changes of a retinal SR (Spiwoks-Becker et al., 2008). This suggests that these ribbon complexes are not just phylogenic relics, but functional organelles involved in intercellular

communication between adjacent pinealocytes. The pineal SRs have been identified to contain glutamate, which is thought to serve as an intrapineal signal molecule stimulating neighboring pinealocytes, resulting in the inhibition of noradrenergic melatonin synthesis while receiving environmental photic stimuli (Redecker & Veh, 1994).

SRs have been observed in various forms in different organs and species. These diverse forms indicate a variety of functions, including a mechanoreceptive activity in some fishes' hair sensory cells, visual function in photoreceptors of the retina, auditory role in the inner hair cells of the mammalian cochlea, as well as a yet obscure neuroendocrine function in pinealocytes. Each of these functions in different organs are correlated to a different set of proteins, all enabling SRs to serve as a scaffold for SVs and participating in their exocytosis (Figure 3).

B- Function of a ribbon synapse in the retina and in pinealocytes - extrapolation

SRs as organelles are abundant in the so-called ribbon-synapses of sensory neurons where they represent a specialization of the cytomatrix at the active zone (CAZ). Though the function of observed SRs in mammalian pinealocytes remain partly unclear, the mechanism of action of ribbon synapses has mostly been studied in another photoreceptive tissue: the retina (Jarsky et al., 2010). The abundant research and data on mammalian retinal SRs allow us to identify specific proteins judged necessary for the proper performance of ribbon synapses. For instance, the first identified major component of the SRs is called RIBEYE. This aggregate of proteins is responsible for tethering the SVs in the presynaptic area (Schmitz et al., 2000). This evolutionary conserved protein is the only known protein specific to SRs. Ergo, detecting the RIBEYE protein in a cell suggests the presence of a SR within that cell. From the database of known identified retinal SR proteins and of conventional neural synapse proteins, we can study the SRs of other neurosensory organs by extrapolation. As we mentioned it earlier, the structure and protein composition of SRs has been studied in various organs such as the retinal photoreceptors and the cochlear hair cells. The variety of SR compositions in different mammalian organs is presented in Figure 3. These schematic representations allow us to visualize the similarities more easily, and to compare the few differences in protein composition responsible for the different SR functions as part of an auditory, visual, or neuroendocrine organ.

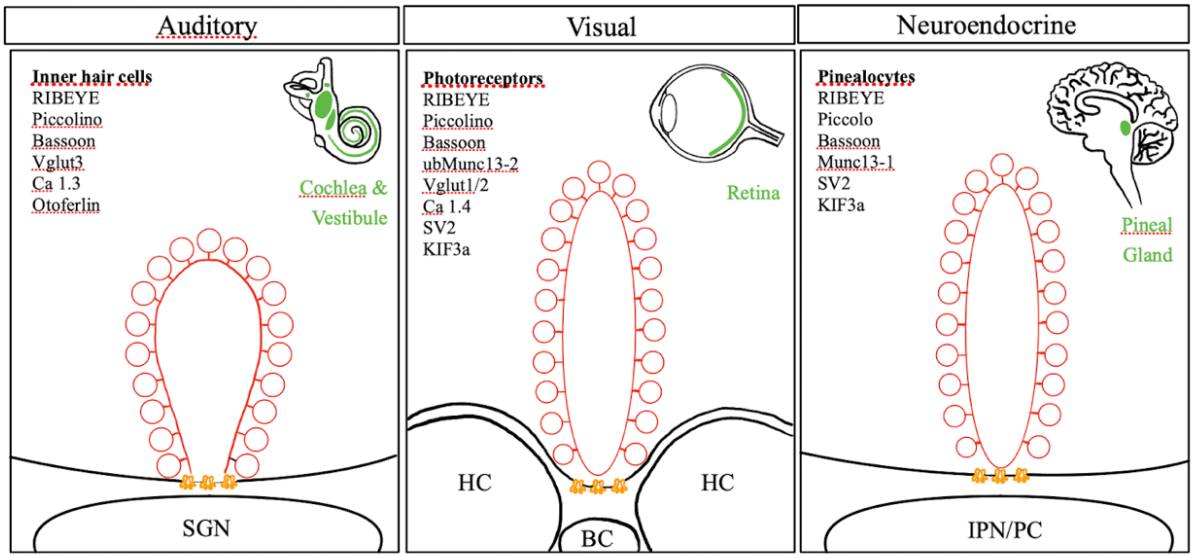

Figure 3: Stereotypic SRs shapes in different neurosensory organs (adapted from Voorn & Vogl, 2020) - *Ribbon synapse morphology and molecular composition differ between biological systems. Schematic drawings of stereotypic ribbon shapes from the indicated sensory or neuroendocrine system they are operating in, to illustrate gross morphological and molecular differences. Please note: Ribbon dimensions are not drawn to absolute scale. BC, bipolar cell; HC, horizontal cell; IPN, intrapineal neuron; PC, pinealocyte; SGN, spiral ganglion neuron.*

Several proteins have been identified from neural synapses, such as conventional CAZ proteins including RIM2, SV2, Munc13-1 and ERC/CAST. Aside from these typical CAZ proteins, photoreceptor cells possess a ribbon-associated compartment harboring Bassoon, Piccolo, RIM1, CtBP1 and KIF3A proteins – where Bassoon and Piccolo proteins have an important scaffolding role in the CAZ. Even though progress has been made over the past years in the characterization of the synaptic proteins, most of their specific functions remains obscure. Some exemptions are notable, like the KIF3A identified as a kinesin motor protein of SR microtubules – making KIF3A a potential candidate actively driving the SVs translocation at the release site –, and Bassoon which plays a major role in anchoring SRs at the CAZ and ensuring proper release of transmitter from the ribbon synapse (Spiwoks-Becker et al., 2008). The Munc13-1 also proved to play a role in priming SVs to a fusion-competent state, as well as potentiating the neurotransmitter release.

Evidence further showed that the presence of calcium channels is critical in the vesicle releasing (exocytosis) function of the retinal ribbon synapse. We can hypothesize that in the pinealocytes, SRs function in a similar way. Meaning that under an inducing signal, Ca^{2+}-channels

of the ribbon synapse will ensure a near-linear relationship between presynaptic membrane potential and exocytosis in the active zone (Jarsky et al., 2010). In the mammalian pineal, the increase of intracellular Ca^{2+} results from the noradrenergic input through α_1-adrenoreceptor activation – and β-adrenoreceptors to a lesser extent –, causing both an entry from extracellular calcium and Ca^{2+} release from intracellular stores (Hernández-Díaz et al., 2001).

Researching the function of SRs proteins can prove to be quite complicated as we need to approach the problem by means of mutants. For example, the normal Bassoon-mediated functions in the SRs were demonstrably perturbed in Bassoon-mutant mice. Other means of researching pineal SRs are based on the detection of previously identified proteins in other known SRs – e.g., in the retina. This can be performed in pinealocytes via postembedding immunoelectron microscopy with molecular markers, immunohistochemistry, or Western blot.

With these methods, Spiwoks-Becker and her team showed that pineal SRs contain key proteins characteristic of SRs. The presence of RIBEYE in pineal SRs was confirmed, and their immunohistochemical studies revealed that pineal SRs are indeed associated with the proteins CtBP1, Bassoon, Piccolo, Munc13-1 and KIF3A. Yet differences were noted between the ribbon complexes (RCs) of pinealocytes and that of sensory cells. The RCs contain RIMs and ERC/CAST proteins in photoreceptor terminals but not in pinealocytes, which suggests a difference in the anchor mechanism between the SR and the cell membrane.

Their research also demonstrated dynamic changes of pineal SRs and their associated proteins: The role of SRs is yet unclear but may be regarded either as a conveyer belt transporting vesicles to the presynaptic membrane, or as a tonic release of neurotransmitter-filled vesicles upon receiving stimulation(Spiwoks-Becker et al., 2008). As seen in rod photoreceptors, mammalian pineal SRs display a diurnal plasticity. I.e., pinealocyte ribbons have been shown to reversibly increase in size and number overnight, while structurally re-organize from a plate- to a horseshoe-like shape (Voorn & Vogl, 2020). The SR-associated CAZ proteins also exhibit diurnal differences: Munc13-1 and the structural proteins Bassoon and Piccolo co-localize with RIBEYE to a greater extent at night. This higher colocalization of the kinesin KIF3A at one specific end of the pineal ribbon suggests a transport of SR material – possibly to re-establish the ribbon – explaining the reversible decrease in size, or loss of material, during daytime. Here, high rates of SR material transport during the night may lead to the accumulation of KIF3A in the morning, which is then slowly

removed during the course of the day. As the pineal SRs proved to contain glutamate possibly paracrinally inhibiting the NAergic melatonin synthesis during daytime (Redecker & Veh, 1994), the diurnal plasticity of pineal SRs would likely be related to glutamate-containing SVs released during the day and replenished over the night in the absence of photic stimuli.

Once placed under total darkness, the experimental animals kept on showing rhythmic changes. This suggests that the changes in the CAZ proteins are driven by the circadian metronome within the suprachiasmatic nucleus (SCN) of the hypothalamus and mediated by the release of noradrenaline in the pineal gland into the dark phase. Since exposure to constant photic stimulation abolishes the diurnal rhythm of noradrenalin release (Korf et al., 1998), it is not surprising that Spiwoks-Becker's research noticed the abolishment of the CAZ protein rhythm under constant light in pinealocytes. The details of this extrinsic pathway of the pineal gland are later discussed into more details in **chapter 2** of this thesis. Since the secretory and electrical activity of the pineal is lower at day than night (Korf et al., 1998), the SR-associated proteins changes showed in Spiwoks-Becker's research corroborate the high significance of pineal SRs in the pineal functions, including melatonin secretion.

The SRs found in the pineal gland of vertebrates, and especially mammals, resemble in appearance and in constitution to the already well-documented SRs of the mammalian retinal photoreceptors. We also can notice the diurnal morphologic plasticity of those ribbons, which mirrors the diurnal rhythmicity of melatonin production and secretion by the pinealocytes. The exact importance and relation of said ribbons to melatonin release remains yet unclarified. By comparing the observable structures of pineal SRs under electron microscopy and their molecular composition via immunohistochemistry yet allows us to extrapolate. It seems likely that pineal SRs do possess a RIBEYE scaffold on which a set of proteins allows for the replenishment and exocytosis of glutamatergic SVs. This exocytosis is permitted, inter alia, by the presence of calcium channels as well as proteins like Munc13-1, which is known to be absolutely required for synaptic vesicle priming (Augustin et al., 1999). Detecting conventional CAZ proteins in the pinealocytes like synaptic vesicle glycoprotein 2 (SV2) – known for their role in stabilizing the transmitter content of vesicles, orienting the releasable pool of SVs, but also in regulating calcium sensitivity

to ensure the efficient, coordinated release of the transmitter (Stout et al., 2019) – further corroborates this extrapolation.

The role of pineal SRs in the melatonin production is yet obscure. For this reason, we discuss directions for further research and experiments in **Chapter 4-B**. Such groundwork could be based on the use of mutants. Specifically, artificially mutated laboratory animals for specific pineal SR proteins. As aforementioned, specific protein mutants of Munc13-1, SV2 or KIF3A could enable a study to focus on whether said-mutants can still produce normal levels of melatonin. The problem we encounter with such a method is the lack of exclusive specificity of those proteins to the pineal SRs. Being present in other organs – more specifically in other neuronal SRs necessary for the normal functions of the animal's physiologic and endocrinologic systems – creating mutants with malfunctioning proteins could as well hinder other mechanisms in the animal's regulation systems. We can however imagine an experiment with explanted pineal gland tissue cultured in vitro from a mutant animal or embryo, where the levels of melatonin production in the culture media could be measured and compared to non-mutant tissue samples. Such an experiment could further clarify the role of pineal SR proteins in the modulation of melatonin production and secretion.

Chapter 2: EXTRINSIC pathway – from retinal photic stimulation to pineal melatonin regulation

A- Discovery of melatonin, its synthesis and circadian rhythm influence

The melatonin was first identified from bovine pineal extracts in 1958. This research was based on lightening effects observed on the skin cells of frogs, toads, and fish. That research suggested that the isolated pineal protein be henceforth termed as 'melatonin', which semantically means 'skin whitening' (Lerner et al., 1958). It was only later ascertained – with research on mammalian cells – that melatonin in fact does have an opposite effect on the skin of most mammals than it does in batrachians and fish, causing a mild darkening of the skin by stimulating skin's melanocytes.

Melatonin is a hormone produced in various cells, such as peripheral reproductive organs including the oocyte, granulosa cells, and the cumulus oophorus (Tamura et al., 2009). These cells' production along with the blood may contribute to the accumulation of melatonin into the follicular

fluid, which proved to show in levels higher than those present in the blood (Reiter et al., 2014). Aside from these secondary sites of production, melatonin is mostly synthetized and secreted by the pineal gland. Its biosynthesis is nowadays well-known (Hossain et al., 2019), where the amino acid tryptophan is converted to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase. A decarboxylase then converts it into 5-hydroxytryptamine (5HT or 'serotonin'). In darkness – e.g., during nighttime – the enzyme AA-NAT (arylalkyl N-acetyl transferase) converts 5HT to N-acetylserotonin, in turn converted by HIOMT (hydroxyindole-O-methyltransferase) into melatonin (Figure 4).

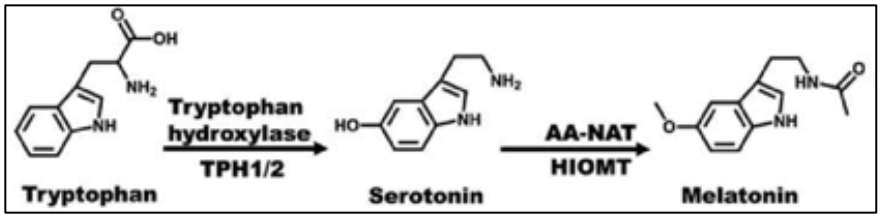

Figure 4: Simplified biosynthesis of melatonin

Since the discovery of melatonin, it has been acknowledged that its blood and urinary levels fluctuate during the day, peaking at darkness/sleep-time and troughing at daytime. Moreover, the circulating blood levels and the urinary content of melatonin are remarkably reflecting one another, allowing us to monitor an individual's melatonin secretion by either blood and/or urine samples (Lynch et al., 1978). The fluctuation of circulating melatonin during the day suggests either a neural/hormonal connection of the pineal gland to the inner body clock – which resides mostly in the central mammalian oscillator, the suprachiasmatic nucleus (SCN) – or a direct influence of environmental stimuli perception by the pineal gland itself.

Concerning the hypothesis of a direct, endogenous, perception of environmental stimuli such as light by the pineal gland, we already discussed this matter in **Chapter 1**. Though the research so far were mainly performed on murine species, we concluded that high order mammals such as ewes and rams are likely to dispose of pineal SRs functioning as photoreceptors in neonatal periods of their existence – i.e., before sympathetic innervation of the pineal gland. This early capacity of pineal SRs might enable the newborn lamb to already have a rhythmic production and secretion of melatonin, until fully functional regulatory systems towards the pineal gland can develop. As the development of the neonate continues, the SRs of pinealocytes are retained, though will later play

a different role in the modulation of pineal functions – enabling a communication of signals among neighboring pinealocytes via neurotransmitters vesicular release – thereby synchronizing the secretory functions of pinealocytes in a paracrine manner.

On the other hand, the connection of the pineal gland to the inner circadian oscillators is termed as "extrinsic pathway" and is discussed in **Chapter 2-B**. Though the melatonin output did not directly reflect the rhythm of internal clock, it was indeed associated with it (Y. Li, 2016).

B- Extrinsic pathway: environmental photic stimulation to the modulation of melatonin secretion

The retinal photoreceptors sense the environmental light stimuli and send the perceived information to the rest of the brain via retinal ganglia. While most of the photic information received by the retina is transmitted to image-forming areas of the brain via the optic nerves, a small portion of retinal ganglia contain melanopsin and have intrinsic photoreceptor capability that send the neural signals to non-image processing areas of the brain (including the pineal gland throughout complex neuronal pathways). This photic information perceived by the retinal photoreceptor cells is partially sent to the suprachiasmatic nucleus (SCN), the inner "clock" of mammalian physiology. The SCN consist of a paired nucleus structure located in the ventral hypothalamus, which each half containing about 10,000 neurons in mice and about 500,000 in humans. From this point, a network of GABAergic projections from the SCN to the hypothalamic paraventricular nucleus (PVN) relay the information, which is then transmitted to the intermediolateral nuclei of the spinal cord (located in the so-called intermediate zone between the dorsal and ventral horns of the spinal cord), connecting to the superior cervical ganglion (SCG). The following ascending signal consisting of noradrenergic (NAergic) sympathetic fibers from the SCG to the pineal gland regulates the production of melatonin by this latter gland (Figure 5). Interestingly, the NAergic innervation of the pineal gland is believed to play a role in the human Alzheimer's disease (Jengeleski et al., 1989).

The pathway by which SCN regulates melatonin production in mammals is well defined. In case of a positive light stimulus onto the retinal photoreceptive cells, the SCN starts secreting

gamma-aminobutyric acid (GABA) which is responsible for the inhibition of the synaptic neurons in the PVN. Consequently, the signal towards the SCG, and thereupon the signal towards the pineal gland is interrupted, effectively inhibiting the noradrenaline (norepinephrine, NA) stimulation of the pineal gland by the SCG. So, in the case of light stimuli received by the retina, the pineal gland stops producing melatonin. On the other hand, in the absence of light (nighttime, closed farm animal stable in intensive production methods…), the SCN produces glutamate, responsible for the stimulation of the PVN. The paraventricular nucleus conveys information along the intermediolateral column of the spinal cord towards the SCG that will now be able to transmit the final signal to the pineal gland through sympathetic postsynaptic fibers by releasing noradrenaline. This NAergic stimulation causes the pinealocytes to produce melatonin by activating the transcription of the mRNA encoding the first enzyme partaking in the melatonin synthesis, AA-NAT (Aulinas, 2000).

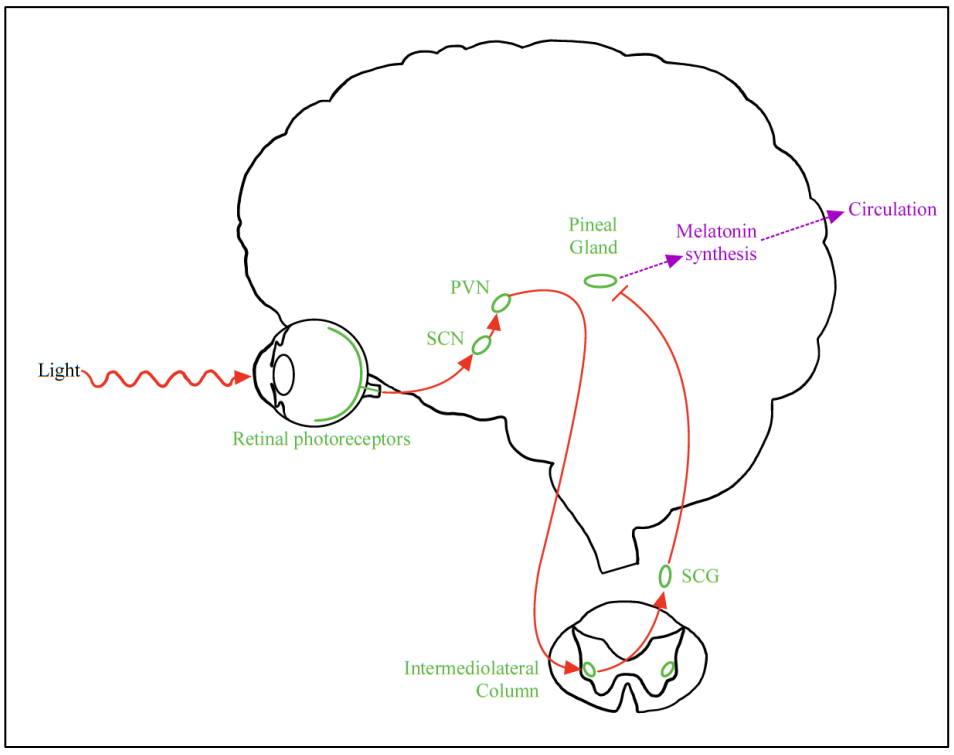

Figure 5: Schematic of the Pineal gland extrinsic pathway (adapted from Ostrin, 2019) – *Inhibition of the pineal melatonin synthesis under environmental light stimuli. SCN, Suprachiasmatic nucleus; PVN, Paraventricular nucleus; SCG, Superior cervical ganglion.*

Light exposure is the most important factor governing the pineal gland's function as a melatonin producing gland. The light exposure can be determined by the intensity and the duration

of the stimulus received by the retina. A suitable light exposure in constant darkness, of sufficient duration and intensity, can be enough to phase shift and resynchronize the melatonin rhythm. As we mentioned earlier, the retinal ganglia leading to the image-forming areas of the brain, and others leading to non-image processing areas of the brain are anatomically and physiologically distinct from each other. Studies have researched the fact that blind subjects may retain an intact retinohypothalamic pathway, enabling them to exhibit normal physiological melatonin rhythm despite a lack of conscious light perception (Czeisler et al., 1995).

An intact innervation of the retina towards the pineal gland is required in order to preserve this rhythmicity. For instance, if any damage was to occur in the retinohypothalamic pathway – especially in the SCN –, a damped rhythmicity is to be expected, though studies showed that a return to normal rhythmicity can be achieved under experimental conditions. According to numerous studies performed on rats, mice, hamsters and cats, a damage or destruction of the SCN results logically in the abolishment of circadian rhythm, rendering the animals insensitive to various light exposures (Silver & Moore, 1998). Silver's earlier research in 1987 also demonstrated that a SCN transplantation – in an animal who's SCN was destroyed, and between animals of the same specie – could reestablish an arrhythmic animal back to a normal circadian rhythmicity. In 1988, Ralph and Menaker further demonstrated that this restored rhythmicity came rather from the donor's SCN than the host itself when they included tau-mutant hamster in the transplantation experiments (Ralph & Menaker, 1988). This 'tau' mutation was found in the golden hamster where a double homozygote carrier of this mutation has its circadian rhythm reduced to 20 hours, as opposed to the usual 24 hours. The secretion of melatonin has been proved to also be reliant on intact noradrenergic innervation. Any damage to the NAergic axons of the SCG leading to the dorsal pole of the pineal gland would once again abolish the circadian rhythmicity. It was nevertheless experimentally established that continuous daily administration of noradrenaline recovers damped melatonin rhythm in constant darkness (Y. Li, 2016).

As to comprehend why a damage to the SCN results in a damped rhythmicity of the individual – that can only be recovered by a full transplantation of a donor's SCN – it is worth briefly describing the composition of the SCN. The SCN is located right above the optic chiasma on the lower part of the third cerebral ventricle and consists of 2 subdivisions respectively called the ventrolateral "core" and the dorsomedial "shell". This distinction of the two parts is related to the high expression of vasoactive intestinal peptide (VIP) in the ventrolateral core, and high expression

of arginine-vasopressin (AVP) in the dorsomedial shell. The complex composition of the neurotransmitters in both parts also differs: on one side, the SCN shell neurons contain γ-amminobutyric acid (GABA), calbindin (CALB), AVP, met-enkephalin (mENK) and angiotensin II (AII) and receive input from galanin (GAL) and VIP immunoreactive fibers. On the other hand, the SCN core neurons synthetize GABA, CALB, VIP, calretinin (CALR), gastrin releasing peptide (GRP), and neurotensin (NT), and receive input from the retina and from fibers that contain neuropeptide Y (NPY) and 5HT (Abrahamson & Moore, 2001). The neurons in the SCN differ in their pace making abilities, neurotransmitter expressions and rhythmicity. The heterogenicity of neuron cells in the SCN yet functions wholly as the circuit that can even make up the loss of rhythm in a single cell caused by 'clock gene' mutation (Ko et al., 2010). This complex physiological composition of the SCN is the reason why only a full transplantation can recover a damped circadian rhythmicity following damage to the ventral hypothalamus.

Chapter 3: Melatonin functions and relation with reproduction

A- The functions of melatonin onto the ovine reproductive system

As we mentioned it before, melatonin is primarily secreted by the pineal gland and its functions are various, the most eminent one being that of mediating physiological responses to seasonality changes and especially that of the reproductive system (Davis, 1997). By receiving environmental photic information through an extrinsic neural pathway, the pineal gland is able to adjust its melatonin production and secretion depending on the ratio of daylight to darkness. This ratio can be specified into two categories: long days (LD) and short days (SD), respectively a ratio of long days-short nights and short days-long nights. Some vertebrate animals are considered seasonal breeders, such as ewes or horses, meaning that their reproduction is only optimal at a specific time of the year. Specifically, sheep are referred to as polyestrus SD-breeders, while horses are referred to as LD-breeder. In the case of sheep, being SD-breeders implies that their melatonin production is more extensive, and that they start showing signs of estrus, in autumn, in days of decreasing daylight. Their 152 days pregnancy occurs during winter and their lambing during spring, with an anestrus phase in the summer. Most studies of melatonin effects on reproduction were performed on rats, mice, or hamsters. These murine species however are continuous breeders as opposed to

seasonal ones. In the case of murine species specifically, their all-year-long polyestrus does not function in the same manner of a sheep's. A second early discovered function of melatonin takes part in the development of the embryos by communicating information about photoperiod and thereby adaptively regulating reproductive development, while also entraining the developing circadian rhythms of the developing offspring (Davis, 1997). The cyclic levels of melatonin in the blood pass through the placenta and aid in the organization of the fetal SCN. In the absence of this synchronizing effect, the offspring may exhibit neurobehavioral deficits (Reiter et al., 2014). The first major reproductive effect of melatonin is then an entrainment of rhythmicity in the ewe and in the fetuses. As seasonal breeders, melatonin participates in the development and establishment of circadian rhythms, which regulate the ovine physiological reproductive systems.

Regarding the role of melatonin in helping the development of fetal cells in prenatal and neonatal life, studies on in vitro maturation of porcine oocytes and their parthenogenetic embryonic development assessed that even low concentration of melatonin in the culture media improved in vitro fertilization and early embryo development, along with increased fertilization rate in a concentration-dependent manner (Shi et al., 2009). The use of melatonin in maturation culture media caused significantly higher rates in blastocyst formation (21 ± 1.5 %). The supplementation of about 10 % follicular fluid – which is rich in melatonin (Reiter et al., 2014) – in the maturation media would benefit the oocytes' in vitro maturation (IVM). Though this research was based on porcine oocytes, we may extrapolate a similar role of melatonin on ovine oocytes maturation. Therefore, melatonin may also play a role in the maturation of embryonic cells in sheep.

Melatonin is also known to possess a role of antioxidant and free radical scavenger. Free radicals can be any molecule containing an unpaired electron in an atomic orbital. The most significant type in mammals are the oxygen free radicals, or reactive oxygen species (ROS). ROS are considered to play an important role in degenerative changes and harmful effects such as carcinogenesis, neurodegeneration (Hardeland et al., 1993) or ageing of the cells. The ageing of cells, and thereby of organisms, results inevitably in decreased reproductive functions inter alia. By the year 1997, research carried out on male and female rats concluded that the night administration of melatonin in drinking water prolongs survival of the mice from 23.8 to 28.1 months in average. They also reported that a melatonin administration in aged individuals induced a 18 % increase in the concentration of mRNA encoding gonadotropin releasing hormone (GnRH),

stating that it had noticeably 'reversed the influence of ageing' onto reproductive functions (S. Li et al., 1997).

ROS are also produced in the ovarian follicles, especially during the ovulatory process (Agarwal et al., 2005; Tamura et al., 2013). As mentioned in **Chapter 2-A**, melatonin tends to accumulate in higher concentrations in the follicular fluid than in circulating blood. Studies demonstrated that melatonin treatment in infertile women increases intra-follicular melatonin levels, thereby reducing oxidative damage, even elevating fertilization, and pregnancy rates (Tamura et al., 2013). The great ability of melatonin as an antioxidant does not only rely on itself, but also it the capacity of its metabolites acting as direct free radical scavengers, such as N-acetyl-5-methoxykynunemanine. Moreover, research proved that melatonin may be considered as an indirect antioxidant by upregulating the gene expression of other antioxidant enzymes like glutathione peroxidase (GSH) and superoxide dismutase (SOD) (Hirata et al., 1974; Tan et al., 2005). The direct and indirect antioxidant capability of melatonin constitutes another major role of melatonin onto the ovine reproductive system, as it ensures persistent healthy reproductive cells (notably granulosa cells, cumulus oophorus, oocytes etc.), allowing for a longer reproductive capacity throughout the years. This antioxidant capability is also, inter alia, responsible for the protection of the developing embryos against ROS, corroborating our hypothesis that follicular melatonin improves oocyte maturation and early embryonic development.

B- Effects of sex hormones – especially estrogen – on melatonin production

Experimental evidence over the past decades indicates that the electrical activity of pinealocytes in mammals can be directly influenced by several hormones of the reproductive system. More specifically, in pregnant animals we can expect a general stimulatory effect on the pineal melatonin secretion by estrogen (estrone, E1) which increases the expression of the HIOMT enzyme, while expecting an overall opposite inhibitory effect by progesterone, testosterone, prolactin or human choriogonadotropin (hCG). On the other hand, in male mammals, we observe a general inhibitory effect caused by estrogen, progesterone, prolactin. The effect of testosterone varies, as the predominant response to a testosterone exposure early in the day results in inhibition, while the exposure of pinealocytes to testosterone in the late afternoon and evening results in an

overall excitation (Semm et al., 1981). These results suggest that mammalian pinealocytes in relation to reproductive systems are under feedback control.

More than a modulation in enzymatic expression, estrogen experimentations have hinted for its direct effect onto pineal nuclear estrogen receptors. Further in-depth studies will yet be required to elucidate whether estrogen acts by modulating the synthesis of α_1- and β-adrenoreceptors, the expression of proteins – enzymes like HIOMT, or other proteins capable of inducing adrenoreceptor signal –, or both (Hernández-Díaz et al., 2001). In 2004, an experimental study corroborated the idea of estrogen directly modulating the pineal sensitivity to adrenergic stimulation in a dose-dependent manner. Namely, it was obsberved that one of estrogen's final target is the pineal β-adrenoreceptor, where the low or high dose of estrogen (estradiol, E2) would respectively inhibit or stimulate the pinealocytes' sensitivity to adrenergic stimulation – in in-vitro as well as in in-vivo situation – (Sànchez et al., 2004). Since we can observe a higher blood estrogen concentration in the active phase of a female's reproductive cycle and during pregnancy, we can speculate that the high circulating estrogen upregulates the maternal ability to produce and secrete melatonin by sensitizing it to adrenergic stimulation of the SCG, which will inherently help in fertilization of the oocyte and in the fetal development. The experiments performed on murine species over the past two decades allow us to hypothesize the existence of complex feedback mechanisms between the mammalian reproductive systems and the pineal melatonin production.

C- Application of exogenous melatonin on ovine animals: optimization of reproductivity

Now that we have mentioned the different roles of melatonin in relation to reproductive systems, as well as the effects of the latter systems onto the pineal melatonin secretion itself, we will discuss the possible future protocols aiming at increasing ovine reproductivity. The purpose of increasing reproductivity can be to ensure the preservation of a pure breed where reproduction rates are considered suboptimal, or also to increase the benefits in a farm selling animal products such as meat, milk, or wool. The question here is to consider if melatonin could be used for its antioxidant and tissue development promoter properties, to improve both follicular development and oocyte quality in sheep superovulation. Superovulation is an important tool in assisted

reproductive technology, usually consisting in injecting porcine follicle stimulating hormone (FSH) twice daily for three to five days, aside from using intrauterine progesterone sponges. Such protocol stimulates the ovine ovaries to produce more viable and recoverable oocytes, allowing us to perform embryo-transfers (ETs), laparoscopic inseminations, or in vitro fertilization (IVF) in purebred sheep or goats. It has been experimentally proved that it is still possible to produce and recover a good number of transferable embryos during natural estrus while avoiding the use of progesterone sponges. This allows for a lower labour and cheaper protocol of superovulation (Mayorga et al., 2011). Though it is already efficient in bovine species, the use of a single FSH injection in degradable polymers – allowing for a sustained hormone release over time, thereby reducing the necessary labour of such protocols – for ovine animals has yet to be commercially produced (Panyaboriban et al., 2018).

As we discussed it in **Chapter 3-A**, melatonin acts directly on the ovary (Tamura et al., 2009) and is also a good free radical scavenger – along with its metabolites – in the ovarian follicle (Tamura et al., 2013) helping in follicular development and reversing the ageing effects (S. Li et al., 1997). Experiments on anestrus ewes – over summer, lasting from March to May as mentioned in **Chapter 3-A** – were directed by giving melatonin implants in addition to controlled internal drug release device with follicle stimulating hormone (FSH) during anestrus. The administration of melatonin in conjunction with FSH during seasonal anestrus proved to have no visible reliable effect on the number of antral follicles, nor on the number of recovered and healthy oocytes intended for IVF (Luther et al., 2005). However, later reproductive ovine studies determined that melatonin implants – administered in March on selected high-prolificacy Rasa Aragonesa aged ewes – can improve the embryos viability after superovulation during the seasonal anestrus period at medium term, i.e., 3 months after implantation (Forcada et al., 2006). Melatonin treatments can elevate fertilization and pregnancy rates in females with fertility disorders, while also contributing to oocyte maturation, embryo development and possibly in the luteinization of granulosa cells (Tamura et al., 2013).

The in vitro application of melatonin to the culture medium has proved to increase the percentage of hatched blastocysts, while reducing the percentage of degenerating embryos by improving the progesterone secretion of the corpus luteum, helping in maintaining the pregnancy after IVF (Abecia et al., 2002).

The reproductivity of ewes in a farm can also be synchronized through the introduction of a ram. Rams produce pheromones which are perceived by the ewes via the "flehmen response", where the animal curls back its upper lip, exposing its gums that can perceive said pheromones and send a signal to the vomeronasal organ. This detection of male pheromones by non-cycling ewes – referred to as "ram effect" – stimulates an ovulation upon introducing a novel ram. Research showed that the use of melatonin implants in non-cyclic ewes – around early March – increases the proportion of entrainment of estrus cyclicity in a flock upon introduction of a novel ram – about a month after implantation, in mid-April – compared to non-melatonin treated females. Moreover, the use of melatonin treatment also seemed to hasten the appearance of first estrus, as well as the time of mating and lambing. Though treated and non-treated ewes did not differ significantly in fertility and fecundity, the use of melatonin implants proved to modify the ovarian response of ewes to the ram effect. We can conclude that melatonin treatments reliably modify mating patterns, and consequently, the lambing curve, which can prove to be of certain use in the out-of-season reproduction management of ovine farms (Abecia et al., 2006). Considering that melatonin treatment in breeding programs of anestrus ewes can be useful, further research tried to determine whether melatonin could also improve the ram's performance. Research with Rasa Aragonesa sheep consisted in exposing rams to two months of long days (LD, 16 hours of light/day of sufficient intensity >300 lux) – between 1 Feb and 31 Mar – followed by a return to natural photoperiod along with receiving three subcutaneous melatonin implants. This LD-protocol ensured that the treated rams were induced into a sexually active state, while the combination of artificial photoperiod with melatonin implants significantly increases the testosterone secretion of rams in spring. Interestingly, the ewes exposed to the treated rams had a higher proportion of lambing compared to the ewes exposed to untreated rams (100 % and 78 % respectively), but also proved to have a higher fecundity (1.44 ± 0.51 lambs/ewe and 1.00 ± 0.69 lambs/ewe respectively). I.e., the use of melatonin implants combined with artificial photoperiod management in rams can increase the number of ewes becoming pregnant and their fecundity (Abecia et al., 2018). While such protocol of photoperiod management and melatonin implantations in rams can be quite expensive and labour-demanding, it can prove to be useful in out-of-season reproduction management in ovine farms.

Although promising results were obtained in the past, further research of melatonin usage in ovine reproduction programs should be performed to establish a reliable protocol, where the expected results – in increased fertilization and pregnancy rates, number of recovered transferable embryos, oocyte quality, embryonic development promoter, ram effect efficiency and female fecundity – can justify the extra labour and expenses generated by a broader melatonin use.

Moreover, superovulation protocols in ovine species usually aim at increasing the number of offspring per pregnancy, through the use of reproductive hormones like the PMSG (Pregnant Mare Serum Gonadotrophin). Due to its LH- and FSH-like activities, PMSG has been used in sheep as a single dose superovulating agent in the past decade (Somanjaya et al., 2021). Though the use of PMSG produces a reliable positive effect on ovine prolificacy and reproductivity, the method of PMSG harvest poses ethical concerns. Indeed, PMSG has yet to be produced synthetically in laboratories. For this reason, the entire collection of PMSG in the world relies on the exploitation of pregnant mares in so-called 'blood farms' – primarily in Uruguay and in Argentina – which is then exported worldwide for its use in porcine, bovine, and ovine industrial farming. As this hormone is only produced in the early equine pregnancy, its collection – mainly produced from day 40 to 140 of the equine pregnancy – results in the systematic abortion of fetuses. Due to ethical and animal welfare concerns raised by these 'blood farms', it is likely that the EU may come to ban the use of this hormone in future years. Though no reliable research has been published yet in the matter of finding a replacement for PMSG, melatonin – due to its effects on fertilization and pregnancy rates, and on superovulation – may very well be a possible solution. The joint use of melatonin with gonadotropin releasing hormones (GnRHs) could prove to be a reliable alternative to the use of PMSG in ovine industrial farming.

Chapter 4: Hypothesis and experiments proposed

A- Supposed reason for the lack of experimental research in ovine species

In 1997, a research facility in Scotland, UK, managed to produce the first ever successful cloning of a higher ranked mammal using sheep's cells. The result of this transfer of an adult ovine cell into an enucleated oocyte was the cloned sheep, Dolly. This worldwide known experiment was followed by a series of new ethical and experimental regulations. As the public and medical authorities in the world commended this achievement, legislations were set to impose clear ethical boundaries for future research to come. At the time, discussions among scientific comities debated over whether there should be limitations on the study, transfer, and use of mammalian embryos. It resulted in a ban of further research on techniques deemed inacceptable such as cloning or the creation of chimeras, that is the artificial combination of two different species' genes, resulting in a viable living specimen. While research based on preimplantation genetic screening and gametes cryopreservation permitted the discovery of ideal culture and storage media for embryos, the prohibition on non-therapeutic embryo research impeded the development of potential diagnostic and treatment technologies. Some world countries fully prohibit and condemn embryo research, while others opt to diminish the possible funding of said research. A lack of consensus regarding the moral status of embryos made future research more difficult, if not sometimes unreasonable(Andrews & Elster, 1998). Due to new research legislations throughout the world since the date of 1997, a scarcity on mammalian embryonic research can primarily be noticed since that date, with a mild recurrence over the past few years. Embryonic research is yet still allowed in the world, though mainly in in vitro conditions. The use of live animals and in vivo testing proves to be quite difficult, so we must speculate the functions and actions of melatonin onto the embryonic and postnatal organisms from mostly in vitro experiments. Due to the lack of research on embryo development in higher order mammals, especially seasonal breeders such as ewes, I will propose grounds for future ovine research in the following section of this thesis.

B- Goals and grounds for future experimentations and research

The aforementioned effects of melatonin on ovine reproductive systems appear promising. However, the yet obscure role of SRs on pineal melatonin synthesis and the possible applications of melatonin to ovine reproductive technologies remain to be clarified. In this context, I will try to summarize the previously mentioned information related to pineal functions, and lay grounds for future experimental research. The following proposed experiments will be based on sheep as the animal model, since their reproductive cycle is mainly controlled by the pineal gland activity (Abecia et al., 2006; Zhang et al., 2013). Previous research in the early nineties described a creation method for in vitro pinealocytes cultures – using an ovine pineal gland suspension (Howell & Morgan, 1991) – permitting a full control over experimental conditions and offering the possibility for a wide range of tests. Firstly, in order to clarify the development of pineal SRs and their role in the modulation of melatonin synthesis, we could study their morpho-functional development in different culture media (cf. proposed experiments **I** and **II**). Secondly, further experimental research can be proposed to study the effects of melatonin on estrus, follicular development, and early embryonal development (cf. proposed experiments **III** and **IV**). A study on melatonin as a replacement to PMSG in superovulation reproductive programs in ewes may also be considered, though would necessitate to be tested on a broad ovine population and, as we mentioned it in Chapter 4-A, may pose certain application difficulties for in vivo experiments, or animal trials (cf. proposed experiment **III**).

I. Determine how different hormonal/neurotransmitters environments may modify the structure of pineal SRs <u>in vitro</u>, and protein composition of pineal SRs

The intrinsic neural modulation of pineal SRs seems to involve glutamatergic signals stimulating neighboring pinealocytes, inhibiting the NAergic melatonin synthesis (Redecker & Veh, 1994). SRs have been identified to contain glutamate with tonic release under photic stimulation, corresponding to the proposed inhibitory role of pineal SRs on melatonin production during the day (Karasek et al., 1983). Moreover, the pineal extrinsic control is also modulated by estrogen levels in a dose-dependent manner (Sànchez et al., 2004). To clarify the SRs' structure and functioning in diverse neuro-hormonal environments, we can first consider the effect of direct cell illumination (Tosini, 2000; Zimmerman & Tso, 1975), where the fluctuating levels of

glutamate could be experimentally measured. We can also consider the effect of diverse concentrations of E2, and melatonin (Houssay & Barcelo, 1972), alone or in combination. To test the impact of neurotransmitters on pineal SRs, we could consider blocking (or triggering) glutamate (McAdoo et al., 2005) and NE (Przybylska-Gornowicz et al., 2016), while assessing the changes in SRs' structure by electron microscopy (Spiwoks-Becker et al., 2008). In experimental conditions, qPCR, ELISA, and Western blot techniques could allow one to measure the levels of E2 and melatonin, while the localization of these hormones could be assessed via immunohistochemical electron microscopy. According to the already mentioned effects of hormones and neurotransmitters on pineal SRs, such an experiment could confirm (or refute) our hypotheses of E2 and direct environmental illumination causing pineal SRs to grow in size and in closer juxtaposition to mitochondria and presynaptic membrane. Moreover, this would confirm (or refute) that the addition of E2 in the culture media would enhance glutamate-SVs exocytosis and melatonin release by pineal SRs.

Grounds for future research in identifying the protein composition of pineal SRs was previously initiated in **Chapter 1-B**, with the use of mutants, and immunohistochemical localization.

II. Determine the morpho-functional development of pineal SRs in a native tissue environment, <u>ex vivo</u>

If the <u>in vitro</u> experiment on pineal SRs confirms our hypotheses, one could in a second time, work with biopsied pineal glands from ovine fetuses (da Silveira Cruz-Machado et al., 2012) due to their relatively preserved integrity and cell-to-cell interactions, in order to confirm the morpho-functional development off pineal SRs in a native tissue environment, <u>ex vivo</u>. As mentioned in **Chapter 1-A**, sporadic investigations on mammalian species suggested that pineal SRs develop paracrine cellular interactions during late prenatal to early postnatal period (Liao et al., 2004; Zimmerman & Tso, 1975). The goal of such an ex-vivo study would be to study the role of astrocytes and microglia in pineal gland formation and homeostasis (Rodriguez et al., 2016). Glial impact includes gliotransmission via calcium and ATP signaling, glia-driven morpho-functional neural maturation – in regulating precursor cells, remodeling blood vessels and sympathetic nerve fibers –, and the modulation of inflammatory processes (Villela et al., 2013). Like in the first proposed experiment, one could study the effect of direct cell illumination, as well as the effect of natural hormones like E2, while modulating the influence of astrocytes and microglia on pineal

SRs. Past research on these cells suggest the use of antimetabolites and Ca^{2+} chelation to block gliotransmission (Dessi et al., 1995), and the use of pro- and anti-inflammatory factors – e.g., TNF, IL-1ß, LPS, IL-4, etc. – (Sultani et al., 2012) to modulate the effect of microglia. Immunohistochemical and ultrastructural evaluations of prenatal and neonatal pineal SR development can be assessed on biopsied pineal tissue samples – with electron microscopy and from samples taken on ovine fetuses of different age – in consecutive developmental stages 7 and 15 days after explantation (Redondo et al., 2001; Regodon et al., 2001). Glial and microglial functions can be assessed with immunofluorescent imaging and PCR techniques (Fields & Stevens-Graham, 2002).

Such an experiment could help in clarifying the role of glial and microglial actions in pineal SRs maturation, where one could expect that amplifying inflammatory processes would suppress melatonin secretion and inhibiting them would do the opposite.

III. Clarify how melatonin acts on follicular development and fertilization <u>in vitro</u>, and estimate the viability of melatonin in superovulation protocols <u>in vivo</u>

Follicular melatonin is not only systemic (from the pineal gland) but is also synthetized directly in the ovary by the granulosa cells into the follicular fluid (FF), as mentioned in **Chapter 2-A** (Tamura et al., 2009). Due to the presence of melatonin receptors in granulosa cells – those cells being the only somatic cells interacting with the oocyte from the follicular formation until the release of the oocyte at ovulation –, we can assume that melatonin is involved in the in vivo oocyte maturation prior to ovulation (Abecia et al., 2018). The role of FF melatonin levels in oocyte maturation was already studied in human and swine species (Shi et al., 2009; Tamura et al., 2013), but is yet missing on a seasonal-breeding animal such as ewes. As an <u>in vitro</u> study of the effects of melatonin on follicular development, one could work with sheep ovaries recovered from slaughterhouses, isolating, and culturing pre-antral follicles until ovulation (the antrum being the forming cavity in the follicle around the oocyte, prior ovulation). By either adding exogenous melatonin, or using melatonin-inhibitors, during the maturation phases of the oocytes and then trying to perform an IVF with ram sperm cells, one could clarify the role of melatonin on follicular development and fertilization. A past similar research suggests considering the development rates of pre-antral follicles, antral formation, rate of ovulation and rate of fertilized embryos as experimental variables (Shi et al., 2009). As an <u>in vivo</u> study, one could collect both FF and blood

samples from cyclic and acyclic ewes. Using immunoassays (RIA or ELISA), one could measure the difference between follicular and systemic melatonin levels, prior to (and after) a hormonal treatment for superovulation with exogenous melatonin. With such an approach, one could clarify the use of exogenous melatonin in ovine reproductive strategies, or even evaluate the viability of melatonin as a replacement for the use of PMSG in superovulation protocols. (Forcada et al., 2006; Smith et al., 1988; Somanjaya et al., 2021; Zhang et al., 2013).

IV. Determine melatonin's potential on early (preimplantation) embryo development and implantation potency

Melatonin already has hinted to have a benefic effect on endometrial morphology and embryo implantation in non-photoperiodic animals such as rats (Dair et al., 2008), although very limited data exist on preimplantation effects of melatonin in ewes. By culturing in vitro ovine pre-implantation embryos (produced from IVF) in varying melatonin concentrations media, one could clarify the role of melatonin on embryo quality and developmental potency (capability) promoter. With the use of technologies like qPCR, one could measure known implantation markers of expanding blastocysts (Somoskői et al., 2020), as to clarify the role of melatonin in ovarian response to superovulatory treatment in non-Histologically, the mammalian pineal gland is composed in minority of fbreeding season (as evoked in Chapter 3-C (Abecia et al., 2006, 2018)), and its potential use for enhancing the implantation, and thereby increasing fertility and fecundity.

Conclusion

The pineal gland production and secretion of melatonin are under both intrinsic and extrinsic influence. In regard to the intrinsic modulation of pineal melatonin secretion, we discussed that SRs structures observed in pinealocytes adopt a photoreceptor-like activity in prenatal and neonatal periods of life of high order mammals. This neonatal function would allow the progeny for photoreception – ensuring circadian rhythmicity and therefore of physiological rhythms – until the full development of sympathetic innervations towards the pineal gland. These pineal SRs then develop into cellular structures capable of intrapineal paracrine functions. This intrinsic paracrine stimulation between neighboring pinealocytes is considered to rely on glutamatergic signals,

resulting in the inhibition of NAergic melatonin synthesis during the day, while under photic stimulation. Knowing previously identified proteins responsible for SRs structures and functions in organs such as the retina, we can study the pineal SRs complexes. The presence of conventional CAZ proteins – like SV2 and Munc13-1 – in pineal SRs corroborates their chemical synapse-like activity. Their presence in pinealocytes was detected using postembedding immunoelectron microscopy with molecular markers, immunohistochemistry, and Western blot. Moreover, proteins like RIBEYE, Piccolo, Bassoon and KIF3A were also detected in pinealocytes, further corroborating their ribbon-associated complexes to a similar composition to that of retinal SRs. Notable differences in anchoring proteins – like the absence of retinal-like ERC/CAST or RIMs in pinealocytes – call for further research on ovine pinealocytes as to clarify the pineal SRs' protein composition and their diurnal plasticity in relation to melatonin production. Elucidative studies in pineal SVs exocytosis remains to be done, but research so far indicates that the pineal SRs exocytosis of vesicles relies on calcium channels signaling, along with proteins like Munc13-1 and KIF3A, respectively responsible for priming and actively driving the SVs translocation to the release site.

While the pineal melatonin production is certainly modulated by intrinsic mechanisms, it also relies on extrinsic pathways. Indeed, we discussed that this hormone's secretion is partly regulated by environmental photic stimulation and is under the circadian clock's – ventral hypothalamic SCN – modulation. The enzymatic synthesis of melatonin from the amino acid tryptophan under dark conditions is inhibited at daytime by the pineal gland perceiving information from the retinal pathway stimuli. This extrinsic pathway modulating the pineal melatonin production is known to start with the perception of environmental light by the retinal rod and cone photoreceptors. This photic information is then transmitted to the hypothalamic SCN, further conveyed to the hypothalamic PVN. The hypothalamus relays this information to the intermediolateral nuclei of the spinal cord, connecting to the SCG. From this latter ganglion, NAergic fibers towards the dorsal pole of the pineal body will modulate the melatonin secretion. The physiological pineal melatonin production depends on both intact hypothalamic (SCN) and intact NAergic (SCG) innervation. Indeed, we mentioned that damage to the circadian SCN results in a damped rhythmicity of melatonin secretion, which is only recoverable under experimental conditions with a full-SCN transplantation from another healthy donor. Moreover, damage to the NAergic axons ascending

from the SCG would once again abolish the melatonin rhythmicity, though such loss of innervation seems partly compensable with daily administration of NA.

Regarding the melatonin's functions in mammals, we mentioned that its production peaks at nighttime and promotes sleep in diurnal animals, earning it the appellation of "hormone of darkness". Melatonin is also responsible for promoting embryonic cellular development – increasing the proportion of hatching blastocysts and improving fertilization rates in a dose-dependent manner – and is believed to entrain the circadian rhythmicity in the developing fetuses. Its antioxidant action, along with its metabolites, participates in "reversing the effect of ageing" of aged reproductive cells, and counters the oxidant effect of ROS in ovulatory processes. Melatonin's higher concentration in FF is thought to elevate fertilization and pregnancy rates, and also upregulates the gene expression of other antioxidant enzymes such as GSH and SOD. Additionally, the reproductive hormones of ovine animals seem to partake in the modulation of pineal functions. Indeed, the presence of ERα and ERβ in pinealocytes at cytoplasmic and nuclear levels modulates several gene expression, as well as modulating the pineal sensitivity to adrenergic stimulation in a dose-dependent manner. E2, testosterone, prolactin and progesterone all modulate the pinealocytes' functions in various ways, hinting that the reproductive systems of sheep and the pineal gland activity are under mutual feedback control. In regard to reproduction technologies and protocols used around the world to improve ovine farming productivity and economic return, the use of superovulation protocols in anestrus season – March to May in general – is widely used. The superovulation protocols – consisting of several FSH administrations along with intravaginal progesterone sponges – can be combined with the preemptive use of melatonin implants. We discussed that such combinations could reduce the need – and therefore the implied costs – for intravaginal sponges. The use of melatonin implants on anestrus ewes also seems to efficiently improve the embryo viability after superovulation, while also elevating the fertilization and pregnancy rates. Regarding the in vitro reproductive technologies such as IVM and IVF – for the purpose of ETs and laparoscopic inseminations – the application of melatonin into the cellular culture media would be beneficial, as it allows for a higher percentage of hatching blastocysts, while reducing the percentage of degenerating embryos by improving the progesterone secretion of the corpus luteum maintaining the ongoing pregnancy. Melatonin implants in anestrus ovine animals also seems to increase the efficiency of the male's ram-effect, while also increasing the ewe's receptivity to that ram-effect by exacerbating their mating patterns and fecundity. The

creation of a clearly defined reproductive protocol with administration of melatonin to anestrus ewes yet remains to be achieved through future experimentations.

Since 1998, we notice a scarcity in embryo research in ovine species. Though not fully condemned, such research would need to acquire the necessary funding. We proposed grounds for future research, aiming to clarify the structure and role of pineal SRs in developing animals. We also suggested targeted research to determine the influence of melatonin on estrus, follicular development, and pre-implantation development in ovine species. At the term of the proposed experiments, one should be able to clarify the role and mechanisms of pineal SRs in the modulation of pineal melatonin secretion. Further understanding of such cellular mechanisms would permit the creation of more efficient cellular culture media, as well as enabling the establishment of reliable reproduction protocols. I.e., one would be able to consider if melatonin can reliably be used for its antioxidant and tissue development promoter properties, in order to improve both follicular development and oocyte quality in sheep superovulation reproduction protocols.

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Értesítési e-mail címe: marissiauxjb@yahoo.fr
telefonszáma: +33601423692

Szakdolgozat címe: Pineal gland & melatonin in ewes: a review in understanding and optimizing their reproductivity

2. A TANSZÉK TÖLTI KI!

A hallgatótól a Élettani és Biokémia Tanszék részéről a mai napon átvettem

- fenti című szakdolgozatának egy bekötött példányát, melyen a hallgató és a dolgozat adatai kívülről is jól olvashatóan fel vannak tüntetve,

- diplomamunka konzultációs lapot.

TDK dolgozat esetében:

- TDK- és diplomamunka azonosságáról szóló nyilatkozatot.

Kelt Budapesten, 2022.11.18.

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átvevő

PH.