THE EFFECT OF THE BIOLOGICAL CLOCK ON THE CIRCADIAN EXPRESSION OF THE mRNA FOR THE ARYLALKILAMINE-N-ACETYLTRANSFERASE IN THE CHICKEN PINEAL GLAND AND RETINA

Ph.D. thesis

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1. Introduction

1.1. Charactheristics of the circadian rhythms

From unicellular organisms to the most developed vertebrates or plants, the phisiology of many organism changes with a daily or circadian rhythm. There is an internal biological clock behind the rhythmic changes, which is synchronized (entrained) by the rhythmically changing enviromental cues. In order to normalize differences in the length of subjective day observed among species the term circadian time (CT) has been introduced. By convention CT 0 corresponds to the subjective dawn and CT 12 corresponds to the subjective dusk. The external cues, which are responsible for synchronization of the external and internal rhythms are called Zeitgebers. If the clock and the physiological processes governed by the clock follow the changes of a given *Zeitgeber*, then the *Zeitgeber* time (ZT) should be used instead of CT.
In the case of multicellular organisms bearing organs even more than one organ may contain biological clock. The organs containing a biological clock, which can be entrained by external cues and are able to coordinate the clock of other organs and therefore are called master oscillators, whereas the organs goverened by the master oscillator are known as slave oscillators.
The biological background of the circadian rhythms is formed by a three-component circadian system. First, there is an input informing the clock about the changes of the external enviroment. The central component of the system is the biological clock known as circadian oscillator. The output is a hormonal or neural signal modulating the circadian functions or behavior of the organism.

1.2 The circadian system of the birds.

The main components of the avian circadian system are the retina, the suprachiasmatic nucleus of the hypothalamus (SCN) and pineal gland. All components of the avian circadian system have their own internal clock, which can work independently from the other clocks. The most important environmental factor, or *Zeitgeber* entraining the rhythm of the master oscillator of the birds is the light. The changes of the enviromental lighting is detected primarly by the retina. The light sensitivity of the avian hypothalamus and retina is known, as well. The SCN is informed by the reina through the retino-hypothalamic tract about the enviromental lighting conditions. The SCN modulates the pineal gland through a multisynaptic pathway. The pineal body of the birds is a follicular organ located under the calvaria and connected to the epithalamus by a stalk. The pinealocytes lining the wall of the follicles are responsible for melatonin production. The pineal gland is innervated mostly by noradrenergic fibres. The noradrenalin acts on the pinealocytes through α_2-adrenergic receptors, thus the noradrenalin exerts an inhibitory effect on these cells. The melatonin production of the avian pineal gland is regulated by the master oscillator of the pineal gland along with the environmental light.
The melatonin produced by the pineal gland acts on specific receptors in the peripheral organs and in different areas of the brain, among which the hypothalamus is the most important.

1.3. The molecular biological background of the biological clock

Our knowledge about the biological clock of the higher vertebrates arises mostly from experiments made with rodents. The positive elements of the biological clock are the mBMAL1 and mCLOCK proteins in the mouse, which belong to the family of the bHLH-PAS domain containing proteins. In rats just rBMAL1 and its mRNA show significant daily changes from the heterodimer pair. A significant change in the daily levels of rCLOCK has not been found, but the C-terminal region of the protein has a glutamine-rich domain, which allows rCLOCK to activate the transcriptional initiation complex. The mCLOCK/mBMAL1 heterodimer stimulates the synthesis of mREV-ERBα proteins and enhances the transcription of the negative components of the clock and clock controlled genes. mREV-ERBα stimulates the synthesis of mBMAL1 from the *mBmal1* gene and the transcription of the *mE4Bp4* gene with positive feed-back. The inhibitory components of the mammalian circadian oscillator are *Per, Cry* and *Tim* genes. Several isoforms of the mPER and mCRY proteins have been described so far. In contrast to its *Drosophila* ortholog the mTim does not show significant circadian rhytmicity. The role of mTIM might be the stabilization of the phosphorylated mPER. The cryptochrome proteins, encoded by *mCRY* genes are still photosensitive in simple organisms. They are able to form complex with mPER and this complex inhibits the stimulatory effect of the mBMAL1/CLOCK proteins. The core of the biological clock is formed by *mBmal1* and *mClock* also including their protein products. Other transcription factors, like mouse *D-site binding protein* and mE4Bp4 are also known as repressors and the activators of the *mPer* gene, respectively.

The biological clock of the mammals encompasses then two feed-back loops. The positive feed-back is established by BMAL1/CLOCK/ REV-ERBα and the negative one is formed by PER/CRY/TIM. The link between the two feed-back loops is the BMAL1 protein.

The clock of the birds is very similar to the mammalian clock regarding its structure, but certain details are different. The positive elements of the biological clock are the cBMAL1 and the cCLOCK proteins in the chicken too, which are similar to their mammalian orthologs regarding their function and structure. An alternative partner for heterodimer formation is cBMAL2 for the cCLOCK, but cBMAL2 inhibits in high concentrations the stimulatory effect of cCLOCK if cBMAL1 is simultaneously present. Although the expression of *cRev-erbα* mRNA has been demonstrated in birds, there are no data indicating any link between *cBmal1* and *cRev-erbα* mRNA expressions. Unlike in mammals *Tim* is not a part of the avian clock and only the *Per2* ortholog of *mPer2* has been identified so far. In addition to cBMAL1/cCLOCK the cMOP4/cBMAL1 heterodimer also stimulates, while E4BP4 homodimer inhibits the expression of the *cPer2* mRNA. All three known isoforms of the CRYs are present in birds too. *cCry4* is the most recently described clock gene, but its role has to be revealed by further studies.

Considering the informations about the biological clock we can conclude, that the biological clock of the birds can be a good model of the circadian oscillator of the vertebrates. We choose the pineal gland and the the retina for our experiments on the biological clock because their cells work as master oscillators in birds. The differences in the regulation of the melatonin synthesis between birds and mammals have to be considered during the evaluation of data obtained from experiments on birds.

1.4.1. The connection of the melatonin synthesis to the endogenous oscillator

The melatonin is an indolamine hormone. Its synthesis is regulated by the endogenous biological clock resulting in higher melatonin levels in the dark phase and lower levels in the light phase. The pineal cells are responsible for the melatonin production in the chicken, although the eyes can contribute to blood melatonin level in some avian species.

The indolamine metabolism has two major fields. The first is the synthesis of serotonin from tryptophan, the second is the conversion of serotonin to melatonin and to deaminated products. The arylalkilamine N-acetyltransferase (AA-NAT) is unique among the enzymes of the melatonin synthesis, because its activity shows robust circadian changes suggesting that this is the key enzyme of the melatonin synthesis in mammals.
The connection between the melatonin levels and AA-NAT levels or activity raises the question if the biological clock regulates the melatonin production by the AA-NAT enzyme. The master clock is located in the SCN in mammals and it regulates indirectly the enzyme activity of the AA-NAT by noradrenergic fibers reaching the pineal gland. The pinealocytes of the bird have a circadian oscillator, which can regulate directly the enzyme-activity of the AA-NAT. Because the elements of the circadian clock are transcription factors, there is a theoretical possibility, that the transcription of the *Aa-nat* gene could be regulated directly by the clock. In other words the *Aa-nat* gene could be regarded as a clock-controlled gene (*Ccg*).

2. Objectives:

1. It has been demonstrated in mammals, that the *Bmal1* and *Clock* genes play a pivotal role in the nocturnal increase of the AA-NAT activity, but limited data are available about the expression pattern of these genes in birds under light-dark photoperiods corresponding to the external lighting. Thus, we investigated in the first part of the present work the circadian expression pattern of these genes taking samples in every second hour, which gives a much better time-resolution than it has been described before.
2. Our experiments should answer the question if there is any difference in the circadian expression pattern of the *cBmal1, cClock* and *cAa-nat* genes in the retina and the pineal gland of the same animal under lighting regimens.
3. We wanted to demonstrate the role of the circadian expressed genes in the melatonin synthesis *in vitro*, depending on the results of the *in vivo* experiments. The *cAa-nat* gene has a crucial role in the melatonin synthesis and it is also regarded as a *Ccg*. Thus, we developed first a superfusion system, in which we could study the changes in the expression of the *cAa-nat* gene and the consequential changes in the melatonon secretion by the inhibition of the clock gene function. The elimination of the clock gene function could be achieved by the use of antisense oligonucleotides containing locked nucleic acid (AONs containing LNA).

3. Animals and methods

3.1. Chemicals

If it is not stated otherwise we obtained all the chemicals from Sigma-Aldrich Kft.

3.2. AON containing LNA

Two antisense LNA containing oligonucleotides complementary to the 5'-region of *cBmal1* mRNA encompassing the start codon were designed and synthesized by Proligo (Boulder, CO) to induce RNase H activation (LNA/DNA/LNA gapmer) or to block the binding of the translation machinery (LNA/DNA mixmer). Inverse AON containing LNA and MQ water were used as controls.

3.3 Animals

Three-week-old-male White leghorn chicken (*Gallus domesticus*) were obtained from a local hatchery (Remenypuszta, Hungary) and housed for 2 weeks on a 14-h light: 10-h dark cycle (LD 14/10; lights on from ZT0 to ZT 14) corresponding to the actual seasonal lighting. For *in vivo* experiments three animals were sacrificed by decapitation in LD every 2 h beginning at the fifteenth 24-h period. Retinas and pineal gland of each animal were immediately removed and stored in TRI Reagent (Sigma Chemical Co., St. Louis, MO) under -70 °C. For each superfusion experiment four animals were sacrificed by decapitation in LD at ZT 5. Animal housing, care and experimental protocols meet the guidelines of European Communities Council Directive of 24 November 1986 (86/609/EEC).

3.4. Transfection in the superfusion system

The superfusion of dissociated pinealocytes was performed as described earlier *(Rekasi et al., 1991.)* with some modifications. After a 24-h recovery, the culture medium was changed to OPTI-MEM I medium without antibiotics and BSA at ZT 6 for 60 min, then the pineal cells were transfected for 4 h between ZT 7 and ZT 10 using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Oligonucleotides and transfection reagents were diluted separately into equal volumes (1 mL each) of OPTI-MEM I medium and mixed briefly. The dynamic cell cultures were exposed to a daily lighting regime of LD 14/10 with light onset at ZT 0, and this was maintained until the end of the transfection experiments. The cells were lysed by the infusion of 1 mL TRI Reagent (Sigma) at the indicated time points every 4 h beginning at the second LD cycle or at the end of the transfection experiments (ZT 17 in the fourth LD cycle). The homogenous lysate was collected into sterile Eppendorf tubes and stored at -70 °C until the subsequent isolation of total RNA as reported *(Rekasi and Czompoly, 2002)*. Each experimental protocol was performed in three superfusion experiments. Measurement of lactate dehydrogenase activity released from the cytosol of damaged cells into the medium was also performed by Cytotoxicity Detection Kit (Roche, Mannheim Germany) according to the manufacturer's instructions to test the possible non-specific cytotoxic effect of the transfection reagents.

3.5. Quantitative analysis of mRNA levels

The changes in the gene expressions were detected by a reverse-transcriptase polymerase chain reaction (RT-PCR) as it has been described *(Toller et al., 2006).*

3.6. Melatonin RIA

Melatonin content from the collected superfusion fluid (200 µl sample size) was assayed with RIA developed in our laboratory *(Rekasi et al., 1991)*.

4. Results

4.1. Temporal profile of retinal and pineal cBmal1, cClock and cAa-nat mRNA expressions in vivo

Temporal changes in *cBmal1*, *cClock* and *cAa-nat* mRNA levels were examined by quantitative RT-PCR analysis of the retinal photoreceptor cells or pinealocytes, which were obtained from the same chicken kept in 14/10 h LD cycle for 2 week *(Figure 1).* All three gene transcripts displayed daily fluctuations in both tissues. *cBmal1* mRNA showed a peak during the light

phase at ZT 8 and ZT 10 in the pineal gland and in the retina, respectively, preceding the amplitude of the nocturnal increase in *cAa-nat* expression at ZT 16 in both tissues. Pineal *cBmal1* and *cAa-nat* mRNAs exhibited an approximately 11- and 19-fold more robust cycling, respectively, than their corresponding retinal transcripts in the same animal. The peak values of *cBmal1* mRNA contents declined more rapidly in the retina than in the pineal gland. In contrast, retinal and pineal *cClock* mRNA levels failed to exhibit well detectable rhythm. They showed only a small variation with low amplitude between ZT 8 and ZT 10, furthermore between ZT 16 and ZT 18. These peaks were coincident with the peaks of *cBmal1* and *cAa-nat* expression both in the retina and in the pineal gland.

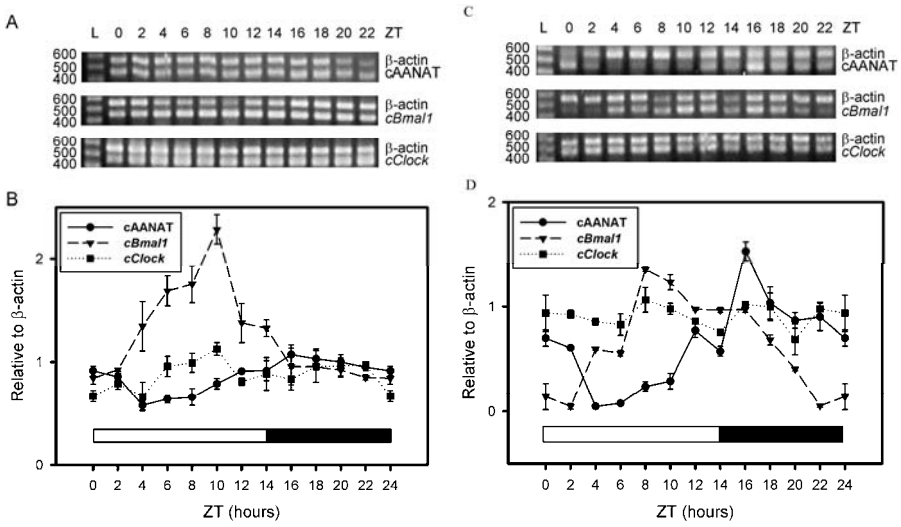

Figure 1. Temporal expression of retinal *cAa-nat*, *cBmal1* and *cClock* mRNAs under a lighting regimen of 14/10 LD, *in vivo*. Chicken were housed in 14/10 LD cycle for two weeks before the experiments. Light was on at Zeitgeber time /ZT/ 0, from this point on tissues were collected at 2-h intervals from 3 animals for each time point. Representative RT-PCR analysis of *cAa-nat*, *cBmal1* and *cClock* mRNAs from retina (A) and pineal gland (C). L: 100-bp ladder. Relative levels of *cAa-nat*, *cBmal1* and *cClock* transcripts in chicken retina (B) and pineal gland (D). Transcripts levels were normalized to the amount of β-actin mRNA in each sample. Each time point represents the mean ± S.E.M. transcript level that was determined from three different groups of animals, each of which assayed in triplicate. The ZT 24 value represents a replotting of the ZT 0 value. The white and black bars in the figure represent light and dark phases, respectively.

4.2. Temporal profile of retinal and pineal cBmal1, cClock and cAa-nat mRNA expressions in vitro

Chicken pinealocytes or retinal cells were cultured for 4 days under 14/10 h LD in a superfusion system, and melatonin secretion from pinealocytes was measured by RIA. The sensitivity of the RIA assay was not high enough to detect the melatonin concentrations from retina in superfusion system. In contrast, from pinealocytes a detectable fluctuation of melatonin level could be already observed in the first LD cycle, however, the first amplitude of melatonin secretion was significantly lower and phase-delayed than the peak values of the subsequent LD cycles. Peak of melatonin secretion was completely entrained to the dark phase in the fourth LD cycle, therefore total RNA for RT-PCR analysis was extracted from both chicken tissues only after the third LD cycle to be comparable with *in vivo* samples.
The retina and pineal gland were found to express robust rhythms of *cBmal1* and *cAa-nat* mRNAs. Peak values of *cBmal1* transcript in retina and pineal gland occur at approximately ZT 9 in LD preceding the amplitude of the nocturnal increase in *cAa-nat* expression at ZT 17 in both tissues. Amplitudes of the *cBmal1* mRNA are 1.7- and 3.4- fold in the retina and pineal gland, respectively, whereas the *cAa-nat* mRNA rhythm amplitudes are 2.4- and 4.6-fold, respectively, in the two tissues.
Neither retinal nor pineal *cClock* mRNA exhibit robust rhythm in LD. However, cycling of *cClock* mRNA cannot be ruled out as there was a small amplitude around 35 %, and 44% in mRNA expression, with apparent levels peaking at ZT 9.

4.3. Circadian expression pattern of cBmal1 and cAa-nat transcripts and diurnal melatonin production in chicken pinealocytes in the superfusion system

These superfusion experiments with chicken pinealocytes were designed similarly to those described under the section 4.2.. The first amplitude of melatonin secretion was significantly lower and phase-delayed than the peak values of the subsequent LD cycles in accordance with the results of the previous experiments. In order to determine the optimal time interval for the transfection with AONs containing LNA *cBmal1* and *cAa-nat* mRNA levels were measured in the second LD cycle between ZT2 and 22 with 4 hour intervals. The pinealocytes are found to express robust rhythms of *cBmal1* and *cAa-nat* mRNAs in the second LD cycle with peaks at ZT14 and ZT 18, respectively. Amplitudes of the mRNA expression of *cBmal1* and *cAa-nat* are 14.6-fold and 2.9-fold, respectively. Based on this observation the transfection of dissociated pinealocytes with AONs containing LNA was performed between ZT 7 and ZT 10 in the second LD cycle of the subsequent experiments. The cells were then lysed by the infusion of TRI Reagent 55 h after the transfection (at ZT 17 in the fourth LD cycle) at which time point the level of both examined mRNAs is quite high in the control experiments.

4.4 Melatonin secretion from chicken pinealocytes transfected with cBmal1 AONs containing LNA in superfusion system

Chicken pinealocytes were transfected in the superfusion system between ZT 7 and ZT 10 of the second LD cycle with *cBmal1* AONs containing LNA either inducing RNase H activation (LNA/DNA/LNA gapmer) or blocking the binding of the translation machinery (LNA/DNA mixmer, *Figure 2*). The inhibitory effect of AONs containing LNA was manifested only in the dark phase of the third LD cycle by which time both LNA/DNA/LNA gapmer and LNA/DNA mixmer almost completely abolished melatonin secretion. The significant inhibitory effect of LNA/DNA/LNA gapmer and LNA/DNA mixmer was persistent until the end of the experiments (ZT 17 of fourth LD cycle). In contrast, the inverse LNA serving as the control for the AONs containing LNA or MQ water which was used as a solvent had only a negligible effect.

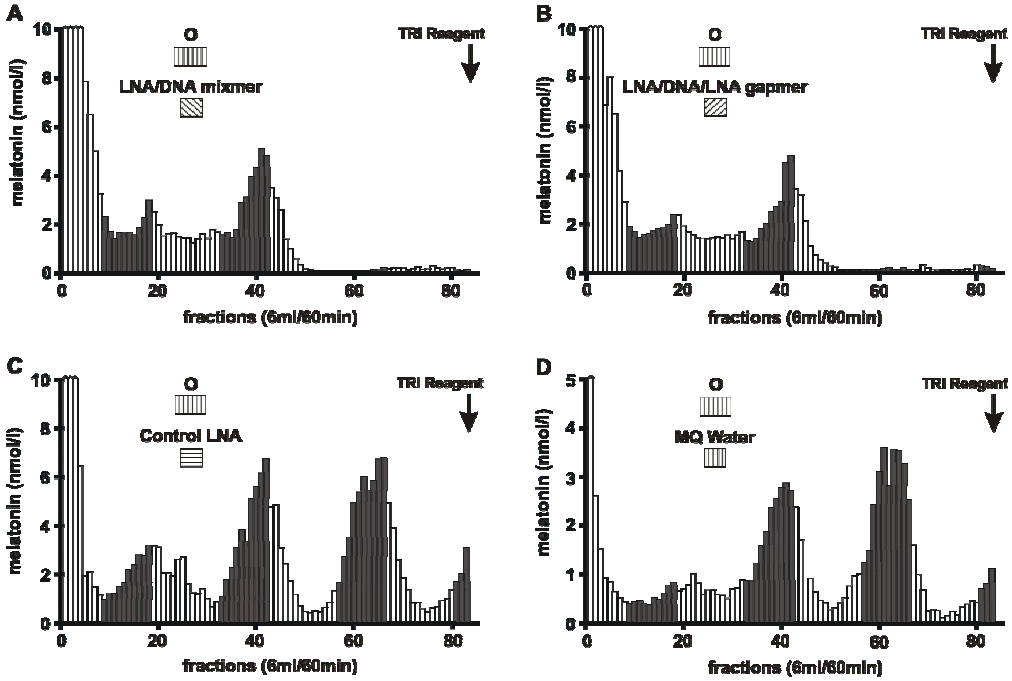

Figure 2. Effect of *cBmal1* AONs containing LNA on melatonin secretion from dissociated pineal cells in the superfusion system. Pinealocytes were cultured for 4 days in 14/10 LD cycle (light on at Zeitgeber time /ZT/ 0). Filled bars indicate when light was off. The culture medium was replaced by OPTI-MEM I without antibiotics and BSA between ZT 6 and ZT 11 in the second LD cycle (O). The cells were transfected with 200 nM of *cBmal1* AONs containing LNA between ZT 7 and ZT 10 in the second LD cycle. The AONs containing LNA were designed either for blocking the binding of the translation machinery (LNA/DNA mixmer, *Fig. 2A*) or for activating RNAse H (LNA/DNA/LNA gapmer, *Fig. 2B*). In the control experiments a mixture of two oligonucleotides containing LNA non-complementary to chicken mRNA sequences (inverse or control LNA, *Fig. 2C*) and MQ water *(Fig. 2D)* were infused to the cells, as positive and negative controls, respectively. Total RNA was extracted by TRI Reagent in the fourth LD cycle at ZT 17, which is indicated by arrow.

4.5. Expression levels of cBmal1 and cAa-nat mRNAs in chicken pinealocytes transfected with cBmal1 antisense oligonucleotides containing LNA in superfusion system

Both LNA/DNA/LNA gapmer, and the LNA/DNA mixmer reduced remarkably the expression of *cAa-nat* gene, the key enzyme of melatonin biosynthesis by 70% and 87%, respectively, whereas inverse LNA or MQ water was ineffective.
The RNase H inducing effect of LNA/DNA/LNA gapmer was tested by an RT-PCR for *cBmal1*, in which reaction the forward primer was designed for the first fragment of *cBmal1* encompassing the postulated cleavage sites of RNase H. *cBmal1* mRNA level was suppressed only by LNA/DNA/LNA gapmer designed for the induction of RNase H activity (by 44%), whereas LNA/DNA mixmer and control LNA did not change significantly the level of *cBmal1* transcript.

5. Conclusions

1. Our experiments showed, in agreement with the results of other research groups, that the *in vivo* and *in vitro* expressions of the *cBmal1* and *cAa-nat* genes cycle with a diurnal rhythm both in the retina and in the pineal gland under LD cycles corresponding to the external lighting. The maximum value of the *cBmal1* mRNA levels preceeds that of the *cAa-nat* mRNA. The *cClock* levels did not show a marked diurnal rhythm.
2. Our results indicated that the zenit and the nadir of the *cBmal1* mRNA levels are coinciding in the pineal gland and the retina. The highest and lowest values of the *cAa-nat* mRNA levels showed also a good correlation in both organs.
3. Chicken pineal cells maintain their ability to secret melatonin with a daily rhythm in the superfusion system indicating that the melatonin secretion of the pineal gland is driven by an endogenous oscillator even after the denervation of the organ. The intact daily expression of *cBmal1* and *cAa-nat* mRNAs may explain the rhythmic melatonin expression. The pattern of *cBmal1* expression suggests that *cBmal1* may play a role in *cAa-nat* expression.
4. The gradual entrainment of melatonin secretion to environmental LD rhythm could prove the presence of the functional pineal photoreceptors*, in vitro*.
5. The inhibition of the translation from *cBmal1* mRNA in pineal cells led to the decrease of *cAa-nat* mRNA levels and melatonin secretion, *in vitro*. This result completed with the observation that peak of *cBmal1* precedes that of *cAa-nat* may suggest that *cAa-nat* mRNA levels are under the regulation of the protein translated from *cBmal1* mRNA. This provides further evidence that *cAa-nat* is a clock controlled gene and the biological clock is responsible for the circadian melatonin rhythm.
6. To our knowledge, we have made transfection first in the superfusion system. Based on our experiences the transfection with AONs containing LNA is not toxic for tissues cultured in the superfusion system. Our results indicate that the transfection with AONs containing LNA might be a useful method for studying the regulation of endocrine organs in the superfusion system.

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