

A Novel Transgenic Mouse for Gene-Targeting Within Cells That Express Corticotropin-Releasing Factor

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Corticotropin-releasing factor (CRF) orchestrates the mammalian endocrine, autonomic, and behavioral stress response and has been implicated in the pathophysiology of illnesses ranging from irritable bowel syndrome to mood and anxiety disorders. CRF is produced and released from a variety of cell types, making it difficult to distinguish the specific role of CRF from other neurotransmitters with which it colocalizes. To clarify the basic biology of the CRF neuron, we must be able to manipulate selectively CRFergic cells. Here we describe a novel transgenic mouse using 3.0 kb of the CRF promoter to drive expression of Cre-recombinase (CRFp3.0Cre). Crossing CRFp3.0Cre with a fluorescent reporter strain results in Cre-dependent green fluorescent protein expression within CRF-producing cells. Thus, CRF cells can be identified for single-cell polymerase chain reaction and electrophysiological procedures. Furthermore, the CRFp3.0Cre transgenic can be combined with other available mouse strains containing a “floxed” gene of interest to allow unparalleled detailed analysis of the CRF system.

Key Words: Amygdala, corticotropin releasing factor, Cre-recombinase, lentiviral vector, promoter specificity, transgenic

Corticotropin-releasing factor (CRF), a 41 amino acid peptide, was discovered in 1981 by Vale and colleagues for its role in activating the hypothalamic-pituitary-adrenal (HPA) axis, a primary endocrine reaction to perceived threat. CRF has since been recognized as a key coordinator of autonomic and behavioral stress-responsivity via extrahypothalamic actions in the brainstem and limbic system, respectively (1–5). Although critical in the face of real threat, overactive or inappropriate activation of CRF and the stress–response systems it coordinates can have severe consequences for mental and physical health (6–9).

Despite its importance in adaptive and pathological stress responsivity, the inner workings of CRFergic neurons are not well understood. CRF is produced in a variety of cell types including neurons and glia (10) and is colocalized with numerous neurotransmitters and neuropeptides (11–15); this heterogeneity complicates efforts to clarify region and cell-type-specific influences of CRF. Here we describe the development of a novel transgenic mouse in which CRFergic cells can be identified and manipulated apart from neighboring cells.

Using our previously outlined method of screening promoters for cell-type-specific expression (16), we amplified 3.0 kb upstream of the translation start site for the CRF coding sequence. This 3.0-kb promoter, inserted into a lentiviral vector backbone, drives expression of Cre-recombinase (LVCRFp3.0Cre; Figure S1 in Supplement 1). Cre, a 35-kDa enzyme, recognizes 34-bp sequences known as “LoxP” sites and excises DNA flanked by LoxP sites (“floxed”) via intrachromosomal recombination

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(17,18). The LVCRFp3.0Cre construct was then used to establish a transgenic mouse in which CRF-producing cells also express Cre (CRFp3.0Cre).

Crossing CRFp3.0Cre mice with fluorescent Cre-reporter strains results in expression of green fluorescent protein (GFP), permitting real-time visualization of CRFergic cells for use in single-cell polymerase chain reaction (PCR) and electrophysiologic procedures. Importantly, CRFp3.0Cre mice can also be crossed with available transgenics containing a “floxed” gene; such dual transgenics will improve our understanding of interactions between CRF and other signaling molecules within CRFergic cells under normal and pathologic conditions.

Methods and Materials

CRFp3.0Cre Vector

The CRF promoter (3.0 kb) was PCR-amplified and topo-cloned into pCR2.1-topo, (Invitrogen, Carlsbad, California) (Figure S1 in Supplement 1). A lentivirus backbone, pCMVGFPPdNhe (19) (Inder Verma, Salk Institute, La Jolla, California) was digested to remove the CMVGFP sequence. The linearized backbone was ligated to the 3.0-kb CRF promoter and to the Cre coding sequence, using T4 DNA ligase (New England Biolabs, Ipswich, Massachusetts).

CRFp3.0Cre Mouse

After preliminary tests of promoter functionality and specificity (Figure S2 in Supplement 1), the LVCRFp3.0Cre construct was linearized, purified by electroelution, and diluted to 2 ng/μL for pronuclear microinjection into FVB mouse cells by the Emory University Transgenic Core Facility. F1 offspring, PCR-positive for CRFp3.0Cre, were crossed with one of three Cre-reporter strains, denoted below as CRFp3.0Cre^{LacZ}, CRFp3.0Cre^{GFP}, and CRFp3.0Cre^{mT/mG}. Procedures are approved by Emory University's Institutional Animal Care and Use Committee.

In Situ Hybridization

CRFp3.0Cre mice were sacrificed with CO₂, brains were fresh-frozen and sectioned at 20 μm on a cryostat. In situ hybridization for Cre and CRF transcripts were performed on serial slides using ³⁵S-UTP labeled riboprobes as described previously (20).

X-Gal Staining

CRFp3.0Cre mice were crossed with a strain containing a Floxed-stop-LacZ construct in the Rosa26 locus (Rosa LacZ, Jackson Laboratories, Bar Harbor, Maine). CRFp3.0Cre^{LacZ} offspring were sacri-

Figure 1. Cre-recombinase transcript and enzymatic activity correspond to corticotropin-releasing factor (CRF) transcript expression: in situ hybridization for CRF (**A, C**) and Cre-recombinase (CRFp3.0Cre) (**B, D**) from the central amygdala (CeA, top) and paraventricular nucleus of the hypothalamus (PVN; bottom) of a CRFp3.0Cre mouse. LacZ staining with X-Gal demonstrates functional Cre activity in the CeA (**E**), bed nucleus of the stria terminalis (**F**), and PVN at low (**G**) and high power (**H**) of a CRFp3.0Cre^{LacZ} mouse.

ficed with chloral hydrate and perfused. Twenty-micrometer-thick brain sections were collected in series on SuperFrostPlus slides (Fisher Scientific, Hampton, New Hampshire). Slides were rinsed in phosphate-buffered saline then incubated overnight in X-Gal solution. At least 10 CRFp3.0Cre^{LacZ} mice were examined; their expression patterns are represented in **Figure 1**.

Immunohistochemistry

CRFp3.0Cre was crossed with a fluorescent Cre-reporter strain containing cytoplasmic EGFP downstream of a floxed-stop construct (CAG-Bgeo/GFP, Jackson Laboratories #003920). Immunohistochemistry for CRF peptide was performed on offspring from these matings (CRFp3.0Cre^{GFP}) to verify colocalization of Cre enzymatic activity with CRF peptide.

Electrophysiology

CRFp3.0Cre^{GFP} mice were decapitated under isoflurane anesthesia (Abbott Laboratories, North Chicago, Illinois). Brains were placed in kynurenic acid-based artificial cerebrospinal fluid (ACSF_{KA}) (21–23). Three hundred fifty micrometers coronal slices

were cut, hemisected, transferred to ACSF_{KA}, and gassed with a 95/5% oxygen/carbon dioxide mixture for 1 hour before being placed in oxygenated control ACSF; recordings began 30 min later.

Single-Cell Reverse Transcriptase-PCR

Cytoplasm was aspirated from 25 GFP-positive and eight GFP-negative cells. For each cell, cytoplasm was expelled into a microcentrifuge tube containing reverse transcription (RT) cocktail (Applied Biosystems, Foster City, California). The RT product was amplified in triplicate as previously described (24) and screened for 18 S rRNA. Only cells positive for 18 S rRNA were subjected to amplification with primers for CRF.

Specific methods for each of these procedures are included in Supplement 1.

Results and Discussion

The paramount importance of CRF in the normal and pathologic response to a threatening situation is clear, yet the inner workings of CRF-containing circuits remain poorly understood.

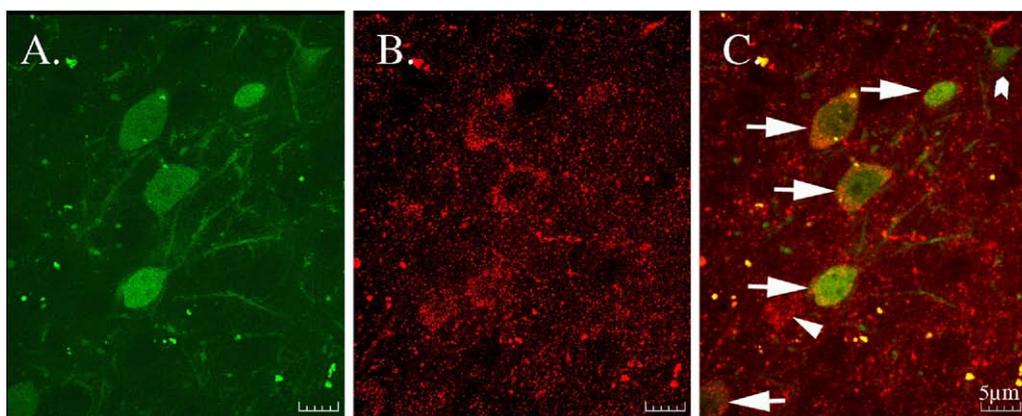


Figure 2. Cre-dependent expression of green fluorescent protein (GFP) colocalizes with corticotropin-releasing factor (CRF) peptide in the central amygdala (CeA). (**A**) endogenous GFP expression in the CeA of a Cre-recombinase (CRFp3.0Cre^{GFP}) mouse (63× magnification); (**B**) immunohistochemistry for CRF (Abcam, rabbit polyclonal anti-CRF); CRF-like immunoreactivity is visualized with AlexaFluor568-tagged secondary antibody. (**C**) Merged image. Arrows demonstrate cells with high levels of coexpression of GFP and CRF. The arrowhead demonstrates the occasional cell that is CRF-positive/GFP-negative. The chevron identifies the occasional cell that is CRF-negative/GFP-positive. See text for discussion.

Achieving restricted transgene expression within CRF-expressing cells in this novel CRFp3.0Cre mouse strain has the potential to expand our understanding of CRF regulation rapidly in a region-specific manner and perhaps contribute to the identification of relevant targets within selective CRF circuits.

As the first step in characterizing this novel strain, expression of CRF transcript was compared with that of Cre in serial slices from F2 and F3-generation CRFp3.0Cre mice. In situ hybridization revealed a high degree of congruence in regional expression of these two transcripts in serial coronal slices ($n = 9$ mice, representative images are shown in Figure 1A–1D).

As a second step in characterizing CRFp3.0Cre mice, Cre enzymatic activity was assessed in CRFp3.0Cre mice crossed with the Rosa-LacZ strain (CRFp3.0Cre^{LacZ}). In these dual transgenics, the pattern of LacZ expression matched that of CRF within the central amygdala (CeA) (Figure 1E), the bed nucleus of the stria terminalis (Figure 1E), and the paraventricular nucleus of the hypothalamus (PVN) (Figure 1F and 1G). A small amount of LacZ-positive cells were scattered within the cortex, consistent

with the pattern of cortical CRF. There was no other concentrated LacZ expression throughout the remainder of the brain and brainstem.

Functional Cre enzymatic activity was also assessed by crossing CRFp3.0Cre mice with a Cre-reporter strain containing a membrane bound EGFP (CRFp3.0Cre^{mT/mG}, Figure S4 in Supplement 1), as well as a reporter strain with cytoplasmic EGFP downstream of a floxed-stop construct (CRFp3.0Cre^{GFP}). Immunohistochemistry for CRF was performed on six slices from four CRFp3.0Cre^{GFP} subjects using a red fluorophore-tagged secondary antibody and compared with endogenous (Cre-mediated) GFP expression. At the cellular level, CRF-like immunoreactivity colocalized with Cre-dependent GFP expression, providing firm support that the 3.0 kb CRF “mini-promoter” targets transgene expression to CRFergic cells (Figure 2). Potential explanations for the occasional CRF-positive/GFP-negative or CRF-negative/GFP-positive cell are discussed subsequently.

In tissue from CRFp3.0Cre^{GFP} transgenics, GFP-expressing neurons have been visualized in real time and targeted for

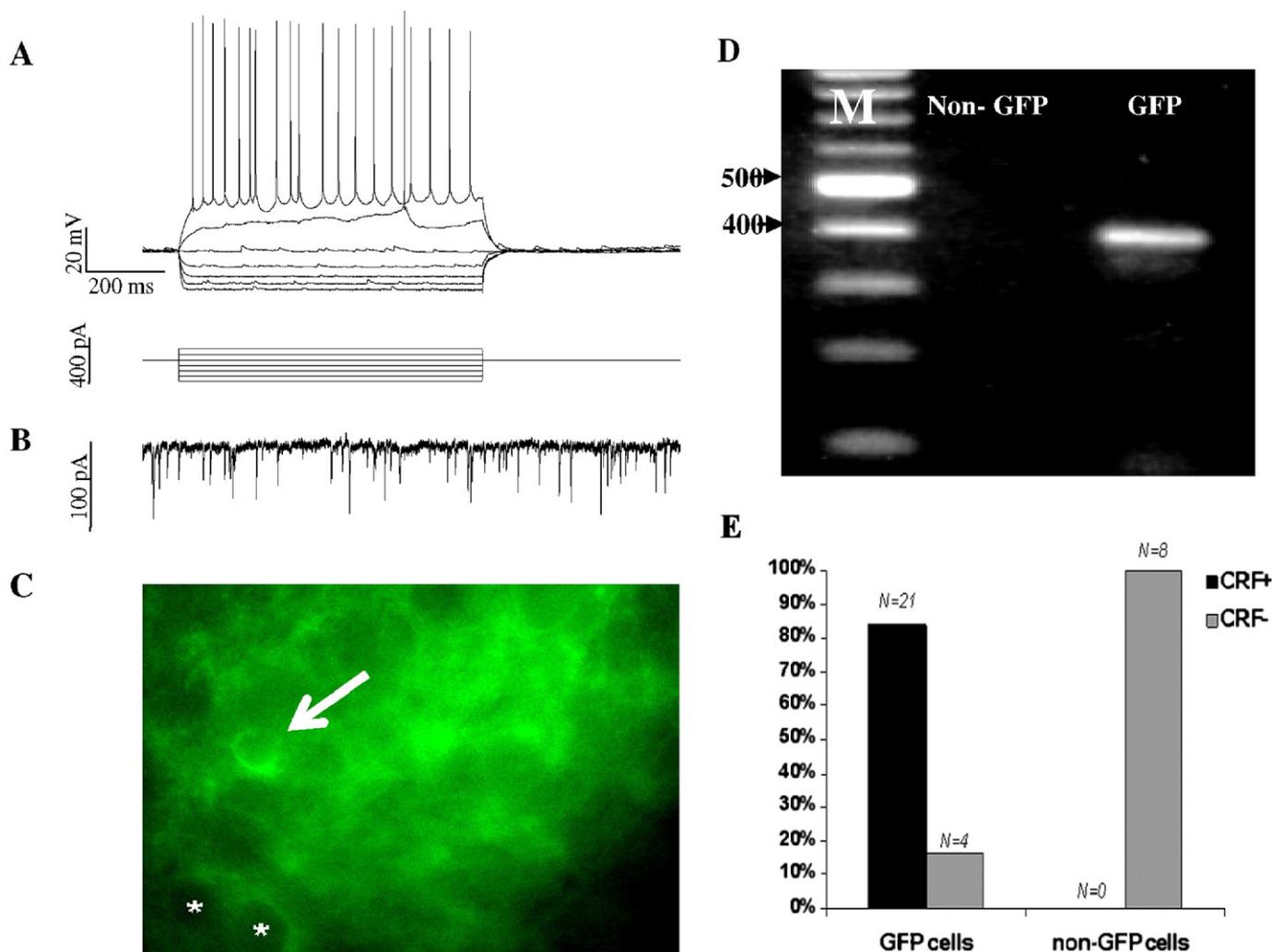


Figure 3. Physiologic and molecular properties of visually identified, polymerase chain reaction (PCR)-verified corticotropin-releasing factor (CRF)ergic cells. (A) voltage response of a CRF-containing neuron to transient (750 msec) hyperpolarizing and depolarizing current injections. (B) CRF neurons are driven by a continuous input of spontaneous excitatory postsynaptic current (EPSC). (C) Photomicrograph of the cell in the recording chamber showing the expression of CRF-driven green fluorescent protein (GFP) in the CeA. Arrow indicates neuron illustrated in A and B. Stars indicate GFP neurons. (D) Single-cell reverse transcriptase (RT)-PCR demonstrating mRNA expression of CRF aspirated from GFP-positive (right lane) but not GFP-negative (center lane) cells in the CeA. The PCR product for CRF is 394 bp; M = 100-bp ladder. (E) Quantification of 33 AA cells aspirated and analyzed with RT-PCR.

electrophysiologic recordings. Acute brain slices were prepared, and recordings were obtained from visually identified GFP-positive (putative CRFergic) CeA neurons in CRFp3.0Cre^{GFP} subjects (Figure 3). Multiple GFP-positive cells have now been recorded from more than 20 CRFp3.0Cre^{GFP} mice; Figure 3A shows a representative trace. These data demonstrate the viability of cell-type-selective promoters to label and characterize electrophysiologic properties of a subpopulation of neurons.

Given the limited quantitative nature of immunohistochemistry, single-cell PCR was our primary method to determine the sensitivity and specificity of the CRFp3.0Cre^{GFP} transgenic line. Of eight GFP-negative cells, none contained CRF mRNA, suggesting high (>90%) specificity of GFP expression for CRF-expressing cells. Of 25 GFP-positive cells, 21 were also CRF-positive, suggesting that approximately 85% of the GFP-expressing cells contain detectable levels of CRF transcript (Figure 3D and 3E).

With all promoter-driven reporter transgenic mice, the main concern is specificity of reporter expression. In the immunohistochemistry experiment, at least one cell with CRF-like immunoreactivity lacks concurrent expression of GFP (arrowhead in Figure 2). This CRF-positive/GFP-negative cell may have recently begun to express CRF and had not had time to produce detectable levels of GFP. In the RT-PCR experiment, lack of CRF transcript in four of the GFP-expressing cells can be explained by the sensitivity and irreversibility of Cre-mediated DNA excision; after Cre-mediated excision has occurred, GFP expression is not dependent on continued activation of the CRF promoter; expression of the GFP construct will continue even if the cell no longer expresses CRF. The detection limits of the single cell RT-PCR approach could also contribute to the finding of GFP-positive/CRF-negative cells.

To summarize, Cre expression in this novel strain exhibits extremely high regional specificity and 85% to 90% sensitivity and specificity for CRFergic cells. This is true even within the CeA, a region of dense CRF expression. Thus, we are confident that the CRFp3.0Cre mouse reliably produces Cre within CRFergic cells. Ongoing experiments using CRFp3.0Cre mice crossed with fluorescent Cre-reporters as well as other “floxed” transgenics continue to support this conjecture.

The utility of the CRFp3.0Cre strain is twofold: 1) when crossed with a Cre-reporter strain, CRF cells are easily identifiable and distinguishable from neighboring cells for the purposes of single cell mRNA isolation and RT-PCR, laser capture, fluorescence-activated cell sorting, and intracellular recording; 2) crossing CRFp3.0Cre with extant mouse strains containing “floxed” gene constructs will extend knowledge of interactions between CRF and other signaling molecules. These properties will be employed to clarify basic cellular biology of CRF-producing neurons and stress-induced alterations in CRFergic cells, which may contribute to the pathophysiologic conditions attributed to CRF overexpression.

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Dr. Nemeroff currently serves on the scientific advisory boards of American Foundation for Suicide Prevention (AFSP); AstraZeneca; NARSAD; and PharmaNeuroboost. He holds stock/equity in Corcept, Revaax, NovaDel Pharma, CeNeRx, and PharmaNeuroboost. He is on the board of directors of the AFSP; NovaDel Pharma, and Mt. Cook Pharma, Inc. Dr. Nemeroff holds a patent on the method and devices for transdermal delivery of lithium (US 6375990 B1) and the method to estimate serotonin and norepinephrine transporter occupancy after drug treatment using patient or animal serum (provisional filing April 2001). In the past year, he also served on the Scientific Advisory Board for Forest Laboratories and received grant support from NIMH, NARSAD, and AFSP.

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Supplementary material cited in this article is available online.

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