REPORT CAEN AWARD

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Introduction:

In my doctoral thesis I proposed to determine the origin of glutamatergic terminals innervating the ventral tegmental area (VTA) that express the receptor for corticotropin-releasing hormone type 2 (CRH-R2), whose activation is essential for stress-induced relapse to drug seeking. The background that gave base to my work is as follows:

After withdrawal of drugs of abuse, relapse occurs in individuals who are confronted with environmental stimuli associated with drug use (Grimm and See, *Neuropsychopharmacology*, 2000; Weiss et al. *Neuropsychopharmacology*, 2001), or a new drug dose (Neisewander et.al. *Neuropsychopharmacology*, 1996; McFarland and Kalivas. *J Neurosci*. 2001), or stressful stimuli (Erb et al. *Psychopharmacology (Berl).* 1996; Ahmed and Koob. *Psychopharmacology (Berl).* 1997) (Shalev et al. *Pharmacol Rev.* 2002; Aguilar et al. *Brain. Res. Rev.* 2009).

It has been shown that stressful stimuli induce the release of CRH in ventral tegmental area (VTA) in control and cocaine-experienced rats. Interestingly, CRH induces the release of VTA glutamate only in cocaine-experienced rats through the activation of CRH-R2. Furthermore, the activation of VTA CRH-R2 is essential for stress-induced relapse to drug seeking. However, there is not available anatomical evidence of the origin of VTA glutamatergic nerve terminals expressing CRH-R2 (Wang B. et al. *J Neurosci*. 2005; Wang B. et al. *Psychopharmacology (Berl),* 2007). VTA receives glutamatergic afferences from cortical and subcortical brain regions. The most prominent subcortical VTA glutamatergic afference is originated in the lateral hypothalamic area (LHA) (Geisler S. *J Neurosci.* 2007). It has been shown that LHA plays a role in addictive behavior.

My thesis project is entitled "**Role of glutamatergic neurons of Lateral Hypothalamus Area in stress-induced relapse to cocaine seeking**", under the supervision of Dr. Katia Gysling Caselli.

My working hypothesis is that "The glutamatergic afferents from the lateral hypothalamus area to the VTA play a key role in stress-indiuced relapse to cocaine seeking". To test this hypothesis, I have proposed the following main objective:

To determine whether stress-induced relapse to cocaine seeking depends on CRH-R2 receptors expressed in neurons of the LHA. I have proposed to knockdown CRH-R2 receptors in LHA neurons injecting a lentivirus with a shRNA for CRH-R2 and analyze stress-induced relapse behavior with the conditioned place preference paradigm (CPP).

I have learned the knockdown technique in the laboratory of Dr. Ulrike Heberlein, during my stay in her laboratory at the Ernest Gallo Clinic and the Research Center of the University of California, San Francisco.

The aims developed during my stay at Dr. Heberlein´s laboratory were:

1. To generate a lentivirus with CRH-R2 receptor shRNA.
2. To get a good viral title in HEK cells to infect PC12 cells.
3. To evaluate CRH-R2 knockdown in PC12 cells by Q-PCR.

Development:

1. To generate a lentivirus with CRH-R2 shRNA:

For the generation of second-generation lentiviruses 3 vectors were used: The transfer vector, containing the shRNA of interest, the packing vector and the envelope vector.

The transfer vector with the CRH-R2 shRNA was donated by Dr. Alon Chen from the Department of Neurobiology, Weizmann Institute of Science, Rehovot, Israel. Lentiviral vectors were constructed to produce lentiviruses expressing short hairpin RNA (shRNA) against the CRH-R2 transcript. The shRNA target sequence from the open reading frame of rat CRH-R2 was cloned into a shRNA expression cassette driven by the H1 promoter in the p156RRLsinPPTCMV-GFP-PREU3Nhe lentiviral construct. shRNA constructs were generated by synthesis of oligonucleotides composed of a 5' end containing a unique XbaI restriction site, a stretch of five adenosines as a template for the Pol III promoter termination signal, a 19-nucleotide rat CRH-R2 sense and antisense strands, separated by a nine nucleotide loop and twenty nucleotides complementary to the 3' end of the Pol III H1 promoter. The following shRNA oligonucleotides were used (in yelow the sense and antisense strands):

5´CTGTCTAGACAAAAAAGGGTCAACTACTCACACTTCTCTTGAAAGTGAGTAGTTGACCCTGGGGATCTGTGGTCTATACA.

Although the transfer vector of the shRNA of CRH-R2 was donated, in the laboratory of Dr. Heberlein, I learned to design shRNAs for CRH-R2 and to clone them into the transfer vector.

The generation of viral particles was performed in HEK 293FT cells, using packaging vector psPAX2 and envelope vector pMD2.G, both from Adgene.

Production of lentiviruses

Recombinant lentiviruses were produced by transient transfection in HEK293FT cells (from Invitrogen). psPAX2 plasmid + pMD2.G plasmid + plasmid DNA (containing shRNA of CRH-R2) + Lipofectamine 2000. Lentiviruses were harvested at 48 hr post-transfection, filtered through 0.45µm-pore filters and concentrated by ultracentrifugation. Aliquots were kept at -80°C.

2. To get a good title viral of HEK cells to infect PC12 cells:

The viral titer was determined by HIV-1 p24 Antigen ELISA Kit. The titer was of 10^7 pg/mL virus.

The PC12 cells that endogenously express CRH-R2 receptors were used to analyze GFP expression by the viral titer and infectivity of the virus. In figure 1 (Fig.1) it is possible to see the infection of PC12 in a 78,4% for Scramble (SCR), and 95,16% for CRH-R2 trough GFP expression.

3. To evaluate CRH-R2 knockdown PC12 cells by Q-PCR:

In vitro validation of lentiviral vectors

The ability of the shCRH-R2 vector to knockdown CRH-R2 expression was assessed by Q-PCR analysis. PC12 cells were infected and the knockdown of the receptor was analyzed by Q-PCR.

I used 2pg per cell of virus infection to get a knockdown of 80% compared to the expression of the endogenous control GAPDH (Fig. 2).

After the successful knockdown test performed in PC12 cells, I can continue in our laboratory with the *in vivo* experiments to analyze the relapse to drug seeking.

My work in the laboratory of Dr. Ulrike Heberlein was completed very satisfactorily, obtaining good experimental results and gaining significant experience in the work with lentivirus vectors. It was rewarding to share experiences of developing scientific research in the United States.

Having done my internship at the Ernest Gallo Clinic & Research Center, also enriched my knowledge in the neurobiology of addiction, because I participated in seminars focused on discoveries at the molecular, cellular and system level regarding addiction to drugs of abuse.

Therefore, I appreciate the possibility to win the CAEN award for the stay in San Francisco, U.S.A.

Figures:

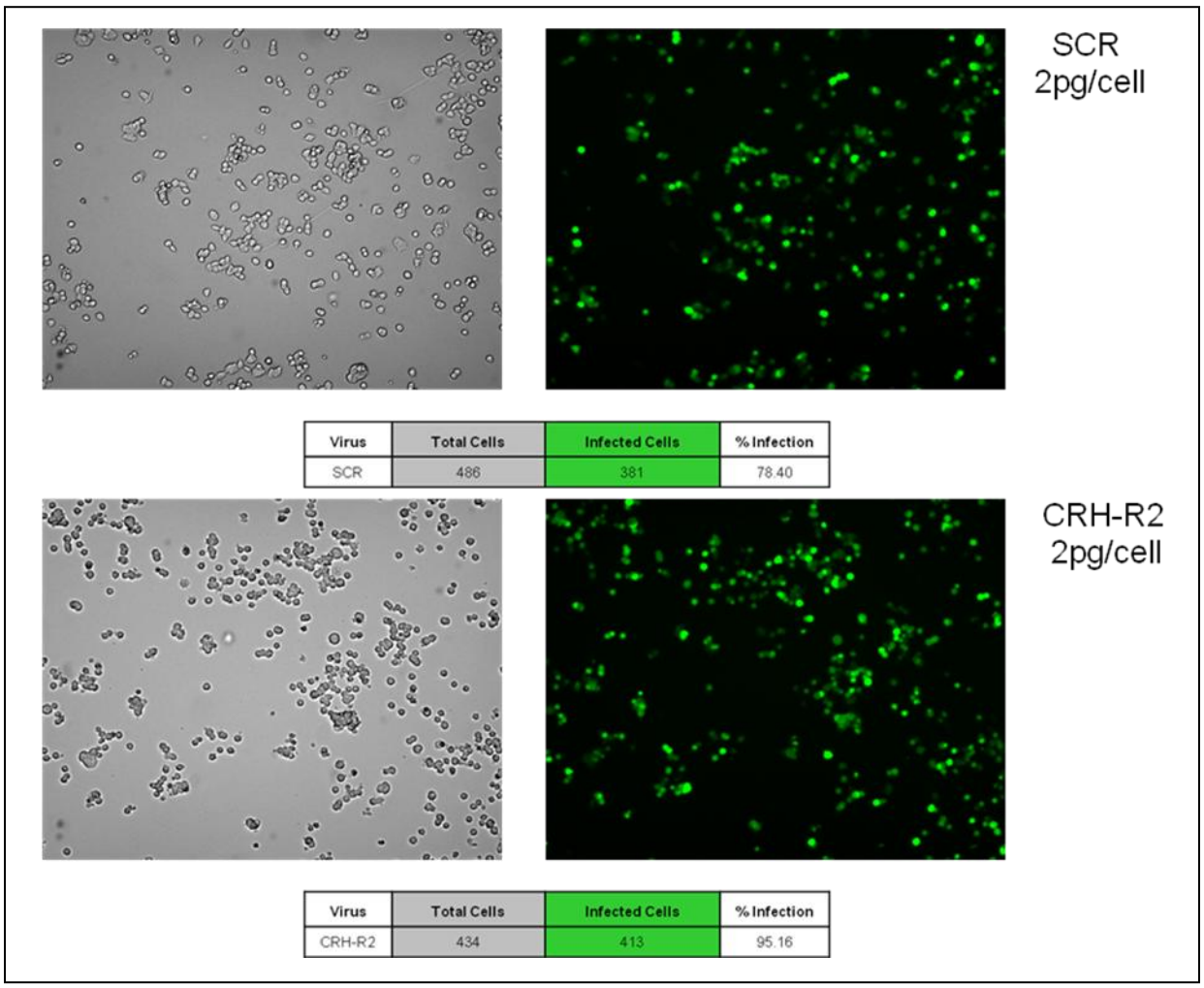
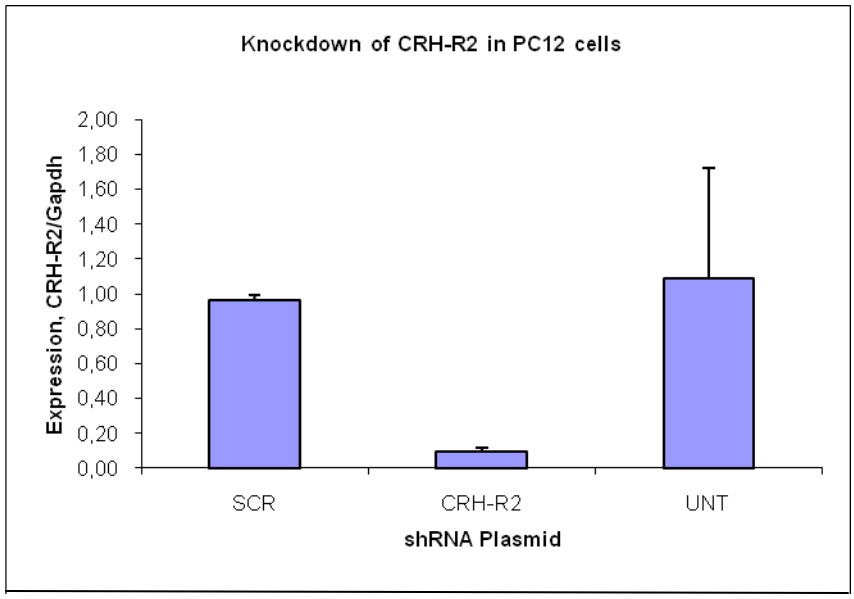


Fig1: Infection of PC12 with 2pg/cell of virus. In the left it is showed the total number of cells, and in the right it is showed the number of infected cells.



	SCR	CRH-R2	UNT
	0,95	0,08	0,64
	0,98	0,11	1,53
AVG	**0,97**	**0,10**	**1,09**
STDEV	0,02	0,02	0,63
NORM	1,00	0,10	1,12

Fig. 2: Knockdown of CRH-R2 in PC12 cells. Infection with 2pg/cell of virus.

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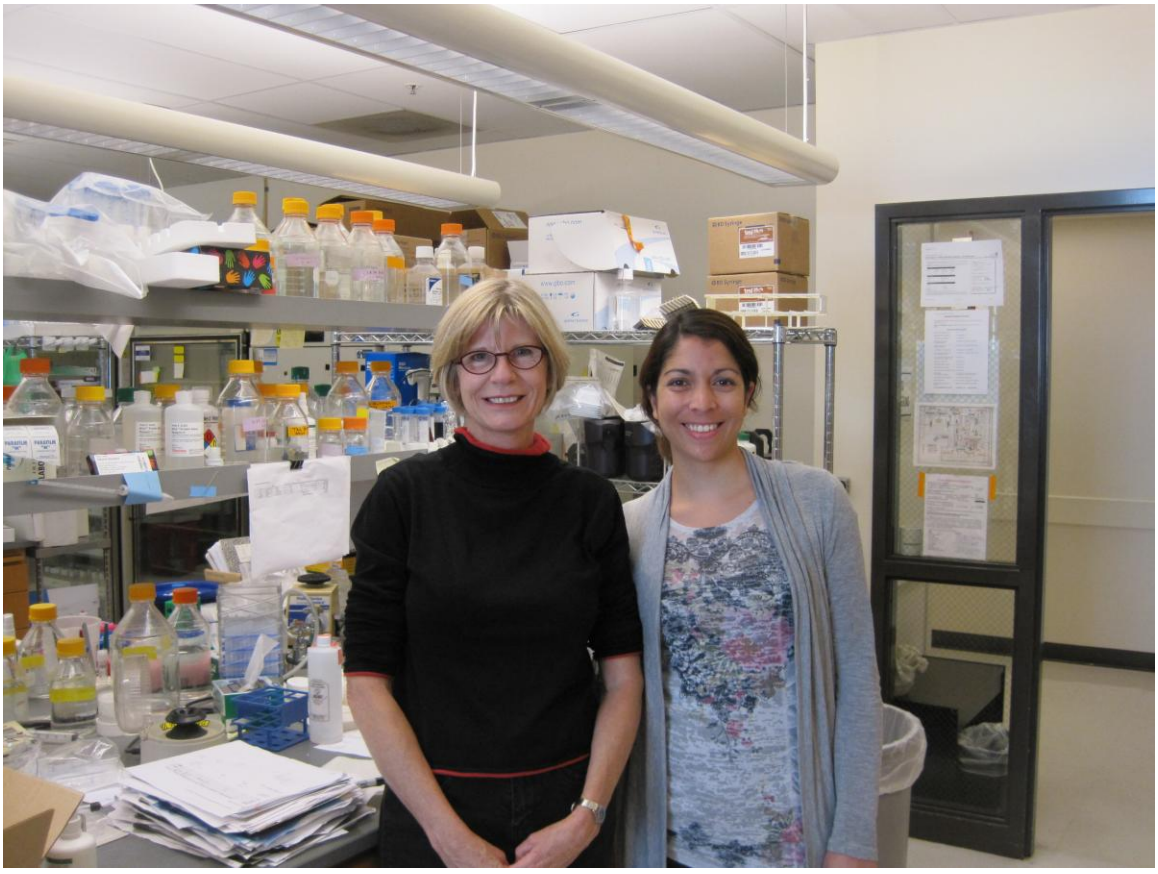
Photograph with Dr. Ulrike Heberlein in her laboratory



Dr. Heberlein and myself (Left to right).