PhD IN BIOLOGICAL CIENCES
CELLULAR AND MOLECULAR PROGRAM
BIOLOGICAL CIENCES FACULTY
PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE

THE UNIVERSITY of WISCONSIN MADISON

VISIT TO ANOTHER LAB REPORT

Design, synthesis and purification of an andeno-asociated virus (AAV) for knocking-down of CoRESTs family proteins

Visit by Julián Sáez (PhD student, Pontifical Catholic University of Chile) to the laboratory of Dr. Corinna Burger at the University of Wisconsin-Madison, USA.

Dates of visit: 03/03/2014 – 08/31/2014

Introduction

Nurr1 (NR4A2) is a constitutively active transcription factor that belong to the orphan nuclear receptors family, it play a protagonic rol in the generation and manteinance of midbrain dopaminergic neurons, through the regulation of key genes, as Tirosine Hidroxilase (TH) (Sakurada et al., 1999), the dopamine transporter (DAT) (Sacchetti et al., 2001) and the Vesicular Monoaminest Transporter-2 (VMAT-2) (Hermanson et al., 2003), among others. It has been shown that the transcriptional activity of Nurr1 is under the control of transcriptional corepressors, such as, PIASγ, SMRT and CoREST (Galleguillos et al., 2004; Jacobs et al., 2009; Saijo et al., 2009). Our laboratory has shown that PIASγ, negatively regulate Nurr1 mediated transactivation (Galleguillos et al., 2004), through a unknown mechanism, but may involve the recruitment of others co-regulators. Previously it was reported that Nurr1 interact with CoREST protein directly (Saijo et al., 2009). CoREST is a transcriptional corepressor that belong to the transcriptional complex LHC (LSD1/HDACs/CoREST), together with the LSD1 protein (histone demethylase lysine-specific) and HDAC1/2 (histone deacetylase 1 and type 2) (Humphrey et al., 2001; You et al., 2001; Shi et al., 2004).

Besides the above, in our laboratory was observed the interaction between PIASγ and CoREST by yeasts two-hybrid assays (not published). However, it is unknown whether the CoREST corepressor and the LHC complex may mediate the strong repressor effect generated by PIASγ on the transcriptional activity of Nurr1 and whether CoREST corepressor may regulate Nurr1 target genes *in vivo*, which are essential for determination and maintenance of the dopaminergic phenotype.

Two other genes, *rcor3* and *rcor2*, with strong homology to *rcor1* (CoREST) have been described in humans and in other species (Tontsch et al, 2001; Zeng et al, 2010), these genes encode for proteins CoREST2 and CoREST3, respectively. For this reason the picture is more complex and determining if this proteins are functionally redundant or exclusives is key to understand the regulatory mechanism in the regulation of Nurr1 function.

Hypothesis and Objective

It is in this last point where the proposed work is focuses and based on the above background has proposed the following hypothesis.

The CoREST family proteins regulate the target genes of Nurr1 in midbrain neurons *in vivo*.

To test this hypothesis I proposed the following objective: **To evaluate the expression of Nurr1 target genes in midbrain neurons in the abscense of CoREST1, CoREST2 and/or CoREST3 proteins *in vivo*.**

From this objective emerge the following specific aims:

- **To design, synthetize and purified AAV vector for delivery of shRNA for CoREST1, CoREST2 y/or CoREST3 in the midbrain of rodents *in vivo***

- To develop RT-qPCR studies of konwn Nurr1 target genes from midbrain of rodents knocked down for CoREST1, CoREST2 y/or CoREST3.

In general to demonstrate this hypothesis, adeno-associated vectors (AAV) encode for shRNA (short hairpin RNA) will be developed. This shRNA will be directed specifically against the mRNA of CoREST1, CoREST2 and/or CoREST3. The general idea is generate the Knock Down (KD) of these proteins *in vivo* by stereotaxic injection of the AAV developed, in the midbrain region of adult rodent's brains and evaluate by RT-qPCR the expression of some Nurr1 targets genes in the absence of CoREST1, CoREST2 and/or CoREST3.

Plan of Work

The plan of work include the design and synthesis of the specific shRNA for the transcripts for CoREST, CoREST2 and CoREST3 proteins. It includes the creation of at least three distinct shRNA for each one and two shRNA "scrambled" or controls. This includes the design of cloning strategies of plasmid vectors encode for the different components of AAV.

On the other hand AAV will be synthesized and purified as described by Zolotukhin (Zolotukhin et al., 2002). HEK293 or HEK293-T cells will transfected by CaCl2 method an mantanied at 37°C for 60 hours prior to harvest and lysate. Protein extracts enriched in the AAV components will obtain and used for concentration and purification processes. This process include the develop of iodixanol gradients columns, then the extracts will be eluted in this column and then an aditional step of purification and concentration will be developed in a column chromatography.

Once obtained the AAV particles, infection and titulation assay will be developed in order to probe the KD efficiency and infective capacity of the different AVV. In first place cell lines will be infected and the knock down evaluated by Western Blot (WB) assays with specific antibodies for each proteins. After the confirmation of the efficiency and functionality of AAV-shRNA, we will proceed to animal studies

The efficiency testing of AAV vectors in animals will be done by stereotaxic injection of the virus in different regions of the brain of adult rodents with special interest in the midbrain. Then it will be evaluated by WB using specific antibodies for CoREST1, CoREST3 and CoREST2 proteins. When there is confirmed a significant reduction in protein levels of protein CoREST we proceed to evaluate by RT-qPCR the mRNA expression of TH, DAT, RET and VMAT-2 genes (known target genes of Nurr1), from midbrain RNA extracts of rodents infected with the specific AAV-shRNA synthesized. In this sense another known CoREST targets genes will be evaluated.

If our hypothesis is correct, we hope to observe a significant upregulation of these Nurr1 targets genes in the midbrain region, suggesting that CoREST protein, negatively regulate the Nurr1 target genes.

On the other hand behavioral study will be done in order to understand the physiological effect of the absence of these corepressors in the rodent brain.

Results

The principal objective to be performed in this visit was, the design , synthesis and purified AAV vector for delivery of shRNA for CoREST1, CoREST2 y/or CoREST3. In this context a group of 4 shRNA sequences for each CoREST protein (CoREST1, CoREST2 and CoREST3) were comercially obtained from QIAGEN. This shRNA cloned in the pGENCLIP vector. One key step in the virus production with th shRNA was the cloning of this shRNA sequences in pTR2-MCS vector that contain the repetitive TR (terminal repeats) sequences, essential for the virus formation. 4 shRNA sequences specific for each CoREST protein was cloned into the pTR2-MCS vector. The efficiency in the knock-down of this shRNA cloned was analyzed in the case of CoREST2. The figure 1 shows the results of the co-transfection of the specific pTR2-sh vectors and the vector encoding for the recombinant myc-CoREST2 protein in HEK293T cells.

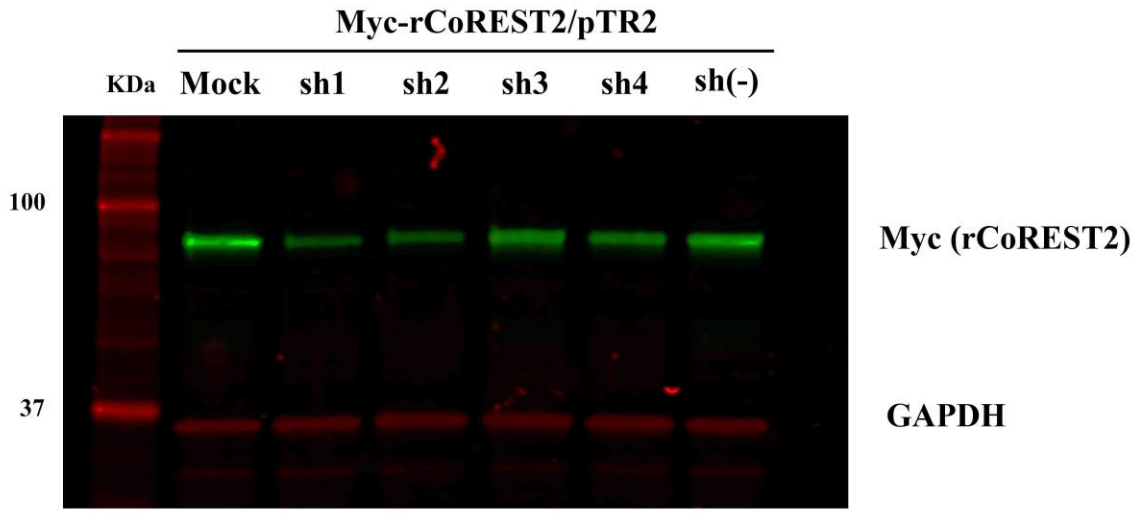

Figure 1: HEK293-T cells were transfected with pTR2-sh vectors (1-4) against CoREST2 and with the myc-rCoREST2 (rat CoREST2). 48 hours post-transfection cells were harvested and total protein extracted with RIPA buffer. Western was performed using anti myc and anti GAPDH antobodies and fluorescent secondary antibodies. Image was visualized in LICOR image system and software.

The figure 1 shows that the short hairpin 1 (sh1) and the short hairpin 2 (sh2) strongly decrease the myc- CoREST2 levels. The sh3 and sh4 have a more slight effect.

In the figure 2, at least 3 independent experiments were quantified

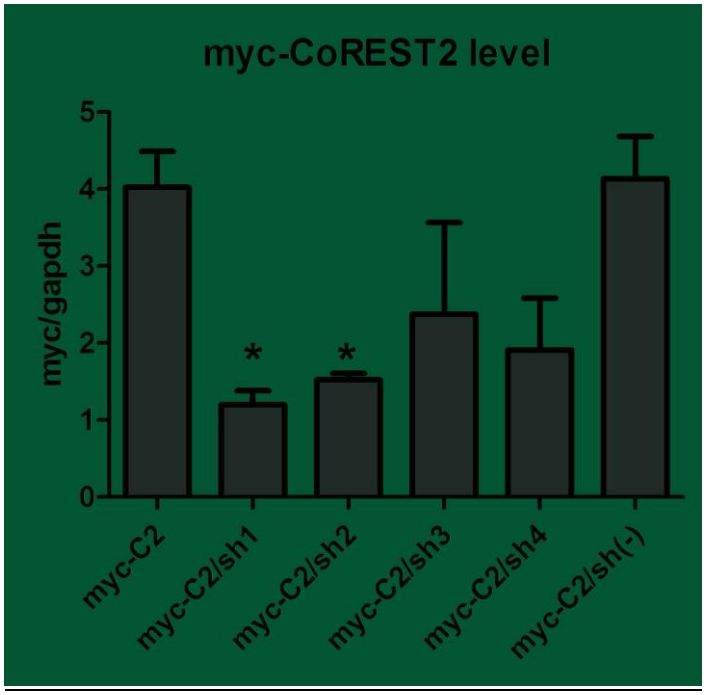

Figure 2: Knock down of CoREST2 in HEK293T cells. Quantification was perfomed using Image J software. Quantification of 3 independents experiments. GAPDH was used as loading control.

To evaluate the specificity of this effect, similar experiments were perfomed using myc-CoREST1 in the assay. The figure 3 shows the quantification of myc-CoREST1 levels form 3 independent experiments . The results show clearly that pTR2-sh against CoREST2 have no effect on myc- CoREST1 recombinant protein.

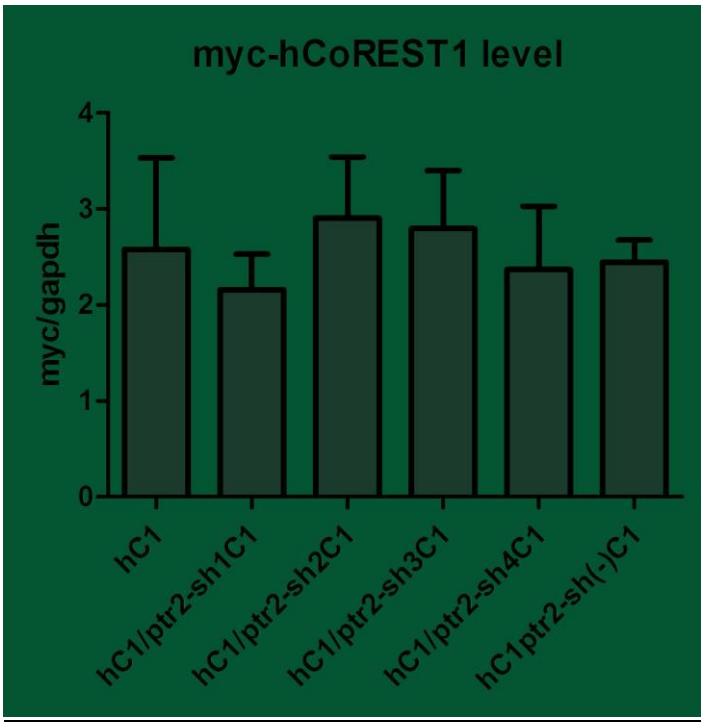

Figure 3: Short hairpins against CoREST2 does not affect to CoREST1: Quantification was perfomed using Image J software. Quantification of 3 independent experiments. GAPDH was used as loading control.

Once obtained the pTR2-sh vectors, the next step was the co-transfection of this vector with pKRAP5 vector that contain the others components of the AAV5 serotype. To obtain the amounts of pKRAP5 vector and the specific pTR2-sh vector maxiprep in Cesium Chloride gradients were performed. For each virus preparation with the specific shRNA, 20 dishes of 15 cm with HEK293-T cells were transfected at 70% confluence using the Calcium Chloride protocol. 48 hours post-transfection the cells were harvested and lyste obtained,
The Protein extracts enriched in the AAV components obtained were used for the concentration and purification processe. This process include the develop of iodixanol gradients columns, then the extracts will be eluted in this column and then an aditional step of purification and concentration was performed in a column chromatography.To demonstrate that the virus obtained are present in correct amounts and with structural integrity, an alicuot of the virus was processed and running in a SDS-PAGE followed by a comassie blue staining for the identification of the virus particules or viral proteins (VP),AAV are characterized for the presence of 3 viral proteins, VP1, VP2 and VP3. Figure 4 shows a representative image of the presence of VP1, VP2 and VP3 in the AAV5 preparation of viruses with short hairpins for CoREST1 and CoREST2, this indicate that the viruses were obtained in good amounts and integrity. The next step is the use of this viruses to generate the knock down of this proteins in the adult rat brain, this experiments are in process in my laboratory in Chile and in parallel the results of this work are been used in an active collaboration with the labororatory of Corinna Burger PhD in the UW-Madison.

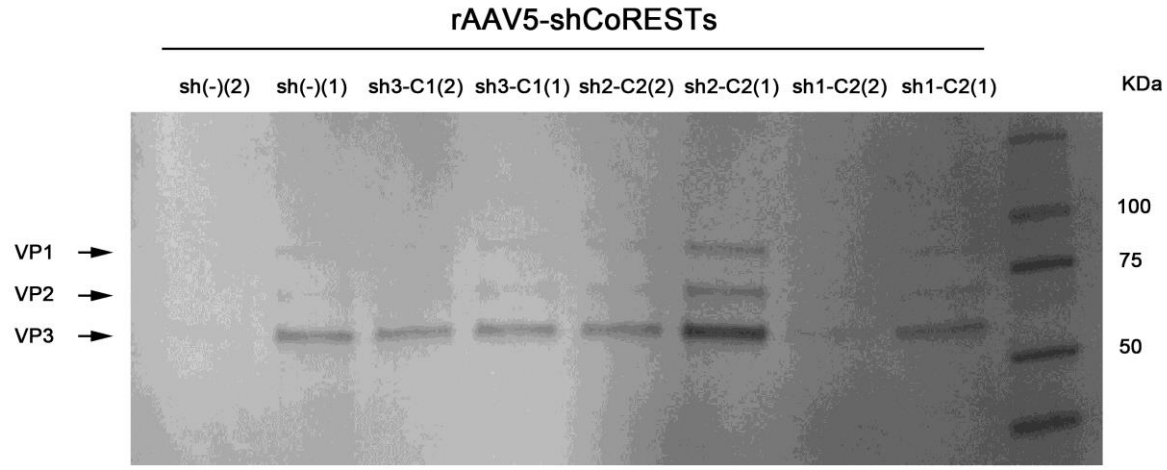

Figure 4: AAV5-shCoRESTs production for CoREST1 and CoREST2: recombinant AAV serotype 5 (AAV5) were obtained for CoREST1 and CoREST2. (1): Virus obtained from the first elution, (2): Virus obtained from the second elution. Sh(-): short hairping negative control, sh1-C2: short hairpin 1 for CoREST2, sh2-C2: short hairpin 2 for CoREST2, sh3-C1: short hairpin 3 for CoREST1.

Conclusion

.- The short hairpins RNA from pGENCLIP were succesfylly cloned into the ptR2-MCS vector

.- The shrRNA for CoREST2 efficiently and specifically reduce the CoREST2 protein levels.

.- AAV5-sh were succesfully produced and purfied with high structure integrity.

References

Galleguillos D, Vecchiola A, Fuentealba JA, Ojeda J, Alvarez K, Gómez A y Andrés ME. (2004). PIASγ Represses the Transcriptional Activation Induced by the Nuclear Receptor Nurr1. JBC. 279(3):2005–11

Hermanson E, Joseph B, Castro D, Lindqvist E, Aarnisalo P, Wallén A, Benoit G, Hengerer B, Olson L y Perlmann T. (2003). Nurr1 regulates dopamine synthesis and storage in MN9D dopamine cells. Exp. Cell Res. 288(2):324-34.

Humphrey G, Wang Y, Russanova V, Hirai T, Qin J, Nakatani Y, Howard B. (2001). Stable histone deacetylase complexes distinguished by the presence of SANT domain proteins CoREST/kiaa0071 and Mta-L1. JBC. 276(9): 6817-24.

Jacobs F, van Erp S, van der Linden AJ, von Oerthel L, Burbach J y Smidt M. (2009a). Pitx3 potentiates Nurr1 in dopamine neuron terminal differentiation through release of SMRT-mediated repression. Development. 136(4):531-40

Sacchetti P, Mitchell T, Granneman J y Bannon M. (2001). Nurr1 enhances transcription of the human dopamine transporter gene through a novel mechanism. J. Neurochem. 76(5):1565-72.

Saijo K., Winner B., Carson CT., Collier JG., Boyer L., Rosenfeld MG., Gage FH., Glass CK.(2009). A Nurr1/CoREST pathway in microglia and astrocytes protects dopaminergic neurons from inflammation-induced death. Cell. 137(1):47-59.

Sakurada K, Ohshima-Sakurada M, Palmer T y Gage F. (1999). Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. Development. 126:4017-26.

Shi Y, Lan F, Matson C, Mulligan P, Whetstine J, Cole P, Casero R, Shi Y. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119(7): 941–53.

Tontsch, S., Zach, O., and Bauer, H. C. (2001). Identification and localization of M-CoREST (1A13), a mouse homologue of the human transcriptional co-repressor CoREST, in the developing mouse CNS. Mech Dev, *108*, 165-169.

You A. Tong J, Grozinger C, Schreiber S. (2001). CoREST is an integral component of the CoREST-human histone deacetylase complex. PNAS. 98(4): 1454–58.

Zeng, W., Kong, Q., Li, C., and Mao, B. (2010). Xenopus RCOR2 (REST corepressor 2) interacts with ZMYND8, which is involved in neural differentiation. Biochem Biophys Res Commun, *394*, 1024-1029.

Zolotukhin S., Potter M., Zolotukhin I., Sakai Y., Loiler S., Fraites TJ Jr., Chiodo VA., Phillipsberg T., Muzyczka N., Hauswirth WW., Flotte TR., Byrne BJ., Snyder RO. (2002). Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. Methods. (2):158-67.