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Diversity of Reporter Expression Patterns in Transgenic Mouse Lines Targeting Corticotropin-Releasing Hormone-Expressing Neurons

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Transgenic mice, including lines targeting corticotropin-releasing factor (CRF or CRH), have been extensively employed to study stress neurobiology. These powerful tools are poised to revolutionize our understanding of the localization and connectivity of CRH-expressing neurons, and the crucial roles of CRH in normal and pathological conditions. Accurate interpretation of studies using cell type-specific transgenic mice vitally depends on congruence between expression of the endogenous peptide and reporter. If reporter expression does not faithfully reproduce native gene expression, then effects of manipulating unintentionally targeted cells may be misattributed. Here, we studied CRH and reporter expression patterns in 3 adult transgenic mice: *Crh-IRES-Cre;Ai14* (tdTomato mouse), Crfp3.0CreGFP, and Crh-GFP BAC. We employed the CRH antiserum generated by Vale after validating its specificity using CRH-null mice. We focused the analyses on stress-salient regions, including hypothalamus, amygdala, bed nucleus of the stria terminalis, and hippocampus. Expression patterns of endogenous CRH were consistent among wild-type and transgenic mice. In tdTomato mice, most CRH-expressing neurons coexpressed the reporter, yet the reporter identified a few non-CRH-expressing pyramidal-like cells in hippocampal CA1 and CA3. In Crfp3.0CreGFP mice, coexpression of CRH and the reporter was found in central amygdala and, less commonly, in other evaluated regions. In Crh-GFP BAC mice, the large majority of neurons expressed either CRH or reporter, with little overlap. These data highlight significant diversity in concordant expression of reporter and endogenous CRH among 3 available transgenic mice. These findings should be instrumental in interpreting important scientific findings emerging from the use of these potent neurobiological tools. (*Endocrinology* 156: 4769–4780, 2015)

Transgenic rodent models enabling gene-based access to specific cell populations provide potent tools for neuroscience research. The use of Cre-driver lines in combination with Cre-dependent methods for the regulation of gene expression, visualization of reporters or optogenetic activation/inhibition has been extremely useful. These combined methods have yielded a large body of innovative discoveries in brain connectivity and in the contributions of specific neuronal populations, and of molecules produced in specific regions, to crucial brain functions, including feeding (1), reward and addiction (2, 3), memory (4), and depression (5).

Transgenic mouse models have been extensively employed in the study of the neurobiology of stress, and have included approaches targeting the stress neuropeptide corticotropin-releasing factor (CRF or CRH) via its deletion or overexpression (6–9). CRH-expressing neurons are highly diverse throughout the central nervous system. For example, in the hypothalamic paraventricular nucleus (PVN), virtually all CRH-expressing cells are non-GABAergic. In contrast, CRH cells are essentially all GABAergic interneurons in adult hippocampus (10–12). In other brain regions, CRH-expressing cells form a mixed

Abbreviations: BNST, bed nucleus of the stria terminalis; CRF, corticotropin-releasing factor; DAT, dopamine transporter; GABA, γ-aminobutyric acid; GFP, green fluorescent protein; ICC, immunocytochemistry; ir, immunoreactive; PB, phosphate buffer; PBS-T, PBS containing 0.3% Triton X-100; PV, parvalbumin; PVN, hypothalamic paraventricular nucleus; WT, wild type.

Table 1. Antibody Table

Peptide/ Protein Target	Antigen Sequence	Name of Antibody	Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody	Species Raised in; Monoclonal Polyclonal	Dilution Used
CRH		Anti-human/rat CRH	Paul E. Sawchenko, Salk Institute	Rabbit; polyclonal	1:20 000–40 000
GFP		Anti-GFP	Sigma, product G6539	Mouse; monoclonal	1:2000
PV		Anti-fish PV	Chemicon, catalog MAB1572	Mouse; monoclonal	1:40 000
Calretinin		Anti-rat calretinin	Chemicon, catalog MAB1568	Mouse; monoclonal	1:20 000
Secondary		Anti-rabbit IgG-horseradish peroxidase	PerkinElmer, NEF812001EA	Goat	1:1000
Secondary		Anti-rabbit IgG-Biotin	Vector Laboratories, catalog BA-1000	Goat	1:400
Secondary		Anti-mouse IgG-Biotin	Vector Laboratories, catalog BA-9200	Goat	1:400
Secondary		Anti-mouse IgG-Alexa Fluor 488	Invitrogen, catalog A11001	Goat	1:400

population, such as in the bed nucleus of the stria terminalis (BNST) (13). The heterogeneity of the CRH-expressing cell populations has necessitated manipulation of the CRH gene promoter itself, and this has been accomplished using a variety of technologies. These have included IRES-CRE (14), BAC technologies (15–17), or direct Cre-Flox targeting of discrete regions of the CRH gene promoter (18). In addition to enabling viral-mediated targeting of these cells (19), the resulting CRH-targeted lines have been crossed to a variety of reporters, including green fluorescent protein (GFP) and Ai9 (tdTomato), to generate mice with "visible" CRH-expressing cells. These neuronal populations are thus rendered amenable to electrophysiology and/or optogenetic or chemical/genetic manipulations (eg, designer receptors exclusively activated by designer drugs).

Collectively, these approaches have confirmed and extended information about the localization, nature, and connectivity of CRH-expressing cells (20) and are poised to revolutionize our understanding of the role of selective populations of CRH cells in a number of fundamental physiological and pathological phenomena (21). These include stress-related anxiety (22), memory problems (23, 24), addiction-relapse (25, 26), posttraumatic stress disorder (22), and potentially other stress-associated conditions such as anhedonia and anorexia nervosa.

Accurate interpretation of studies using cell type-specific transgenic mice is vitally dependent on the degree of congruence between the expression of the endogenous, native peptide and of the transgene or reporter. If Cre or reporter expression does not reproduce native gene expression faithfully, for example, if Cre and reporter expression occur in cells that do not express CRH and vice versa, then, the effects of manipulating unintentionally targeted cells may be misattributed. This issue is especially significant in the case of CRH for peptide-expressing cell populations in the PVN, the amygdala, BNST, and hippocampus. Therefore, we focus here on these neuronal populations.
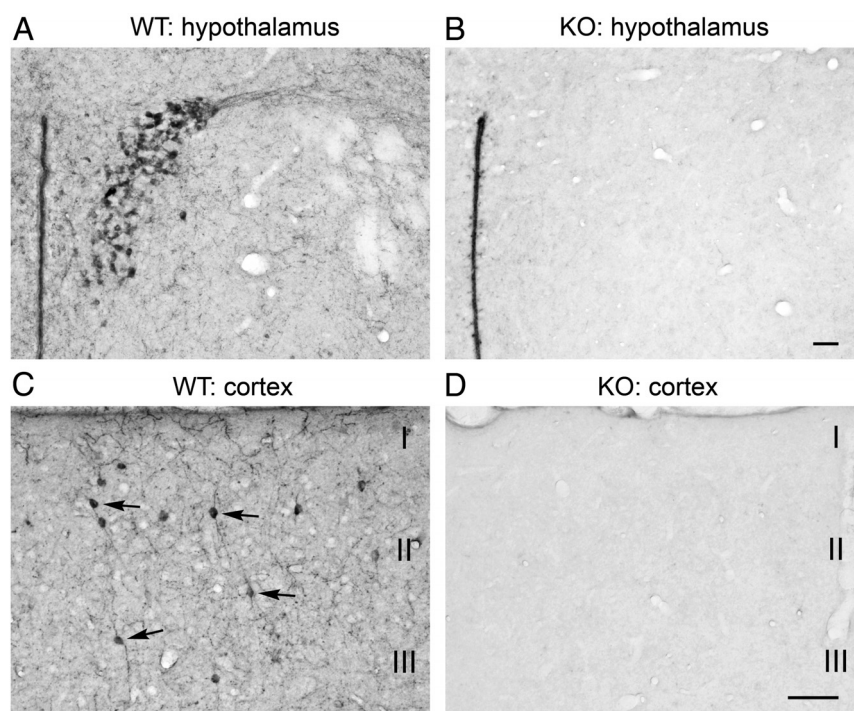

Figure 1. CRH-ir neurons in adult C57BL/6J mouse (WT) vs CRH-null mouse (KO). A and B, In the hypothalamus, CRH-ir neurons are apparent in the parvocellular subregion of the PVN in WT mice, but no signal is detected in KO mice. C and D, Abundant CRH-ir neurons with a bipolar shape (arrows) are evident in layers II and III of the neocortex (motor area) in WT mice but not in KO mice. Scale bars, 100 μm.

Materials and Methods

Animals

All experiments were carried out according to National Institutes of Health guidelines for the care of experimental

animals, with approval by the University of California Institutional Animal Care and Use Committee. Male C57BL/6J mice (3–4 mo) and transgenic mice were housed on a 12-hour light, 12-hour dark schedule (lights on at 7 AM) with ad libitum access to food and water.

Male adult (3–4 mo) mice of 3 transgenic lines were used in these studies

Crh-IRES-Cre;Ai14 *tdTomato mouse (14, 20)*

The tdTomato (*Crh-IRES-Cre;Ai14*) mouse was generated by crossing B6(Cg)-Crh[tm1(cre)Zjh]/J (*Crh-IRES-Cre*) mouse and B6.Cg-Gt(ROSA)26Sor[tm14(CAG-tdTomato)Hze]/J (*Ai14*) mouse. The *Crh-IRES-Cre* mouse and *Ai14* mouse were obtained from The Jackson Laboratory (stock numbers 012704 and 007914, respectively). These mice were maintained as colonies of homozygous mice, with 1 backcrossing to the C57BL/6J background strain after their arrival. Pairs of either homozygous *Crh-IRES-Cre* or *Ai14* genotypes were mated, and the resulting F1 heterozygous *Crh-IRES-Cre;Ai14* male offspring were evaluated.

Crfp3.0CreGFP *transgenic mouse (18)*

The generation of the CRFp3.0CreGFP transgenic mouse has been described in a previous publication (18). Briefly, a CRFp3.0Cre vector was first created by using a lentivirus back-bone, pCMVGFPdNhe (27), and the linearized backbone was ligated to a 3.0-kb CRF promoter and to a Cre coding sequence, using T4 DNA ligase (New England Biolabs). To generate CRFp3.0Cre mouse, 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. The CRFp3.0Cre F1 offspring were crossed with a fluorescent Cre-reporter strain containing cytoplasmic enhanced green fluorescent protein downstream of a floxed-stop construct (CAG-Bgeo/GFP, 003920; The Jackson Laboratory), and their F1 offspring CRFp3.0CreGFP were used in the current studies.

Crh-GFP BAC *transgenic mice (15, 28)*

The generation of CRH-GFP transgenic mice, in which GFP expression was under the transcriptional control of the CRH promoter, has been described (15). Briefly, the CRH-GFP transgenic mice expressing τ-topaz GFP under the transcriptional control of the CRH promoter were generated using BAC transgenic technology (29). The GFP transgene was introduced into the ATG site of the Crh BAC (BAC ID number 397J12) by homologous recombination. The GFP transgene included a τ-GFP fusion protein, followed by a poly(A) signal. Tau, a bovine microtubule binding protein, was used to increase axonal labeling by GFP. BAC filters (BAC mouse II) were obtained from Genome Systems. The CRH-GFP construct was cloned into the shuttle vector PSV1 for the BAC modification. The shuttle vector has 0.6 kb upstream and 0.5 kb downstream arms of CRH sequence flanking the GFP transgene. The shuttle vector was transformed into a DH10B *Escherichia coli* host harboring the CRH BAC. After 2 homologous recombination events, the modified CRH BAC construct was selected, and microinjected into the pronucleus of fertilized oocytes from a CBA/C57BL/6 F1 mouse strain to generate 4 transgenic founder lines using the Rockefeller University transgenic facility. Founder animals were mated with C57BL/6J (The Jackson Laboratory) mice to generate F1 progeny, which was used in the current studies.

Antibody characterization

The antibodies used in this study are described below and in the antibody table (Table 1). For CRH, this was a rabbit anti-human/rat CRH antiserum (Code PBL rC68) provided as a gift from the antiserum resource center (Dr Paul E. Sawchenko, Director, Salk Institute, La Jolla, CA). The antiserum had been absorbed with 2-mg human α-globulin and 1-mg alpha-melanocyte stimulating hormone per milliliter of serum. Detailed assessment of its specificity is provided in the Results section.

Tissue preparation

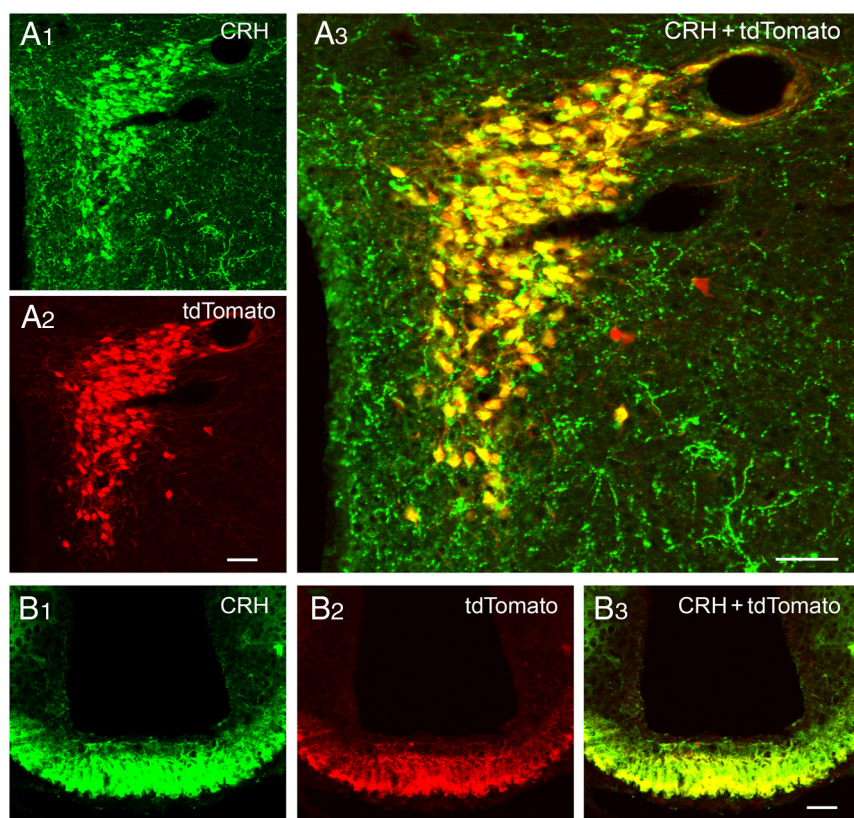To prepare fixed brain tissue, mice (n = 4–5 per strain) were anesthetized as

Figure 2. The expression of tdTomato reporter in the PVN and median eminence in the *Crh-IRES-Cre;Ai14* tdTomato mouse. A, In the PVN, the vast majority of reporter-expressing neurons (tdTomato) in the parvocellular subregion coexpress endogenous CRH. B, In the median eminence, parvocellular CRH-expressing neurosecretory neuron axons terminated within the external layer. A similar pattern was apparent for tdTomato-expressing terminals. Scale bars, 50 μm.

much as is possible under stress-free conditions with sodium pentobarbital (40 mg/kg). This approach prevented stress-induced release of native CRH from somata to axons and obviated the need for colchicine. Mice were transcardially perfused via the ascending aorta with 0.9% saline solution followed by perfusion with 4% paraformaldehyde solution made in 0.1M phosphate buffer (PB) (pH 7.4; 4°C). Brains were postfixed in the perfusion-used fixative for 2–4 hours (4°C) and immersed in 15%, followed by 25% sucrose for cryoprotection. Brains were blocked in the coronal or sagittal planes and sectioned at 20-μm thickness using a cryostat. In each plane, 1 in 4 serial sections were subjected to CRH-immunocytochemistry (ICC), and an adjacent series of sections was stained with cresyl violet or 4′,6-diamidino-2-phenylindole. The others were used for double labeling ICC. Perfusion-fixed BAC mouse brains were shipped in 25% sucrose in 0.1M PB, courtesy of Professor J. M. Friedman and Dr T. Alon (Rockefeller University, New York, NY).

ICC of brain slices

CRH-ICC was performed on free-floating sections using standard avidin-biotin complex methods, as described previously (11). Briefly, after several washes with PBS containing 0.3% Triton X-100 (PBS-T) (pH 7.4), sections were treated with 0.3% H_2O_2/PBS for 30 minutes, then blocked with 5% normal goat serum for 30 minutes in order to prevent nonspecific binding. After rinsing, sections were incubated for 36 hours at 4°C with rabbit anti-CRH antiserum (1:40 000) (Table 1) in PBS containing 1% BSA and washed in PBS-T (3 × 5 m). Sections were incubated with biotinylated goat-anti-rabbit IgG (1:400; Vector Laboratories) in PBS for 2 hours at room temperature. After washing (3 × 5 m), sections were incubated with the avidin-biotin-peroxidase complex solution (1:200; Vector Laboratories) for 3 hours, rinsed (3 × 5 m), and reacted with 0.04% 3,3′-diaminobenzidine containing 0.01% H_2O_2.

To assess the coexpression of potentially low levels of CRH in reporter-expressing neurons, concurrent visualization of CRH peptide and GFP was performed using the tyramide signal amplification technique (30). Sections were incubated overnight (4°C) with CRH rabbit antiserum (1:20 000), then treated with horseradish peroxidase-conjugated anti-rabbit IgG (1:1000; PerkinElmer) for 1.5 hours. Fluorescein or cyanine 3-conjugated tyramide was diluted (1:150) in amplification buffer (PerkinElmer) and was applied in the dark for 5–6 minutes. After CRH detection, sections were exposed to GFP antiserum overnight at 4°C, and immunoreactivity was visualized using anti-mouse IgG conjugated to Alexa Fluor 488 (1:400; Invitrogen).

Concurrent immunolabeling of CRH and parvalbumin (PV) or calretinin was performed as described in detail previously (11). Briefly, sections were first incubated for 2–3 days at 4°C with rabbit anti-CRH antiserum (1:40 000) in PBS containing 1% BSA, yielding a diffuse brown 3,3′-diaminobenzidine reaction product. Sections were then rinsed in PBS-T, preincubated in 5% normal goat serum, and exposed to mouse anti-PV (1:40 000; Chemicon) or anti-calretinin antibodies (1:20 000; Chemicon) overnight at room temperature, followed by the biotinylated second antibody and avidin-biotin-peroxidase complex solutions as described above. To visualize PV or calretinin antibody binding, sections were rinsed, transferred to a 1× acidic buffer (pH 6.2), and then incubated in reaction buffer containing benzidine dihydrochloride and H_2O_2 (Bioenno Tech) for 5–6 minutes. The reaction stopped by rinsing in 0.01M PB containing 0.1% Triton X-100 (pH 6.2).
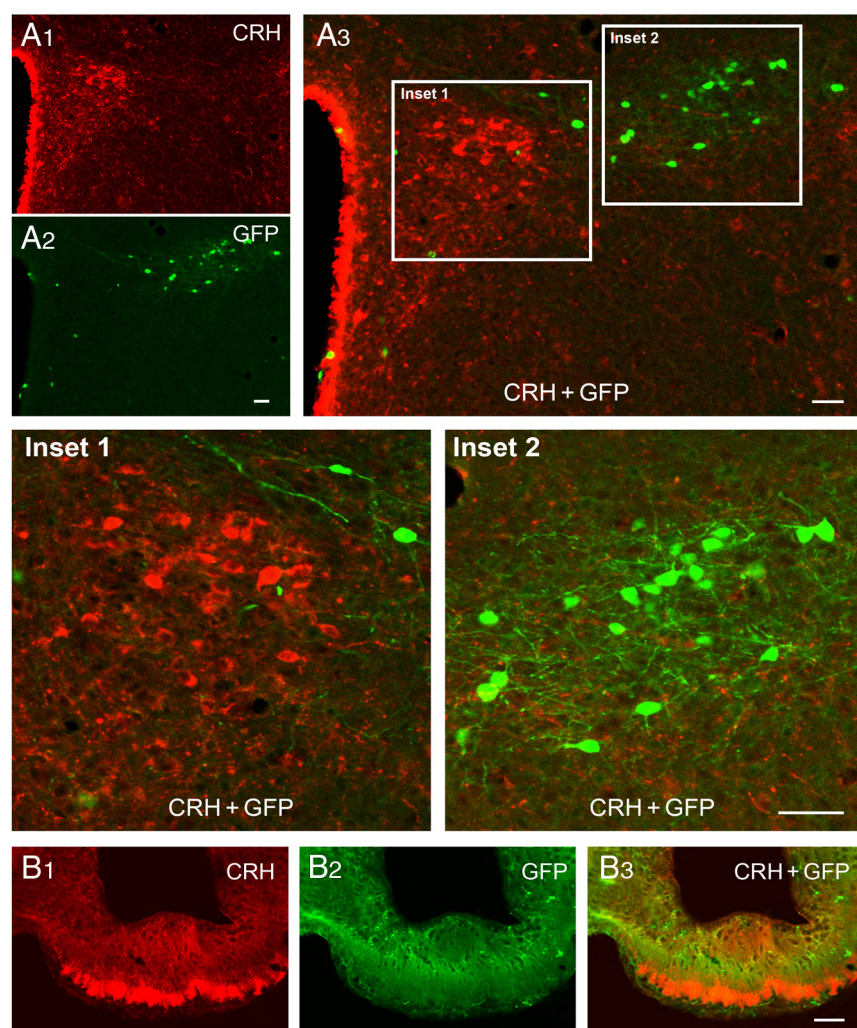

Figure 3. Expression patterns of the GFP reporter and endogenous CRH in the PVN of the Crf.p3.0CreGFP mouse. A, Dual-labeling ICC for CRH (red) and the GFP reporter (green). Two distinct populations of neurons were visualized in the hypothalamus: CRH-ir neurons were located in the dorsomedial parvocellular division, whereas GFP reporter-expressing neurons tended to reside more laterally. Boxed areas in A3 were magnified. B, In the median eminence, a partial overlap of CRH-expressing terminals and GFP reporter-expressing terminals was observed in external layer of this structure. Scale bars, 50 μm.

Imaging and analysis

Brain sections were visualized on a Nikon Eclipse E400 epifluorescence microscope equipped with fluorescein, rhodamine, and 4',6-diamidino-2-phenylindole filter sets. Light microscope images were obtained using a Nikon Digital Sight camera controlled by NIS-Elements F software (version 3.0; Nikon Instruments, Inc). Confocal images were taken using an LSM-510 confocal microscope (Zeiss) with an Apochromat ×63 oil objective (numeric aperture, 1.40). Virtual z-sections of less than 1 μm were taken at 0.2- to 0.5-μm intervals. Image frame was digitized at 12 bit using a 1024 × 1024 pixel frame size. To prevent bleed-through in dual-labeling experiments, images were scanned sequentially (using the "multitrack" mode) by 2 separate excitation laser beams: an Argon laser at a wavelength of 488 nm and a He/Ne laser at 543 nm. Z-stack reconstructions and final adjustments of image brightness were performed using ImageJ software (version 1.43; NIH). For the cell counting ex-ample in the PVN of the tdTomato mouse, we first used ×20 confocal images. A square lattice system over the entire parvocellular PVN was used, and cells further verified under ×63 magnification. For each animal, 2–3 sections per PVN were counted, and a total of 4 *Crh-IRES-Cre;Ai14* (tdTomato) mice were used to calculate the final cell numbers and overlap ratios.

Results

Validation of the anti-CRH serum and expression pattern of the peptide in adult mouse

We employed here the anti-human/rat CRH serum (rC68) created by Dr Wylie Vale (31). This antiserum has been well characterized by numerous groups (eg, 20, 32). To definitively establish the specificity of the antiserum, we followed the recommendations established by Saper and Sawchenko (33) and conducted ICC on naive C57BL6/J mice in comparison with mice lacking CRH (CRH-null, courtesy of Professor J.A. Majzoub, Harvard, Boston, MA). Expression of CRH was clearly apparent in the PVN, within subregions of the nucleus containing the parvocellular group (Figure 1A). These findings are in line with elegant work in the rat (34) and, more recently, in the mouse (35). No immunoreactive (ir) signal was evident in the CRH-null mice (Figure 1B). A similar pattern was apparent in the cortex (Figure 1C): CRH was abundantly expressed in bipolar neurons consistent with interneurons, as described before (36, 37). These neurons were not visible in the CRH-null mouse (Figure 1D).

Diversity of reporter-expressing neurons in the hypothalamus and median eminence of transgenic mice

Because of the crucial role of hypothalamic CRH in initiating the neuroendocrine response to stress, we focused initially on the concordance of native peptide and reporter expression patterns in the PVN. In the tdTomato (*Crh-IRES-Cre;Ai14*) mouse, the distribution pattern of both reporter and native CRH re-
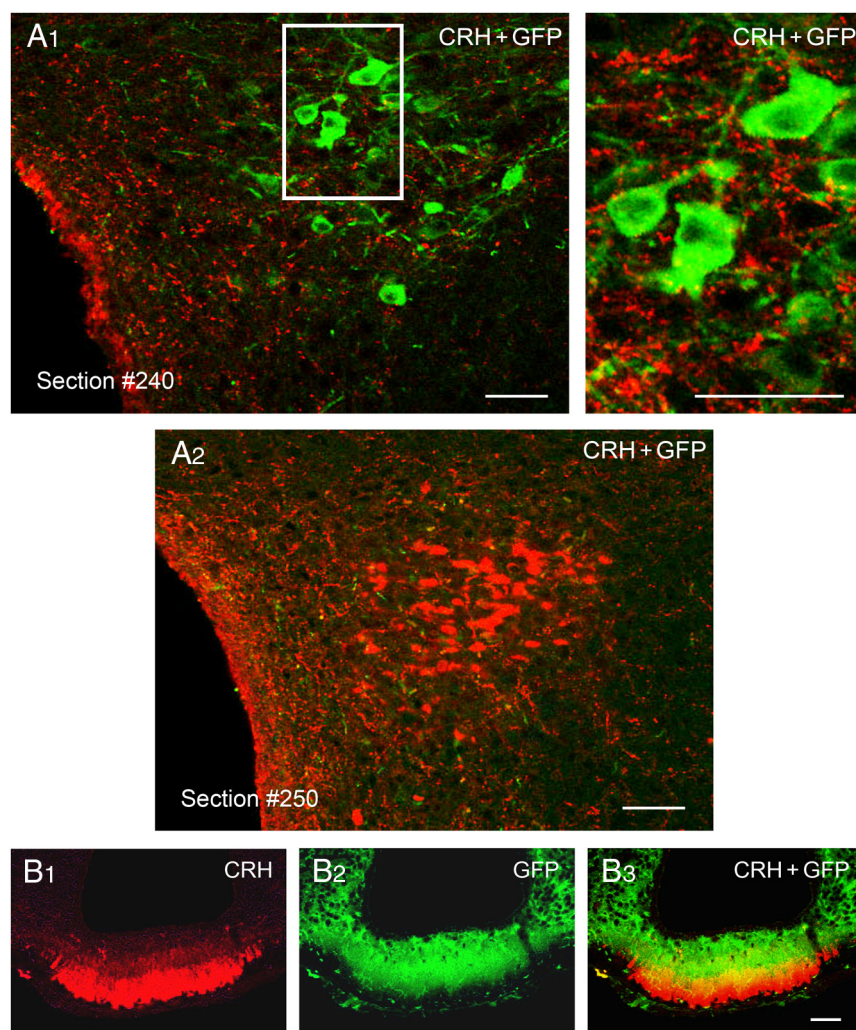

Figure 4. Expression patterns of the GFP reporter and endogenous CRH in the hypothalamus in the BAC transgenic mouse. A, A group of GFP reporter-expressing neurons was detected at the anterior level of the hypothalamus, at which CRH-expressing terminals were abundant, but no CRH-ir parvocellular cell bodies; 200 μm posterior to this level, CRH-ir parvocellular cells in the anterior PVN were apparent. However, no GFP reporter-expressing neurons were detected. B, In accordance with the termination of CRH-expressing cell in naïve mouse and rat, CRH-ir terminals were apparent in the external layer of the median eminence. In contrast, the GFP reporter signal was visible in the inner layer of the structure. Scale bars, 50 μm.

sembled the distribution in wild-type (WT) C57BL6/J mice, and there was excellent congruence of the reporter signal and CRH-ir (Figure 2, A1–A3), in line with the report by Wamsteeker Cusulin et al (20). Specifically, in the parvocellular subdivision of the PVN, CRH expression was observed in 93.3 ± 1.2% of the tdTomato neurons, and 95.1 ± 1.2% CRH-expressing somata coexpressed tdTomato. These data are well in accord with (20), in which colchicine was used. In that analysis, CRH immunoreactivity was observed in 80.5 ± 1.1% of the tdTomato neurons, and 96.0 ± 0.3% of somata containing CRH coexpressed tdTomato. As noted by those authors, both the reporter and native CRH were transported to the external layer of the median eminence (Figure 2B).

In the Crf.p3.0CreGFP mouse, dual ICC for CRH (red) and the GFP reporter revealed a more complex picture (Figure 3). Native CRH and the reporter were clearly visible within the same hypothalamic subregion, eg, the dorsomedial parvocellular division (11, 38). In general, reporter-expressing neurons tended to reside more laterally than those expressing native CRH, and there was limited overlap of the 2 cell groups. Both the native peptide and the reporter seem to be transported to the external later of the median eminence, consistent with the neuroendocrine identity of these cell populations (Figure 3B).

Evaluation of the hypothalamus of the Crh-BAC transgenic mouse after dual labeling ICC for CRH and the GFP reporter identified both CRH-expressing and reporter-expressing neuronal populations. However, these tended to reside in distinct rostrocaudal levels (eg, sections 240 and 250, which were 200 μm apart) (Figure 4). In general, reporter-expressing neurons appeared larger (magnocellular), and, unlike the native peptide, reporter was apparent in the inner layer of the median eminence (Figure 4B).

Heterogeneity of reporter-expressing neurons in the amygdala of transgenic mice

In the adult naïve C57BL6/J mouse (Figure 5A), CRH-expressing neurons reside primarily in the central nucleus of the amygdala (39), where they contribute greatly to the central responses to stress, as well as anxiety and depression (eg, 40–43). CRH-ir cell bodies (Figure 5A, inset) were less prominent than a dense networks of ir fibers (Figure 5A), consistent with previous reports in rodents (10, 24, 44–46). This pattern was largely recapitulated in the *Crh-IRES-Cre;Ai14* tdTomato mouse (Figure 5B), where the large majority of cell bodies and fibers seemed to coexpress the reporter and the native peptide. In the Crfp3.0CreGFP mouse, coexpression of CRH-ir and the GFP reporter was common in both neurons (arrowheads in Figure 5C, right panel) and fibers. In the Crh-BAC
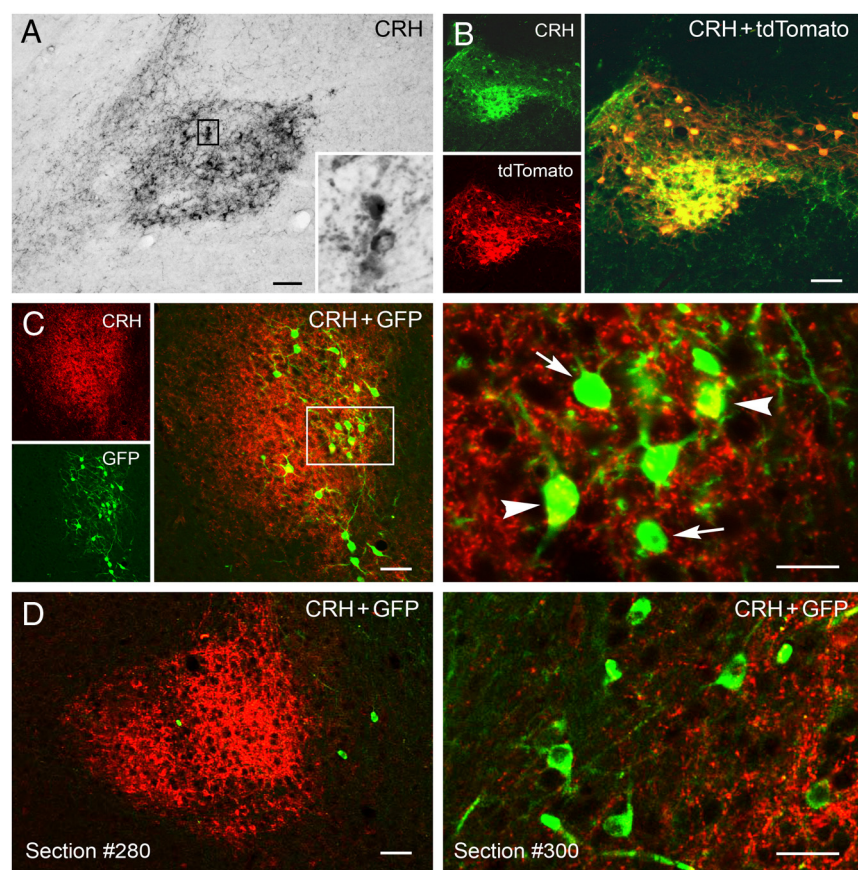

Figure 5. Diversity of reporter-expressing neurons in the central amygdala of transgenic mice. A, In the adult C57BL/6J mouse, CRH-expressing neurons resided primarily in the central nucleus of the amygdala, where ir cell bodies (inset) and a dense mesh of CRH-ir fibers/terminals were apparent. Scale bar, 100 μm. B, The distribution pattern of CRH expression (green) in the central amygdala was largely recapitulated in the *Crh-IRES-Cre;Ai14* tdTomato mouse. The vast majority of tdTomato reporter-expressing neurons coexpressed the native peptide. Scale bar, 50 μm. C, In the Crfp3.0CreGFP mouse, both native CRH (red) and reporter-expressing neurons (green) were apparent. Images shown are within the posterior central amygdala. Confocal high magnification and thin serial sections (0.2–0.5 μm of thickness) revealed a partial overlap of native CRH and reporter for both cell bodies and fibers. A magnification of the boxed area in the middle panel is shown on the right, scanned at 0.5 μm of virtual sections. This method enabled visualization of clear colocalization (arrowheads) of CRH and reporter. Arrows denote lack of overlap. Scale bars, 50 μm (middle) and 20 μm (right). D, In the Crh-BAC transgenic mouse, sections of the central amygdala that harbored most CRH-ir soma and fibers had few reporter-expressing neurons (left). GFP reporter-expressing neurons were apparent in the posterior level of central amygdala, yet the signal did not overlap with CRH-ir neurons (right). Scale bars, 60 μm (left) and 30 μm (right).

transgene, sections of the central amygdaloid nucleus that harbored most CRH-expressing cell bodies and fibers had few GFP-positive cells (Figure 5D, left panel). Most reporter-expressing neurons resided in more caudal sections (Figure 5D, right panel).

Heterogeneity of reporter-expressing neurons in the BNST of transgenic mice

In the naïve adult mouse, the BNST harbors one of the largest concentration of CRH-expressing neurons (39); and these contribute to the integration of stress and emotional functions (eg, 42, 47, 48). The distribution of CRH-ir neurons and fibers in both anterior and posterior BNST subdivisions was evident in WT adult mouse (Figure 6, A1 and A2, respectively). In the *Crh-IRES-Cre;Ai14* tdTomato mouse, a dense network of CRH-ir fibers was noted medially, and most neurons and fibers in the anterior division seemed to coexpress the reporter and the native peptide (Figure 6B, note arrowheads in the enlarged inset). The same dense network of CRH-ir fibers was observed in adult Crfp3.0CreGFP mice, with a more limited coexpression of native peptide and reporter, seen better in the posterior subdivision (Figure 6C, arrowheads). We did not have access to BNST sections from the Crh-BAC transgenic mouse.

Diversity of reporter-expressing neurons in the hippocampus of transgenic mice

Hippocampal CRH-expressing interneurons have been reported originally by Sakanaka et al (37), and we have characterized their ontogeny and distribution in immature and adult rat (11, 12, 32). These neurons play a role in stress-related memory changes, and especially in cognitive defects observed after both early-life and adult stress (23, 49). In the adult C57BL6/J mouse, CRH-ir neurons were clearly apparent in the pyramidal cell layers of both areas CA1 and CA3 (Figure 7A and insets) as well as in strata radiatum and oriens. Dual ICC showed a similarly heterogeneous population of CRH-ir neurons in the *Crh-IRES-Cre;Ai14* tdTomato mouse (Figure 7B). The majority, but not all, of CRH-ir cells coexpressed the reporter (arrowheads). Very few hippocampal neurons of any type expressed GFP in the Crfp3.0CreGFP mice (Figure 7C). This was not a result of absence of CRH, because both cell bodies and fibers expressing the native peptide were visible in these mice. The reduced reporter expression might derive from the relatively short promoter used for the generation of the transgene, which may not enable tissue-specific hippocampal expression (39, 50). In dual-labeled hippocampal sections from Crh-BAC transgenic mice, both CRH-ir neu-
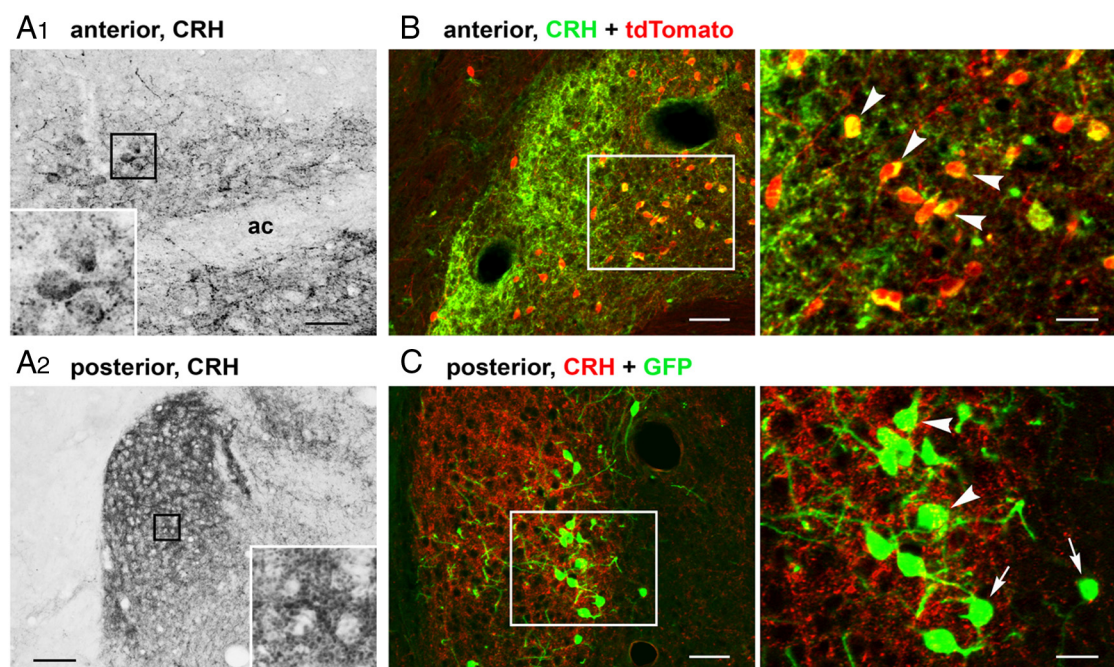

Figure 6. Expression patterns of native CRH and of reporters in the BNST. A, CRH-ir neurons and fibers in the anterior (A1) and posterior (A2) BNST of adult C57BL/6J mice. Cell bodies (inset in A1) of CRH-ir neurons were apparent in the dorsolateral subdivision of anterior BNST, whereas dense networks of ir axon terminals (inset in A2) were found in the posterior region. ac, anterior commissure. Scale bars, 100 μm (A1) and 200 μm (A2). B, In the anterior BNST, the distribution pattern of CRH expression in naïve mouse was recapitulated in the *Crh-IRES-Cre;Ai14* tdTomato mouse. Most reporter-expressing neurons in the dorsolateral subdivision coexpressed the native peptide (arrowheads). Scale bars, 50 μm (left) and 20 μm (right). C, In the posterior BNST, a group of reporter-expressing neurons was observed in the Crfp3.0CreGFP mouse, with a limited coexpression (arrowheads) of native peptide. Arrows point reporter expression only. Scale bars, 50 μm (left) and 20 μm (right). Boxed areas in B and C were magnified to show the colocalization. BNST sections were not available for the CRH-BAC mouse.
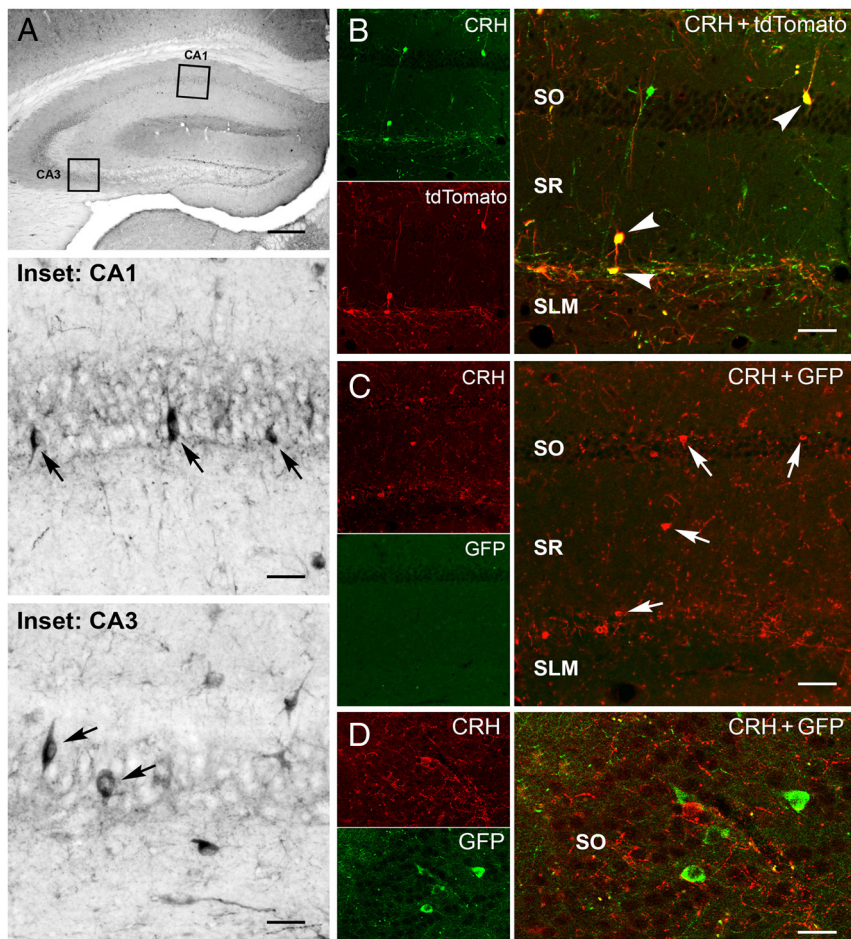
Figure 7. Patterns of CRH- and reporter-expressing neuronal distribution in the hippocampus of naïve and 3 transgenic mice. A, The distribution and structure of CRH-ir neurons in the hippocampus of adult C57BL/6J mouse. Boxed areas in the top panel were magnified in the insets. CRH-expressing neurons (arrows) in CA1 and CA3 pyramidal cell layers appear eccentric, bipolar, and possess a network of terminals surrounding the unlabeled pyramidal cells. Additionally, a heterogeneous population of elongated and multipolar interneuronal-like cells expressing CRH are visible. Scale bars, 500 μm (top) and 32 μm (middle and bottom). B, In the *Crh-IRES-Cre;Ai14* tdTomato mouse, the large majority of CRH-ir neurons coexpress the tdTomato reporter (see more detailed analysis in Figure 8). Arrowheads point the colocalization. SO, stratum oriens; SP, stratum pyramidale; SLM, stratum lacunosum-moleculare. Scale bar, 50 μm. C, In the Crfp3.0CreGFP mouse, reporter-expressing neurons were sparse in area CA1 as well as in area CA3 (data not shown). Arrows point to CRH-ir neurons. Scale bar, 50 μm. D, In the Crh-BAC transgenic mouse, both CRH-ir neurons/fibers and GFP reporter-expressing neurons/fibers were clearly apparent. However, most GFP-positive cells appeared pyramidal in structure, and there was no overlap with CRH-ir neurons. Scale bar, 30 μm.

rons and fibers as well as GFP reporter-expressing neurons and fibers were clearly apparent. However, most GFP-positive cells appeared pyramidal in structure, and overlap with native CRH was minimal (Figure 7D).

A more detailed analysis of the *Crh-IRES-Cre;Ai14* tdTomato mouse (Figure 8A) suggested that although the diverse, heterogeneous interneuronal populations coexpressed the native peptide and the reporter (arrowheads), pyramidal-like cells tended to express the reporter only, in the absence of endogenous CRH (Figure 8A, arrow). CRH-expressing interneuron populations in the hippocampus have been described in rat, but not in naïve, WT

mouse. Therefore, we evaluated the coexpression of CRH- and several interneuronal markers in hippocampi from both WT and the *Crh-IRES-Cre;Ai14* tdTomato mouse. As shown in Figure 8B, a subset of CRH-ir neurons in area CA1 coexpressed PV, as found in the rat (11, 32), and a similar coexpression of the reporter and PV was observed in the transgenic mouse (Figure 8C). In the dentate gyrus, a robust population of CRH-ir neurons coexpressed calretinin (Figure 8D), and the same colocalization was found with the reporter in *Crh-IRES-Cre; Ai14* tdTomato mice (Figure 8E). Together, these findings indicate that adult mice express robust levels of CRH in GABAergic hippocampal interneurons, and that the *Crh-IRES-Cre;Ai14* tdTomato mouse recapitulates this finding faithfully, rendering it a useful tool for exploring the role of CRH-expressing cells in hippocampus.

Discussion

The current work examines the distribution of native, endogenous CRH and of transgenic reporters in 3 genetically engineered mouse lines. This investigation reveals divergent patterns of reporter distribution among the different transgenes as well as variance by brain region. CRH has been demonstrated to play crucial roles not only in the peripheral stress response, but in normal and pathological cognitive and emotional functions involving neuronal networks and structures, including the amygdala, BNST, cortex, and hippocampus (21, 22). Therefore, the exquisite resolution and mechanistic power of transgenic mice where CRH-expressing neurons can be manipulated, offer experimental tools with major importance. However, the use of these instruments requires strong validation of the congruence of reporter and native peptide expression.

Historically, CRH distribution was validated in rat (11, 34). More recently, significant differences have been reported in the relative hypothalamic location of CRH-expressing parvocellular neurons in relation to the oxytocin and vasopressin-expressing magnocellular neurons in
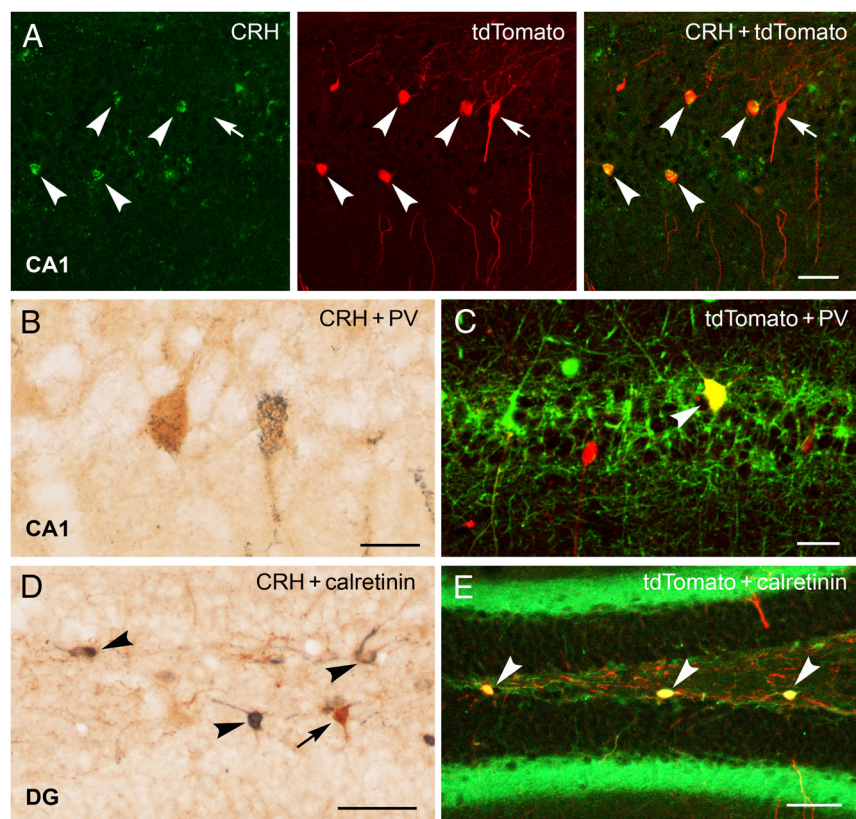
Figure 8. Concordant identities of CRH-expressing hippocampal neurons in naïve mice and of expressing hippocampal neurons in the tdTomato mouse. A, In the *Crh-IRES-Cre;Ai14* tdTomato mouse, interneuron-like reporter-expressing neurons coexpressed CRH (arrowheads) in the pyramidal cell layer of area CA1 (as well as in CA3). However, those with soma shape and dendritic processes typical of pyramidal cell were devoid of CRH expression (arrows). B, A subset of CRH-ir neurons (brown) in the pyramidal cell layer of area CA1 of naïve C57BL/6J mice coexpressed PV (blue granular deposits). C, In the *Crh-IRES-Cre;Ai14* tdTomato mouse, a coexpression (arrowhead) of PV (green), and the reporter was apparent in similar cells. D, In the dentate gyrus of adult C57BL/6J mice, a robust population of CRH-ir neurons (brown) coexpressed calretinin (blue). E, In the *Crh-IRES-Cre;Ai14* tdTomato mouse, the colocalization (arrowheads) of calretinin (green) and the reporter is visible in the same cell population. Scale bars: 50 μm (A), 25 μm (B and C), and 100 μm (D and E).

mice vs rats (35). In addition, CRH expression follows a clear developmental pattern (39, 51, 52). Therefore, to avoid potential developmental and species-related confounders, we used adult mice and employed an antiserum validated by the use of null mice as the reference group to assess the fidelity of reporter expression in 3 available transgenic mouse lines.

We found several types of reporter/CRH distributions: an almost complete overlap of native peptide and the tdTomato reporter was observed in the *Crh-IRES-Cre; Ai14* tdTomato mouse in all 4 brain regions examined, in line with previous observations in the hypothalamus (20). The results position this transgenic line as an excellent, potent investigational tool. Still, a number of pyramidal-looking cells in the hippocampus expressed the reporter but not CRH. A priori, it was conceivable that the reason for such discrepancy might be developmental: pyramidal

cells might express CRH during development together with the reporter, but a developmental shut-off of CRH expression might fail to repress the reporter. We think this possibility is excluded, because detailed ontogenetic studies of CRH expression in the hippocampus failed to show pyramidal cell expression at any age. In addition, in adult mice, ample CRH expression was found, again, exclusively in interneurons.

A second possibility for lack of overlap of native CRH and a reporter might derive from poor sensitivity of the methods used for detection. We employed tyramide amplification and detected ample native CRH expression in the expected neuronal populations in all 3 transgenic lines, suggesting that when cells do express CRH, this expression is detectable. The salient results of the current studies are not a global absence of expression. Rather, the cells expressing the reporter in some brain regions and mouse lines were simply different from those expressing CRH.

A third intriguing possible source of diminished overlap of CRH and reporter expression is a reporter-specific selectivity of expression patterns within the same CRH-targeted line. Such a scenario may be operational in a recent publication that used a variant of the Crh-BAC mouse line (Tg(Crh-cre)KN282Gsat) (17). In that work, the use of different reporter lines (mTomato-GFP vs tdTomato) appeared to result in the labeling of anatomically distinct neuronal populations within the pyramidal cell layer of the hippocampal CA1 (Figure 1 vs Figure 2) (17). Specifically, in BAC CRH-cre mice expressing the mTomato-GFP, labeled neurons were pyramidal in shape and possessed complex dendritic arbors (Figure 1) (17). Surprisingly, when a different reporter (tdTomato) was used on the same BAC CRH-cre line, the reporter-expressing cells were not pyramidal in shape. Instead, labeled neurons were bipolar and multipolar, typical of interneurons (Figure 2) (17). Although neurons expressing both the GFP and tdTomato were reported to be GABAergic and to contain a number of interneuron-associated proteins (eg, PV, cholecystokinin, and somatostatin), the

apparent differences in the anatomy of reporter-expressing neurons (combined with their relatively low coexpression with CRH) support the notion that the different reporters may be selectively expressed in distinct populations or subpopulations of hippocampal neurons. Clearly a more detailed analysis is required to determine whether this is indeed the case, yet the possibility should be considered when interpreting data from the same mouse line crossed to different reporters.

The findings described here highlight the power and also the challenges and potential pitfalls in the use of transgenic mice. They are in line with recent reports regarding dopaminergic neurons of the ventral tegmental area studied using mouse lines with Cre-recombinase under the control of different promotors, tyrosine hydroxylase and dopamine transporter (DAT). In Cre-TH mice, significant reporter expression occurred in nondopaminergic cells within and around the ventral tegmental nuclei, whereas when the DAT promotor was used to drive Cre-recombinase expression (DAT-Cre), dopamine-specific transgene expression was reported (53). The observation that distinct Cre-drivers may promote transgene expression in different neuronal populations highlights the importance determining how well transgene expression replicates that of the native, target gene. Indeed, a number of studies have begun to address the issue of differences between Cre recombination patterns and the endogenous expression of the target gene, including a recent report using in situ hybridization to assess whole-brain gene expression patterns in over 100 Cre-driver mouse lines (54). In addition, the characterization of Cre-reporter expression across the brain in BAC transgenic mouse lines has been conducted and made publically available by GENSAT.

The current work highlights that in certain transgenic lines, congruence of reporter and endogenous gene might take place in one brain region and less so or not at all in others. This renders certain transgenic lines suitable for the study of specific neuronal populations and not others. Although these considerations will be paramount in future studies, the observations made here might also help explain controversies among excellent existing studies. For example, immunohistochemical and electrophysiological studies in C57BL/6J mice found no evidence for the expression of extrasynaptic δ-containing GABA$_A$ receptors in CRH-expressing neurons of the PVN (55, 56). However, studies using a variant of the crh-BAC mouse (Tg(Crh-cre)KN282Gsat BAC crossed with mTomato-GFP) demonstrated the functional expression of δ-GABA$_A$ receptors in CRH reporter-expressing neurons (16). The possibility that 2 distinct neuronal populations, or different subsets of the same neuronal population, were investigated in these studies may provide a plausible explanation for the apparent discrepancy.

In conclusion, we report here on diversity of transgenic mouse lines targeting CRH in terms of the coexpression of reporter and endogenous CRH. In addition to its roles in stress, CRH contributes crucially to learning and memory, anxiety and excitability and function of neuronal networks, including the amygdala, BNST, cortex, and hippocampus. Therefore, awareness and consideration of the diversity of reporter lines should facilitate interpretation and reconciliation of divergent scientific findings, and thus help move forward exciting and important investigations of the role of CRH in the normal and diseased brain.

Acknowledgments

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