Published in final edited form as:

Mol Cell Endocrinol. 2013 May 22; 371(0): 160–165. doi:10.1016/j.mce.2012.12.005.

Site-specific modulation of brain glucocorticoid receptor and corticotropin-releasing hormone expression using lentiviral vectors

Gloria Laryea^{a,c}, Melinda G. Arnett^{b,c}, Lindsay Wieczorek^{d,1}, and Louis J. Muglia^{b,c,*}

^aNeuroscience Graduate Program, School of Medicine, Vanderbilt University, 465 21st. Avenue South, Nashville, TN 37232, USA; gloria.n.laryea@vanderbilt.edu ^bDepartment of Pediatrics, University of Cincinnati College of Medicine, 3333 Burnet Avenue Cincinnati, OH 45229; melinda.arnett@cchmc.org ^cCenter for Preterm Birth Research, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229 USA; melinda.arnett@cchmc.org ^dProgram in Neuroscience, Washington University, 660 S. Euclid, St. Louis, MO 63110, USA. lindsay_wieczorek@med.unc.edu

Abstract

The glucocorticoid receptor (GR) and corticotropin-releasing hormone (CRH) are important molecular regulators of an individual's ability to respond to stressful stimuli in an adaptive manner. Impaired signaling of both GR and CRH often leads to dysfunction of the hypothalamic-pituitary-adrenal axis, which underlies the etiology of many affective disorders such as anxiety and depression. Studies focusing on how GR and CRH influence the stress response are limited as they generalize to broad brain regions, thus hindering identification of how specific CNS nuclei contribute to maladaptive stress responses. Our objective is to distinguish the site-specific involvement of GR and CRH in limbic regions involved in the stress response. With that intent, we use lentiviral (LV) vectors in combination with transgenic mouse lines, enabling us to modify expression of GR or CRH in a very localized manner. This paper describes the generation of several distinct LV vectors and transgenic mice models that will help further elucidate the site-specific actions of GR and CRH.

Keywords

Glucocorticoid receptor; corticotropin-releasing hormone; lentiviral vectors; psychiatric disorders; stress; hypothalamic-pituitary-adrenal axis

1. Introduction

Defining the genes and gene networks involved in mediating the stress response will enhance understanding of the underlying pathophysiology of mental health disorders. The

© 2012 Elsevier Ireland Ltd. All rights reserved

*Author to whom correspondence should be addressed; louis.muglia@cchmc.org; Tel.: +1-513-803-7902; Fax: +1-513-636-8724..

¹UNC Chapel Hill, Center for Alcohol Studies, CB#7178, Chapel Hill, NC, 27599-7178, USA.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

hypothalamic-pituitary-adrenal (HPA) axis is a major part of the neuroendocrine system that responds to stress. Regulation of the HPA axis is critical for adaptation to environmental changes. The principal regulator of the HPA axis is corticotrophin-releasing hormone (CRH), which is made in the paraventricular nucleus (PVN) of the hypothalamus and is an important target for negative feedback by glucocorticoids (GCs) – the key effector of the HPA axis. They exert their effects through ubiquitous nuclear hormone receptor superfamily members, in particular GR (NR3C1) (Samarasinghe et al., 2012).

Disruption of normal HPA axis activity is a major risk factor for neuropsychiatric disorders where decreased expression of GR and altered CRH activation has been documented (Holsboer and Ising, 2010; Pariante, 2009). The use of transgenic and gene knockout mice has helped to define roles for particular genes in mediating altered stress responsiveness and neuroendocrine function (Stenzel-Poore et al., 1992; Tronche et al., 1999; van Gaalen et al., 2003). However, these data are limited due to their inability to regulate spatial and temporal disruption of gene expression. Viral vectors have become the most commonly used gene delivery vehicles due to their high transduction efficiencies (Naldini et al., 1996a). In particular, lentiviral (LV) vectors have become powerful tools in basic and translational research representing one of the most effective gene delivery vehicles allowing for stable long-term transgene expression in both dividing and non-dividing cells (Naldini, 1998; Naldini et al., 1996b).

Our lab has generated five LV vectors in an effort to investigate the role of GR and CRH in specific brain nuclei. In this paper, we will discuss their design, production and possible future application in mice.

2. Methods and Materials

2.1. Animals

All animal protocols were in accordance with National Institutes of Health guidelines and were approved by the Animal Care and Use Committees of Vanderbilt University (Nashville, TN) and Cincinnati Children's Medical Center (Cincinnati, OH). Mice were housed on a 12 h/12 h (Vanderbilt) or 14 h/10 h (Cincinnati Children's Medical Center) light/dark cycle with *ad libitum* access to rodent chow and water. For use with the tetracycline-inducible system, some mice were given *ad libitum* access to doxycycline (doxy) chow (0.0625% doxy; Test Diets) to control transgene expression

2.1.1. Generation of Floxed GR mice—These mice were generated as previously described (Brewer et al., 2003). Briefly, *loxP* sites were inserted into a *SacI* site upstream of exons 1C and 2, and a PGK neo cassette flanked by *loxP* sites was inserted into intron 2 of the GR gene, *Nr3c1*. This plasmid was electroporated into mouse embryonic stem cells and clones demonstrating homologous recombination underwent blastocyst injection. Mice arising from successful germline transmission were backbred more than 10 generations onto a C57BL/6 background.

2.1.2. Generation of Tetop-CRH mice—These mice were generated as previously described (Kolber et al., 2010). Briefly, the CRH gene was placed under the control of a tetracycline-responsive cytomegalovirus minimal promoter (tetop) and inserted into a pUHC13-3 backbone vector. The resulting tetop-CRH plasmid was injected into inbred C57BL/6 oocytes.

2.1.3. Generation of CaMKII α -tTA mice—These mice were generated as previously described (Mayford et al., 1996). These mice express the tetracycline transactivator under the control of the CaMKII α promoter (Jackson Laboratories).

2.2. Lentiviral Vectors

For each LV vector, individual plasmids were designed as described below and sent to the Hope Center Viral Vector Core (Washington University, St. Louis, MO) for packaging. Briefly, plasmids were packaged using three helper plasmids in 293-T cells into replication deficient virions. Viral vector titers were determined by transduction of HT1080 cells and assayed for reporter expression using flow cytometry or real time PCR. We were able to reliably obtain a titer of 10^7 – 10^9 infectious units/mL for each LV.

2.2.1. LV-Cre and LV-GFP Vector—GFP- and Cre-containing (pLV-GFP, pLV-Cre) plasmids were derived from corresponding components of each DNA, placing either Cre-recombinase (with a nuclear localization signal) or GFP under the control of the elongation factor-1 α promoter (Ef1 α ; Fig 1a,b). Previously described (Kolber et al., 2008).

2.2.2. LV-GR Vector—We inserted full-length mouse GR cDNA (generously given by J. Bodwell, Dartmouth, NH) into the BamHI site of pLenti-III-EF1 α (Applied Biological Materials, Richmond, BC, Canada) to generate pLV-GR, an mGR expression vector (Fig 1c).

2.2.3. LV-rtTA Vector—To generate the pLV-rtTA plasmid, we used a LV plasmid that is under the control of a modified moloney murine leukemia virus long terminal repeat (MNDU3; pMNDU3 plasmid generously given by M. Sands, Washington University in St. Louis) (Haas et al., 2003). We directly inserted the reverse tetracycline transactivator (rtTA) from Clontech's rtTA-advanced plasmid (PT3899-5) into the LV backbone with EcoRI and BamHI cuts.

2.2.4. LV-tetop-CRH Vector—We inserted tetop-CRH (Kolber et al., 2010), the CRH gene under the control of the tetop promoter, into the XhoI and EcoRV sites of pLenti-III-EF1 α (Applied Biological Materials, Richmond, BC., Canada).

2.3. Cell Culture

The LV-GR was transiently infected into Chinese hamster ovarian (CHO)-K1 cells (ATCC, CCL-61). Cells were seeded in a 9-well plate at 3×10^5 cells/ml and maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Merelbeke, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), and 1% pen strep (Gibco, New York, NY). Cells were cultivated at 37°C in a humidified atmosphere containing 5% CO₂. CHO-K1 cells were infected with LV-GR (7 μ l, 7.4×10^8 TU/ml) or LV-GFP in triplicate while control wells contained CHO-K1 cells only. Whole cell extracts were prepared after a 72-hour incubation using total protein extraction buffer (PE buffer [10mM Hepes, 60mM KCl, 1mM EDTA], 250mM dithiothreitol, 100% NP-40 and protease inhibitor mixture [0.5 μ g/ml aprotinin, 1 μ g/ml pepstatin and leupeptin]). Analysis of the integrated proviral genome was determined by Western blotting.

2.4. Western Blotting

60 μ g of whole cell extracts were resolved through sodium dodecyl sulfate polyacrylamide gel electrophoresis using 4–12% gradient Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad CA), transferred to a Hybond enhanced chemiluminescence nitrocellulose membrane (GE Healthcare Life sciences, Pittsburg, PA) using a semidry transfer system (Bio Rad, Hercules, CA), and blocked with 5% dry milk in PBS and 0.1% Tween-20 (Sigma Aldrich, St. Louis, MO). Blots were probed with anti-GR antisera (M-20; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1/2,000 dilution overnight at 4°C. Binding of horseradish peroxidase labeled goat anti-rabbit antibody (sc-2004) was determined using

SuperSignal West Chemiluminescent substrate (Pierce, Rockford, IL). Blots were stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) and reprobed with an anti- β -actin antibody (Sigma Aldrich, St. Louis, MO) as an internal control to ensure equal loading of protein.

2.5. Luciferase Assay

We transfected CHO cells with a pTetop-luciferase (pTetop-luc) plasmid in conjunction with pLV-rtTA. After administering doxy at 0, 10, and 100 ng/mL concentrations, we used a luminometer to measure relative light units (RLU) as an indicator of luciferase expression.

2.6. CRH Immunohistochemistry

Mice were anesthetized with 2.5% Avertin, and transcardially perfused with 4% paraformaldehyde. The brains were immersed in 20% DEPC sucrose solution and sectioned at 30 μ m into PBS with 0.25% Triton X-100 (PBS-T); sections were stored at 4°C until use. 0.3% hydrogen peroxide in PBS was used to quench endogenous peroxides, and sections were blocked in 10% normal goat serum (NSG) in PBS-T to prevent non-specific binding. Sections were incubated in primary antibody, 1:1000 rabbit anti-CRH (Bachem, San Carlos, CA) at 4°C overnight. Then, sections were labeled with biotinylated goat anti-rabbit IgG secondary (1:200, Vector Laboratories, Burlingame, CA) at room temperature for an hour, followed by an hour in avidin/biotin complex solution (Vector Laboratories, Burlingame, CA), and detected with diaminobenzidine (DAB) staining. The sections were air-dried on slides overnight, dehydrated with ethanol and xylene, and cover-slipped with Permount (Fisher Scientific, Pittsburg, PA). Stained slides were visualized under a light microscope (Zeiss Axio Imager) and images were prepared using Adobe Photoshop.

2.7. Hematoxylin and Eosin (H & E) Staining

Sections adjacent to those used for CRH immunohistochemistry were placed on slides and air-dried overnight. Slides were processed as follows: 2 min wash in 70% ethanol; 5 min wash in distilled water; 10 min staining in hematoxylin (Polysciences Inc, Warrington, PA); 3 washes in distilled water; 1 min in saturated lithium chloride; 3 washes in distilled water; 2 min in 70% ethanol; 2 min in 80% ethanol; 90 sec staining in eosin (Polysciences Inc, Warrington, PA); 2 min in 70% ethanol; 2 min in 80% ethanol; three 2 min washes in 95% ethanol; three 2 min washes in 100% ethanol; three 2 min washes in xylene; coverslipped with Permount (Fisher Scientific, Pittsburg, PA).

2.8. Stereotaxic Injection

We gave mice a 10 mg/kg dose of ketoprofen (Fort Dodge, IA) and an hour later, anesthetized them with a 90 mg/kg ketamine (Ketaset, Fort Dodge IAO) / 10 mg/kg xylazine (TranquiVed, Vedco, St. Joseph MO) solution and mounted them on a stereotaxic frame (Kopf Instruments, Tujunga, CA). We drilled small holes at the coordinates provided below and injected the LV at either a rate of 0.2 μ l/min into the central nucleus of the amygdala (CeA) or 0.5 μ l/min into the striatum. The mice were monitored for recovery and given a 10mg/kg dose of ketoprofen each day for 3 days post-surgery. Fourteen days after the injection, brains were harvested and processed for immunohistochemistry. Coordinates: CeA (1.25mm bregma, $\pm$ 2.75 lateral, 4.75 ventral); striatum (0mm bregma, 2.5 lateral, 3.8 ventral).

3. Results and Discussion

GR and CRH modulation in the brain of animal models have provided extensive evidence for their involvement in neuroendocrine, behavioral and autonomic responses to stress. Forebrain GR disruption, for instance, increases HPA axis activity, induces anhedonia in the

sucrose preference test, causes despair behavior in the forced swim and tail suspension tests, and alters regulation of anxiety behavior (Boyle et al., 2005, 2006). Mice with forebrain GR disruption treated with the antidepressant imipramine, showed a treatment response in despair phenotypes but not anxiety phenotypes. An important question that arises is whether GR activity differs with respect to disease state and whether GR activity in distinct brain regions contributes in different proportions to anxiety versus depression, or any other affective disorder state for that matter. The same case can be made for CRH. Elevated forebrain CRH produced different neuroendocrine and behavior responses depending on the length of time of CRH overexpression and the specific developmental period in which this overexpression occurred (Kolber et al., 2010; Vicentini et al., 2009). Some of the forebrain regions with altered GR or CRH included the basolateral and central amygdalar nuclei, hippocampus, striatum, PVN of the hypothalamus, and nucleus accumbens. The ability to define how these regions contribute to the observed phenotypes will help discern which regions are important targets for specific psychiatric disorders. We can evaluate this using LV vectors, directly delivered to brain nuclei of interest, in mice with specific transgenes to alter expression of GR and CRH.

3.1. GR Modulation

To modulate GR expression in a region specific manner, our lab has previously generated mice with floxed GR gene as well as a LV containing Cre recombinase, LV-Cre (Kolber et al., 2008). Injections of LV-Cre into floxed GR mice delete the receptor in a very localized manner. In our case, the CeA was a primary site of interest due to its role processing emotional stimuli, particularly fear. Bilateral injections of LV-Cre into the CeA of floxed GR mice reduced GR expression by approximately 60%. We further demonstrated that this disruption of CeA GR diminished freezing in the contextual conditioned fear test that correlated with a reduction in CeA CRH mRNA 60 min after conditioned fear training. These data provided evidence that the CeA is involved in memory acquisition and consolidation, in part through signaling of GR.

In contrast to knocking out GR, we generated a LV that expresses full-length mouse GR cDNA under the control of the Efl α promoter (LV-GR) (Fig 1c), which allows us to rescue GR expression in restricted limbic regions. We validated its function by infecting CHO-K1 cells with LV-GR or LV-GFP (LV expressing GFP, a control) or no virus. We performed Western blots of protein extracts probing with anti-GR antisera and anti- β -actin antibody as an internal control. The no virus and LV-GFP controls both showed no GR protein while our LV-GR infected sample highly expressed GR (Fig. 2). This LV-GR virus is thus able to increase/express GR.

3.2. CRH modulation

We used the tetracycline inducible method to enable modulation of CRH expression along developmental periods. We generated transgenic mice with the tetop promoter driving CRH gene expression (tetop-CRH).

To enable inducible expression of CRH in a site-specific manner, we developed an HIV-1-derived replication-defective LV vector that possesses rtTA (LV-rtTA) (Fig. 1d). The rtTA forms a Tet-on system and when bound to a tetop promoter can activate expression of any downstream gene of interest. This expression is inducible: turned on in the presence of a tetracycline derivative, doxy, or off in its absence. The rtTA has high inducibility (within hours of doxy administration) and low background (Gossen et al., 1993; Urlinger et al., 2000).

To determine the *in vitro* efficacy of the virus, we transfected CHO cells with a combination of pTetop-luc and pLV-rtTA (pMNDU-rtTA). We administered doxy at various concentrations and detected the luciferase expression as measured by the relative light units (RLU) (Figure 3). In the presence of both pTetop-luc and pLV-rtTA, doxy significantly increases the RLU, which was minimal with no doxy present, indicating that doxy allowed rtTA to bind to tetop and drive luciferase expression.

To determine the *in vivo* efficacy of our virus, we performed unilateral stereotaxic injections into the striatum of tetop-CRH mice that were either on or off doxy with 3 μ l of LV-rtTA (7.36×10^9 infectious units/mL). Two weeks after viral infection, we harvested the brains and performed immunohistochemistry to evaluate CRH protein expression. The data indicate that CRH is expressed only in the presence of both LV-rtTA and doxy in mice with the tetop-CRH transgene (Figure 4a). To evaluate the tissue in the injected region, we performed H & E staining. The H & E staining in Figure 4b illustrates mononuclear cell infiltration and cell toxicity in the LV-rtTA injected region. This data raised concerns of a possible host-inflammatory response induced by the rtTA protein.

To eliminate this confound, we decided to use mice that express tTA during development and thus are inherently tolerant to it (CamKII α -tTA mice). Consequently, we generated an LV delivery system, LV-tetop CRH (Fig. 1e), complementary to the CamKII α -tTA transgenic mice, by inserting the tetop-CRH gene into an LV vector, pLenti-III-EF1 α (Applied Biological Materials Inc., Richmond, BC, Canada).

To test *in vivo* efficacy, we unilaterally injected the striatum of CamKII α -tTA mice that were either on or off doxy with 3 μ l of LV-tetopCRH (titer 8.62×10^7 infectious units/ml). We chose striatum because it has no endogenous CRH expression and thus it is easier to visualize CRH introduced by the virus over background staining. We evaluated CRH protein expression two weeks after viral infection. In comparison to the Tet-on system – LV-rtTA in the presence of doxy turns on CRH gene expression – in this system (Tet-off), LV-tetopCRH binds to the tTA in the mice with the CamKII α -tTA transgene and drives CRH expression only in the absence of doxy. Thus, placing the mice on a doxy diet would turn off CRH expression. This allows us to retain the ability to spatially and temporally restrict CRH expression.

The immunohistochemistry data indicate that CRH is expressed only in the presence of LV-tetopCRH (Fig. 5a). Mice on a doxy diet or non-injected mice do not show this striatal CRH expression (Fig. 5b&c). H & E staining in Figure 5 d–f illustrates that the cells in the LV-tetopCRH injected region do not display the same toxic effect observed in the LV-rtTA injected mice. The data support the functionality of this new model.

Previous literature supports the use of LV vectors to define the role of CRH in brain regions such as the CeA and bed nucleus of the stria terminalis (BnST) – two brain regions involved in mediating anxiety and fear behaviors (See (Laryea et al., 2012) for review). CeA CRH elevation in male mice was found to affect anxiety behavior: reduce it with long-term elevation (Regev et al., 2011) but increase it with short-term elevation (Regev et al., 2012). On the other hand, BnST CRH overexpression was involved in inducing despair behavior (Regev et al., 2011). In comparison, CeA CRH elevation in rats demonstrated pronounced maladaptive alteration in measures of anxiety and despair, as well as HPA axis function, similar to that observed in human disorders (Flandreau et al., 2012; Keen-Rhinehart et al., 2009). These studies indicate how different the effects of CRH overexpression are dependent on region, length of over-expression, gender and/or species of organism.

The inducibility of our tetracycline system will enable CRH overexpression for short periods of time during development to evaluate how CRH activity at specific time periods can result

in different phenotypic outcomes. The strength of our model is the ability to terminate CRH overexpression after a length of time to allow for molecular and circuit changes that may occur and influence future sensitivity to stress. For instance, does brief CRH overexpression in amygdalar nuclei early in development prime and provide resilience to fearful stimuli later in life? Or does it predispose pathological states of anxiety or despair? Perhaps the developmental period during which CRH overexpression occurs will predict whether it is beneficial or destructive when exposed to subsequent stressors.

3.3. Conclusions

One of the limitations in treatment of psychiatric disorders is the limited knowledge of their underlying molecular etiology. We believe that LV constructs in combination with transgenic mouse models provide an avenue for evaluating molecular pathways specific to different affective disorders. The use of this paradigm to study other stress-related molecules and monoamines will possibly prove beneficial in understanding the causation of individual differences in the manifestation and prognosis of mood disorders.

Acknowledgments

This work was supported by NIH grant MH079010. We would like to thank Lisa Muglia and Margaret Pan for manuscript review.

Abbreviations

GR	glucocorticoid receptor
CeA	central nucleus of amygdala
HPA	hypothalamic- pituitary-adrenal
BLA	basolateral nucleus of amygdala
CRH	corticotropin-releasing hormone
BnST	bed nucleus of the stria terminalis
CaMKIIα	calcium/calmodulin-dependent kinase
LV	lentivirus
PVN	paraventricular nucleus of the hypothalamus
GC	glucocorticoids
tTA	tetracycline transactivator
rtTA	reverse tetracycline transactivator
Tetop	tetracycline-responsive promoter
Doxy	doxycycline
CHO	Chinese hamster ovarian cell
RLU	relative light units
H & E	hematoxylin and eosin

References

- Boyle MP, Brewer JA, Funatsu M, Wozniak DF, Tsien JZ, Izumi Y, Muglia LJ. Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behavior. *Proc Natl Acad Sci U S A*. 2005; 102:473–478. [PubMed: 15623560]

- Boyle MP, Kolber BJ, Vogt SK, Wozniak DF, Muglia LJ. Forebrain glucocorticoid receptors modulate anxiety-associated locomotor activation and adrenal responsiveness. *J Neurosci*. 2006; 26:1971–1978. [PubMed: 16481429]
- Brewer JA, Khor B, Vogt SK, Muglia LM, Fujiwara H, Haegele KE, Sleckman BP, Muglia LJ. T-cell glucocorticoid receptor is required to suppress COX-2-mediated lethal immune activation. *Nat Med*. 2003; 9:1318–1322. [PubMed: 12949501]
- Flandreau EI, Ressler KJ, Owens MJ, Nemeroff CB. Chronic overexpression of corticotropin-releasing factor from the central amygdala produces HPA axis hyperactivity and behavioral anxiety associated with gene-expression changes in the hippocampus and paraventricular nucleus of the hypothalamus. *Psychoneuroendocrinology*. 2012; 37:27–38. [PubMed: 21616602]
- Gossen M, Bonin AL, Bujard H. Control of gene activity in higher eukaryotic cells by prokaryotic regulatory elements. *Trends Biochem Sci*. 1993; 18:471–475. [PubMed: 8108860]
- Haas DL, Lutzko C, Logan AC, Cho GJ, Skelton D, Jin Yu X, Pepper KA, Kohn DB. The Moloney murine leukemia virus repressor binding site represses expression in murine and human hematopoietic stem cells. *J Virol*. 2003; 77:9439–9450. [PubMed: 12915559]
- Holsboer F, Ising M. Stress hormone regulation: biological role and translation into therapy. *Annu Rev Psychol*. 2010; 61:81–109. [PubMed: 19575614]
- Keen-Rhinehart E, Michopoulos V, Toufexis DJ, Martin EI, Nair H, Ressler KJ, Davis M, Owens MJ, Nemeroff CB, Wilson ME. Continuous expression of corticotropin-releasing factor in the central nucleus of the amygdala emulates the dysregulation of the stress and reproductive axes. *Mol Psychiatry*. 2009; 14:37–50. [PubMed: 18698320]
- Kolber BJ, Boyle MP, Wiczorek L, Kelley CL, Onwuzurike CC, Nettles SA, Vogt SK, Muglia LJ. Transient early-life forebrain corticotropin-releasing hormone elevation causes long-lasting anxiogenic and despair-like changes in mice. *J Neurosci*. 2010; 30:2571–2581. [PubMed: 20164342]
- Kolber BJ, Roberts MS, Howell MP, Wozniak DF, Sands MS, Muglia LJ. Central amygdala glucocorticoid receptor action promotes fear-associated CRH activation and conditioning. *Proc Natl Acad Sci U S A*. 2008; 105:12004–12009. [PubMed: 18695245]
- Laryea G, Arnett MG, Muglia LJ. Behavioral Studies and Genetic Alterations in Corticotropin-Releasing Hormone (CRH) Neurocircuitry: Insights into Human Psychiatric Disorders. *Behav Sci (Basel)*. 2012; 2:135–171. [PubMed: 23077729]
- Mayford M, Bach ME, Huang YY, Wang L, Hawkins RD, Kandel ER. Control of memory formation through regulated expression of a CaMKII transgene. *Science*. 1996; 274:1678–1683. [PubMed: 8939850]
- Naldini L. Lentiviruses as gene transfer agents for delivery to non-dividing cells. *Curr Opin Biotechnol*. 1998; 9:457–463. [PubMed: 9821272]
- Naldini L, Blomer U, Gage FH, Trono D, Verma IM. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci U S A*. 1996a; 93:11382–11388. [PubMed: 8876144]
- Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*. 1996b; 272:263–267. [PubMed: 8602510]
- Pariante CM. Risk factors for development of depression and psychosis. Glucocorticoid receptors and pituitary implications for treatment with antidepressant and glucocorticoids. *Ann N Y Acad Sci*. 2009; 1179:144–152. [PubMed: 19906237]
- Regev L, Neufeld-Cohen A, Tsoory M, Kuperman Y, Getselter D, Gil S, Chen A. Prolonged and site-specific over-expression of corticotropin-releasing factor reveals differential roles for extended amygdala nuclei in emotional regulation. *Mol Psychiatry*. 2011; 16:714–728. [PubMed: 20548294]
- Regev L, Tsoory M, Gil S, Chen A. Site-specific genetic manipulation of amygdala corticotropin-releasing factor reveals its imperative role in mediating behavioral response to challenge. *Biol Psychiatry*. 2012; 71:317–326. [PubMed: 21783178]

- Samarasinghe RA, Witchell SF, DeFranco DB. Cooperativity and complementarity: synergies in non-classical and classical glucocorticoid signaling. *Cell Cycle*. 2012; 11:2819–2827. [PubMed: 22801547]
- Stenzel-Poore MP, Cameron VA, Vaughan J, Sawchenko PE, Vale W. Development of Cushing's syndrome in corticotropin-releasing factor transgenic mice. *Endocrinology*. 1992; 130:3378–3386. [PubMed: 1597149]
- Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, Bock R, Klein R, Schutz G. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet*. 1999; 23:99–103. [PubMed: 10471508]
- Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proc Natl Acad Sci U S A*. 2000; 97:7963–7968. [PubMed: 10859354]
- van Gaalen MM, Stenzel-Poore M, Holsboer F, Steckler T. Reduced attention in mice overproducing corticotropin-releasing hormone. *Behav Brain Res*. 2003; 142:69–79. [PubMed: 12798267]
- Vicentini E, Arban R, Angelici O, Maraia G, Perico M, Mugnaini M, Ugolini A, Large C, Domenici E, Gerrard P, Bortner D, Mansuy IM, Mangiarini L, Merlo-Pich E. Transient forebrain over-expression of CRF induces plasma corticosterone and mild behavioural changes in adult conditional CRF transgenic mice. *Pharmacol Biochem Behav*. 2009; 93:17–24. [PubMed: 19358863]

Highlights

- We generated lentiviral vectors that modulate glucocorticoid receptor expression.
- We also generated lentiviral vectors that overexpress CRH.
- The tetracycline-inducible system allows spatial and temporal regulation of CRH.
- We demonstrate utility of these lentiviral vectors in vitro and in vivo studies.

Figure 1. Constructs of lentiviral vectors packaged into replication deficient virions

Each LV vector contains an HIV-based system including 3' and 5' long terminal repeats (LTR), a 5' self-inactivating (SIN) element to prevent replication and a woodchuck post-transcriptional regulatory element to enhance protein expression (a.) LV-Cre (b.) LV-GFP (c.) LV-GR (d.) LV-rtTA (e.) LV-tetopCRH. ψ = packaging signal; RRE = Rev response element; cPPT = central polyurine tract; gag = group-specific antigen; EF1 α , MNDU3, and tetop = promoters; nls = nuclear localization signal; eGFP = enhanced green fluorescent protein; GR = glucocorticoid receptor; rtTA = reverse tetracycline transactivator; CRH = corticotropin-releasing hormone.

Figure 2. GR protein levels after LV-GR and LV-GFP infection of CHO-K1 cells
Lysates were separated by SDS PAGE and immunoblots were probed with an anti-GR antibody recognizing the N-terminus. LV-GR virus stably expresses full-length mGR protein representing efficient transduction in CHO-K1 cells. Control samples represent CHO-K1 cells without LV infection. B-actin antibody served as an internal control to ensure equal protein loading.

Figure 3. *In vitro* analysis of pLV-rtTA

CHO cells were co-transfected with pTetop-luc and pLV-rtTA in the presence and absence of doxy to test the efficiency of the pLV-rtTA plasmid. Luciferase expression was then measured. pLV-rtTA displays robust expression in the presence of doxy with low levels of expression in the absence of doxy. RLU = relative light units, doxy = doxycycline

Figure 4. CRH immunoreactivity and hemotoxylin & eosin (H&E) staining in tetopCRH mice injected with LV-rtTA

(a.) CRH protein (dark brown pigment) is present when mice are on doxy chow. (b.) H & E staining displays inflammatory cell infiltration within the injected region.

Figure 5. CRH immunoreactivity and hemotoxylin & eosin (H & E) staining in CamKII α -tTA mice injected with LV-tetopCRH

(a.) CRH protein (dark brown pigment) is present when mice are off doxy chow. (b. & c.) CRH protein is absent when mice are on doxy chow or in non-injected controls. H & E staining shows normal cells in the (d.) absence or (e.) presence of doxy as well as in (f.) non-injected controls.