

## Overexpressing Corticotropin-Releasing Hormone in the Primate Amygdala Increases Anxious Temperament and Alters Its Neural Circuit

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### ABSTRACT

**BACKGROUND:** Nonhuman primate models are critical for understanding mechanisms underlying human psychopathology. We established a nonhuman primate model of anxious temperament (AT) for studying the early-life risk to develop anxiety and depression. Studies have identified the central nucleus of the amygdala (Ce) as an essential component of AT's neural substrates. Corticotropin-releasing hormone (CRH) is expressed in the Ce, has a role in stress, and is linked to psychopathology. Here, in young rhesus monkeys, we combined viral vector technology with assessments of anxiety and multimodal neuroimaging to understand the consequences of chronically increased CRH in the Ce region.

**METHODS:** Using real-time intraoperative magnetic resonance imaging-guided convection-enhanced delivery, five monkeys received bilateral dorsal amygdala Ce-region infusions of adeno-associated virus serotype 2 containing the CRH construct. Their cagemates served as unoperated control subjects. AT, regional brain metabolism, resting functional magnetic resonance imaging, and diffusion tensor imaging were assessed before and 2 months after viral infusions.

**RESULTS:** Dorsal amygdala CRH overexpression significantly increased AT and metabolism within the dorsal amygdala. Additionally, we observed changes in metabolism in other AT-related regions, as well as in measures of functional and structural connectivity.

**CONCLUSIONS:** This study provides a translational roadmap that is important for understanding human psychopathology by combining molecular manipulations used in rodents with behavioral phenotyping and multimodal neuroimaging measures used in humans. The results indicate that chronic CRH overexpression in primates not only increases AT but also affects metabolism and connectivity within components of AT's neural circuitry.

**Keywords:** AAV2, Central nucleus of the amygdala, DTI, FDG-PET, fMRI, MRI-guided neurosurgery

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Nonhuman primate models are critical for understanding mechanisms underlying the development and expression of human psychopathology (1,2). This is supported by the remarkable correspondence between nonhuman and human primates in brain structure and function that underlies their similarities in behavior, rearing methods, psychosocial development, and cognition (2,3). The imperative to identify new molecular treatment targets to treat psychiatric disorders (4,5), along with the evolutionary linkage between nonhuman and human primates, provides a compelling rationale to develop techniques in nonhuman primates that can alter the function of candidate genes in a targeted brain-region-selective manner. Furthermore, the ability to use multimodal imaging methods to understand the impact of region-specific molecular

manipulations in a relevant primate model allows for understanding the mechanisms associated with altered neural circuits in psychiatric illnesses.

To date, our nonhuman primate studies have focused on establishing a model for investigating mechanisms underlying the development of extreme early-life anxiety (2,6–15). First, we developed and standardized the no eye contact (NEC) condition of the human intruder paradigm to assess individual trait-like differences in anxiety-related behavior in response to potential threat (16). This trait-like disposition, termed anxious temperament (AT), is a prominent childhood risk factor for the later development of anxiety disorders, depression, and comorbid substance abuse (17–21). With our nonhuman primate model, we identified the altered neural circuitry that

underlies the development of AT and found that it is similar to that observed in humans with anxiety disorders (3,8,10,22). This neural system-level information provides the critical groundwork for directly testing regionally specific molecular hypotheses potentially important in the pathophysiology of AT.

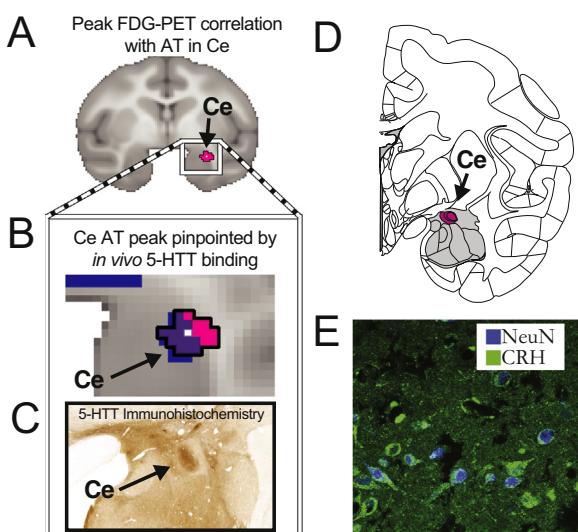
When extreme, AT or its major behavioral component, behavioral inhibition, markedly increases a child's risk for the later development of stress-related psychopathology (17–19,23). By combining measures of threat-related behavioral inhibition (increased freezing and decreased vocalizations) and pituitary-adrenal activation (threat-induced cortisol), along with <sup>18</sup>fluoro-deoxyglucose positron emission tomography (FDG-PET), in rhesus monkeys, we characterized a non-human primate developmental model of AT (6,14). Although the AT neural circuit is distributed across prefrontal, limbic, and brainstem regions, neuroimaging studies in monkeys and humans point to the dorsal amygdala, a region containing the central nucleus of the amygdala (Ce), as a fundamental component of the circuit (8,10,11) (Figure 1). The Ce is of particular interest because it is the major outflow region of the amygdala with its downstream projections mediating the hypothalamic and brainstem contributions to the stress response (24,25). The Ce is causally involved in mediating AT, as neurotoxic lesions of the rhesus monkey dorsal

amygdala, which encompass the Ce, decrease the expression of behavioral inhibition and pituitary-adrenal activity (26).

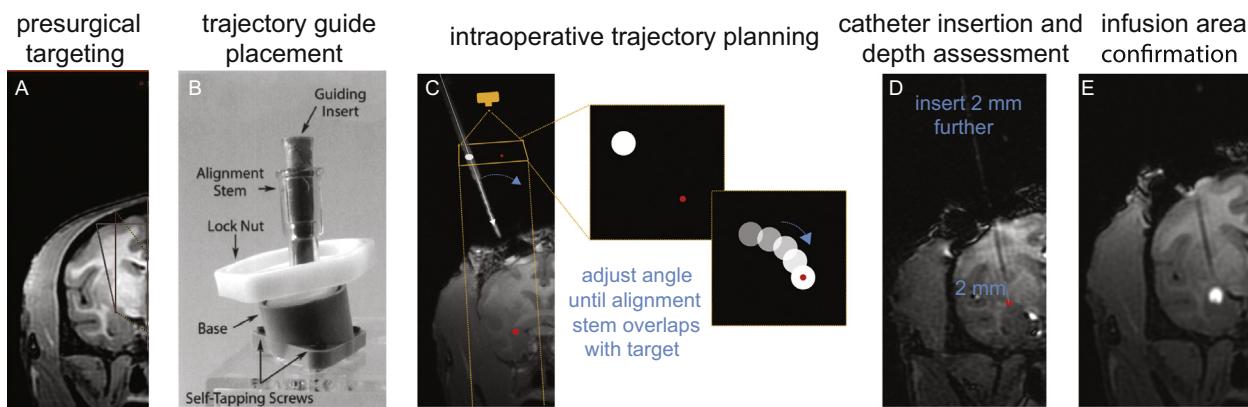
The Ce is predominantly composed of gamma-aminobutyric acidergic neurons that also contain numerous modulatory neuropeptides and receptors (27). Within the Ce, there is expression of corticotropin-releasing hormone (CRH) (Figure 1), its two receptors, and its binding protein (28–31). CRH-containing neurons in the paraventricular nucleus of the hypothalamus play a prominent role in mediating the stress-related pituitary-adrenal response. Interestingly, extrahypothalamic brain CRH neurons, including those in the Ce, can be regulated differently (32) and are important in coordinating the autonomic, emotional, behavioral, and cognitive components of the stress response (33,34). Rodent studies have demonstrated that stress increases the expression of CRH in the paraventricular nucleus of the hypothalamus and Ce (35), whereas corticosterone administration decreases expression of CRH in the paraventricular nucleus of the hypothalamus, while concomitantly increasing the expression of Ce CRH (32). In addition to its role in modulating adaptive stress responses, overactivity of the CRH system is hypothesized to be an important pathophysiological mediator of symptoms associated with anxiety and depressive disorders (36). Recent reports suggest that structural variation of genes in the CRH family may contribute to the expression and pathophysiology of human depression and anxiety disorders (37–43), as well as to extreme monkey AT and its associated altered brain metabolism (44).

In addition to the translational value of nonhuman primate models, it is important to emphasize that primates significantly differ from rodents in the distribution and organization of brain CRH systems (31). Rodent studies have used transgenic, viral vector, and other neuronal modulatory strategies to model CRH molecular alterations hypothesized to underlie stress-related psychopathology (45–50). However, these mechanistic studies have not been translated to primate species. Therefore, using a viral vector gene delivery strategy to chronically increase the expression of CRH in the dorsal amygdala of young rhesus monkeys, we aimed to identify the role of amygdala CRH systems in the expression of primate anxiety.

Here, we demonstrate that regional chronic overexpression of a putative anxiogenic neuropeptide results in increased anxiety-like behaviors along with anxiety-related changes in primate brain function. Using a viral vector infused with convection-enhanced delivery and guided by real-time intraoperative magnetic resonance imaging (RT-IMRI), we aimed to chronically overexpress CRH in the Ce region of the amygdala. This approach was combined with multimodal functional and structural brain imaging to test the hypothesis that increased dorsal amygdala CRH would increase AT, as well as glucose metabolism in the Ce. To allow for new insights into how increased amygdala CRH may influence brain-wide neural alterations underlying stress-related psychopathology, we also examined the impact of chronic dorsal amygdala CRH overexpression on functional and structural connectivity with other relevant brain regions. This study provides a framework for further development of preclinical nonhuman primate strategies to evaluate novel, region-specific molecular targets for the treatment of human psychopathology.



**Figure 1.** Endogenous corticotropin-releasing hormone (CRH) expression in the anxious temperament (AT)-related central nucleus of the amygdala (Ce) region. **(A)** Using <sup>18</sup>fluoro-deoxyglucose positron emission tomography (FDG-PET) imaging, we found that metabolism within the Ce predicted individual differences in AT in young rhesus monkeys. **(B)** The Ce region was defined by its overlap (purple) with serotonin transporter (5-HTT) ligand binding determined with PET imaging (blue). **(C)** The serotonin transporter binding characterized by PET mirrors its neuroanatomical distribution observed with immunohistochemical methods. **(D)** Within this critical primate Ce region, we found **(E)** a moderate amount of endogenous CRH immunoreactivity (green) located in neurons, as defined by the overlap with NeuN expression (blue). [Panel A reprinted with permission from Oler et al. (8). Panel C reprinted with permission from O'Rourke and Fudge (98). Panel D adapted with permission from Paxinos et al. (99).]



**Figure 2.** Real-time intraoperative magnetic resonance imaging guided targeting and infusion monitoring. **(A)** Prior to surgery, a structural magnetic resonance image was obtained to visualize the target and plan the trajectory. **(B)** A pivot point-based magnetic resonance imaging compatible external trajectory guide was mounted on the skull. **(C)** Precise targeting was performed by imaging a plane orthogonal to the long axis of the external trajectory guide, as if it were visualized by a camera from above. The inset boxes represent the plane in which the trajectory guide (white dot) is visualized and aligned in relation to the target injection point (red dot). **(D)** The depth of the catheter is advanced to approximately 2 mm above the target. Another magnetic resonance image was acquired to make precise measurements between the catheter tip and the target before advancing the catheter to its final position. **(E)** Immediately after the adeno-associated virus type 2-corticotropin-releasing hormone/gadolinium infusion was complete, a final magnetic resonance image was acquired to verify the infusion delivery region. [Panel B reprinted with permission from Emborg *et al.* (54)].

## METHODS AND MATERIALS

### Overall Study Design

Behavioral measures of AT and glucose-based measures of brain metabolism (FDG-PET) were assessed during NEC before surgery and again approximately 2 months later in five CRH-overexpressing monkeys and at similar intervals in their five matched unoperated control monkeys. Additionally, magnetic resonance imaging (MRI) measures of structural connectivity with diffusion tensor imaging and functional intrinsic connectivity with resting functional MRI, were acquired before and after surgery. Paired-sample *t* tests controlling for age were used to test for group differences in post-pre measures that resulted from CRH overexpression (i.e., CRH group [post-pre] – control group [post-pre]).

The surgeries were performed using RT-IMRI guidance to localize the target. To estimate the dispersion of adeno-associated virus type 2 (AAV2)-CRH, we infused the viral vector concurrently with the MR visible marker gadobenate dimeglumine (MultiHance, Bracco Diagnostics, Monroe Township, NJ). To characterize the pattern of CRH expression, animals were euthanized approximately 1 year after surgery and immunohistochemical staining for CRH was performed approximately 10 months after the behavioral assessments.

Note that space limitations do not allow for a complete description of the methods in the body of the article and much of the methodological detail concerning the surgery and brain imaging analyses (FDG-PET, functional MRI, and diffusion tensor imaging) can be found in the *Supplement*.

### Subjects

First, two cynomolgus monkeys (*Macaca fascicularis*) were used in pilot studies to 1) demonstrate the effectiveness of the AAV2-CRH virus to direct CRH overexpression *in vivo*, and 2) to visualize endogenous CRH expression in the Ce (*Supplement*). Next, 10 young male rhesus monkeys (*Macaca*

*mulatta*) (1.76–2.63 years old, 2.77–5.25 kg) were used in the CRH overexpression experiment. These animals were screened with the human intruder paradigm and were selected to be in the midrange for freezing responses. Five animals received bilateral infusions (two infusions per hemisphere) of AAV2-CRH into the dorsal amygdala Ce region. The other five nonoperated control animals were age-matched and pair-housed with each of their corresponding transfected monkeys. Because of considerations related to performing unnecessary procedures on nonhuman primates and because we have used nonoperated control animals to successfully examine lesion-induced effects on primate anxiety in previous studies (26,51,52), we chose to use nonoperated cagemates as control subjects. Animals were housed and cared for at the Harlow Center for Biological Psychology and the Wisconsin National Primate Research Center on a 12-hour light/dark cycle, in a temperature- and humidity-controlled vivarium. For all imaging and surgical procedures, the animals were fasted overnight. The experiments were performed according to the federal guidelines of animal use and care (53) and with approval of University of Wisconsin-Madison Institutional Animal Care and Use Committees.

### Characterizing Anxious Temperament and Glucose Metabolism During NEC

AT was characterized using the NEC condition of the human intruder paradigm and was computed as the combination of standardized freezing, cooing, and cortisol measures (6,14). NEC assessment coincided with the administration of FDG followed by small-animal positron emission tomography scanning (see the *Supplement* for further details).

### Real-Time Intraoperative MRI

Placement of the MRI-compatible trajectory guide bases followed previously reported methods [see (54,55) for details]

modified for Ce targeting (Figure 2). The intraoperative targeting was performed using a platform for real-time MR-guided prospective stereotaxy (56) that was initially developed by the University of Wisconsin (57–60). A detailed description of the RT-IMRI methods can be found in the *Supplement*. The rhesus Ce is approximately 5 mm long in the anterior-posterior plane and approximately 1 to 2 mm in the dorsal-ventral and medial-lateral planes. To cover as much of the Ce as possible while minimizing treatment to surrounding regions, two 12- $\mu$ L infusions were performed per hemisphere (one anterior Ce target, one posterior Ce target), for a total of 24  $\mu$ L per hemisphere. After each infusion, the catheter was removed, and after all infusions were complete, the animal was transported back to the surgical suite and the craniotomies were closed.

### Assessing CRH Overexpression

Approximately 1 year after AAV2 infusions, the animals were euthanized. The brains were extracted and fixed overnight in 4% paraformaldehyde, cryoprotected, and then sectioned at 40  $\mu$ m. CRH immunoreactivity was assessed as described in the *Supplement*. Every twelfth section through the brain was immunostained for CRH. Adjacent coronal brain sections were processed for acetylcholinesterase, a cholinergic marker that facilitates anatomical identification of amygdala nuclei and subnuclei (61).

For each animal, the distribution of CRH-positive cells was charted through the rostrocaudal extent of the Ce region using camera lucida techniques. Sections were initially drawn under bright-field illumination at 1.6 $\times$ , to include labeled cells and landmarks such as blood vessels and fiber tracks. The distribution of labeled cells was then confirmed under 10 $\times$  bright-field illumination. Adjacent acetylcholinesterase-stained sections were then viewed under dark-field illumination, using landmarks in the charted sections for alignment. This permitted overlay of acetylcholinesterase-determined boundaries of amygdala nuclei and subnuclei for each section. Finally, paper maps were digitized using a drawing tablet in conjunction with the program Adobe Illustrator CS2 (Adobe Systems, San Jose, CA).

To compare the relative distribution of labeled cells resulting from viral-mediated CRH expression and/or endogenous CRH expression across animals, we overlaid individual maps from each animal upon one another in transparent layers, matching rostrocaudal levels as closely as possible. Labeled cells for each pair of animals were color-coded to assist in the analysis.

## RESULTS

### Characterization of CRH Overexpression

Postmortem immunocytochemical analyses demonstrated marked overexpression of CRH in the dorsal amygdala region of the experimental animals compared with the levels of endogenous CRH in the control animals. The RT-IMRI gadobenate dimeglumine infusion scan from each animal was compared with the animal's pattern of CRH overexpression defined by immunocytochemistry (Figure 3). As can be seen in Figure 4A, in all experimental animals, CRH overexpression was evident in the dorsal amygdala including

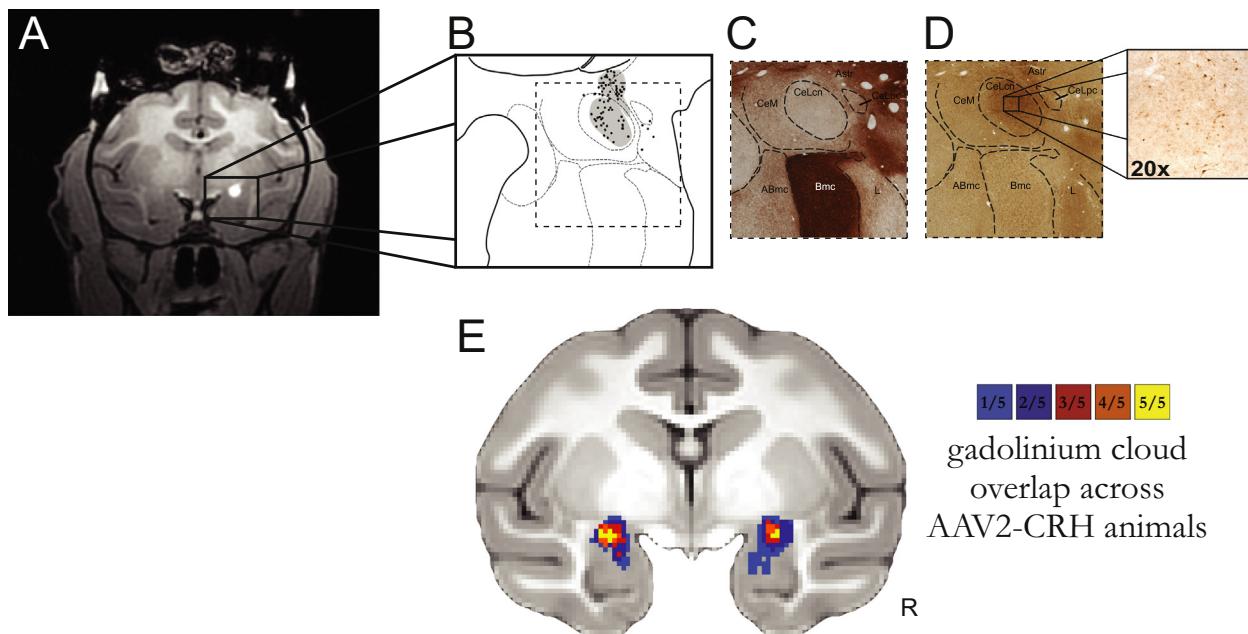
regions of the lateral and medial divisions of the Ce as well as in surrounding areas such as the dorsal regions of the accessory basal nucleus, the magnocellular region of the basal nucleus, the amygdala striatal transition zone, and portions of the ventral putamen (Table 1). We found that across all transfected animals the extent and location of the infusions as determined by RT-IMRI matched the areas of CRH overexpression determined with immunocytochemistry (compare Figure 4A and Figure 3E). This demonstrates the ability to use *in vivo* imaging methods to estimate the extent of infusion as well as AAV2-CRH transfection after 1 year.

### Effects of CRH Overexpression on AT and Brain Metabolism

As predicted, compared with control animals, the CRH overexpressing animals demonstrated a significant increase in AT (CRH group [post-pre] – control group [post-pre]) ( $p < .05$ , one-tailed; Figure 5). A complementary unpaired between-groups analysis (controlling for age as in the paired analysis) revealed significant group differences in the AT phenotype ( $t = 2.040$ ,  $p = .0405$ , one-tailed; see *Supplemental Figure S3*). It is noteworthy that CRH concentrations in cerebrospinal fluid (CSF) did not significantly differ between experimental and control animals (see *Supplemental Methods and Results*). Corresponding analyses of FDG-PET data revealed that CRH overexpression also resulted in significant increases in dorsal amygdala metabolism ( $p < .01$ , uncorrected; Figure 6A). Additional whole-brain voxelwise analyses revealed that the CRH overexpression animals had significantly greater increases in metabolism in orbital preisocortex/anterior insular cortices (OPro/AI) and hippocampus ( $p < .01$ , two-tailed, uncorrected; Figure 6A and *Supplemental Table S1*). It is important to underscore that these regions have been implicated as part of the AT network, as well as in human anxiety disorders. To identify regions across both control and experimental animals in which post-pre changes in AT were predicted by changes in metabolism, we looked within brain regions that demonstrated an effect of CRH overexpression (Figure 6B, yellow outline). This analysis revealed that regardless of treatment condition, changes in AT from the presurgical to postsurgical assessment were predicted by individual differences in metabolic increases in regions of the dorsal amygdala, OPro/AI, and hippocampus (red,  $p < .05$ , two-tailed, uncorrected; Figure 6B and *Supplemental Table S2*). These findings suggest that the effects of dorsal amygdala CRH overexpression on increasing AT involve activation of this distributed neural circuit.

### Effects of CRH Overexpression on Functional Connectivity and White Matter Integrity

The functional MRI data demonstrated that chronic CRH overexpression altered resting functional connectivity. The dorsal amygdala seed region for the functional connectivity analyses was determined by the overlap of the infusion area detected with gadobenate dimeglumine (see yellow region in Figure 3E) with that of the metabolic region that was affected by CRH overexpression (see yellow region in Figure 6A, right). Results demonstrated that CRH overexpression altered functional connectivity such that connectivity with the right dorsal



**Figure 3.** In vivo estimation and postmortem verification of dorsal amygdala corticotropin-releasing hormone (CRH) overexpression. **(A)** The gadolinium clouds in the dorsal amygdala, central nucleus of the amygdala (Ce) region, during and immediately following adeno-associated virus type 2 (AAV2)-CRH delivery provided an estimate of the location and extent of the infusions. **(B)** Camera lucida drawings of CRH expression from postmortem tissue reflected the extent of viral infusion as estimated from the intraoperative gadolinium signal. Gray regions in **(B)** represent neuropil staining and the black dots represent CRH overexpressing cells. **(C)** Acetylcholinesterase staining defined the boundaries of the amygdalar nuclei. Note the relative absence of acetylcholinesterase staining in the central nucleus, lateral central subdivision (CeLcn). **(D)** Adjacent sections were used for CRH immunohistochemistry demonstrating marked overexpression in the dorsal amygdala, Ce region. **(E)** Quantification of gadolinium diffusion extent and cross-subject overlap. Based on the intraoperative gadolinium images, we estimated the infusion extent in standard magnetic resonance imaging space. This allows for an estimation of the across-subject overlap of CRH expression and provides a link to the postmortem CRH expression data (compare with *Figure 4A*). The overlap of the gadolinium injection clouds across all five experimental animals demonstrates the replicability of the magnetic resonance imaging-guided targeting procedure. The colors represent the number of animals with gadolinium signal at each voxel. Note the bilateral overlap across all experimental animals within the Ce region (yellow). ABmc, accessory basal nucleus, magnocellular subdivision; Astr, amygdalostriatal transition zone; Bmc, basal nucleus, magnocellular subdivision; CeM, central nucleus, medial subdivision; Celpc, central nucleus, lateral central subdivision; L, lateral nucleus; R, right.

amygdala seed in various regions differed between groups ( $p < .01$ , two-tailed, uncorrected; *Supplemental Table S3*). Of note, increased connectivity was found between right dorsal amygdala and regions encompassing bilateral portions of OPro/AI.

Analyses of the diffusion tensor imaging data were performed to examine effects of long-term CRH overexpression on white matter microstructure. Voxelwise analyses were performed on measures of diffusivity and fractional anisotropy (FA). Investigation of mean diffusivity, axial diffusivity, and radial diffusivity demonstrated significant alterations in various regions (*Supplemental Tables S4–S6*). Of particular interest, CRH overexpression in the dorsal amygdala was associated with significant increases in mean diffusivity, axial diffusivity, and radial diffusivity in a region of the brain that overlaps with the extended amygdala/bed nucleus of the stria terminalis ( $p < .005$ , two-tailed, uncorrected). Such an increase in mean diffusivity, axial diffusivity, and radial diffusivity can be indicative of decreased density of microstructure but can also be explained by increased levels of CSF. Since this region is proximal to the ventricles, this possibility should be considered.

Analysis of FA, an overall indicator of white matter integrity, demonstrated that dorsal amygdala-CRH overexpression

resulted in decreased FA in various regions including portions of the medial/midline thalamus ( $p < .005$ , two-tailed, uncorrected; *Figure 7* and *Supplemental Table S7*). This region of medial thalamus encompasses the ventral edge of the medial dorsal thalamus, portions of the central medial and paracentral thalamic nuclei, as well as the magnocellular division of the ventral anterior thalamic nucleus. Fiber tractography enabled an investigation of the connectivity of this region with the rest of the brain (*Supplement* and *Supplemental Figure S4*).

## DISCUSSION

In this study, we validated methods combining RT-IMRI with convection-enhanced delivery to reliably locate and accurately infuse AAV2-CRH into the primate Ce region. This demonstrates the feasibility of translating rodent mechanistic studies that directly manipulate gene function in the brain to primates and implicates overactive brain CRH systems in the pathophysiology of excessive primate anxiety. These findings further point to the dorsal amygdala, Ce region, as a key site involved in determining individual differences in dispositional anxiety and the phenotype that represents the risk to develop stress-related psychopathology. This study is the first to use viral vector strategies in nonhuman primates to directly manipulate



**Figure 4.** Quantification of dorsal amygdala corticotropin-releasing hormone (CRH) expression. **(A)** Postmortem analyses demonstrated overexpression of CRH in the dorsal amygdala and surrounding regions in the experimental animals, compared with **(B)** the levels of endogenous CRH observed in the cagedate control animals (top = anterior, bottom = posterior). Each pair of animals is represented by a different color in the composite image, and each dot represents a CRH-expressing cell body. Note that endogenous CRH expression levels in control animals were found in the most posterior regions of the central nucleus of the amygdala (only the left hemisphere is presented) and were substantially lower than that induced by adeno-associated virus type 2 (AAV2)-CRH transfection. AAA, anterior amygdaloid area; ABmc, accessory basal nucleus, magnocellular subdivision; AC, anterior commissure; Astr, amygdalostriatal transition zone; Bmc, basal nucleus, magnocellular subdivision; CeLcn, central nucleus, lateral central subdivision; CeLpc, central nucleus, lateral paracapsular subdivision; CeM, central nucleus, medial subdivision; H, hippocampus; L, lateral nucleus; M, medial nucleus; nbm, nucleus basalis of Meynert; P, putamen; V, ventricle.

CRH molecular systems hypothesized to be involved in human psychiatric disorders. Our primate model provides a unique opportunity to assess the effects of gene manipulation on primate behavior in conjunction with the same *in vivo* measures of brain function and structure that are used to assess human neuropsychiatric patients.

While much of the mechanistic work focused on the role of CRH in anxiety and fear has been performed in rodents, a few studies have been done in primates. Because of the similarities in brain function and structure, behavior, and social functioning between nonhuman primates and humans, rhesus monkeys provide an important and valuable model for

**Table 1. Relative Density of CRH-Labeled Cells in Both Hemispheres, Summing Across All Rostrocaudal Levels**

Case	CeLcn/pc	CeM	Bmc	ABmc	Astr	Ventral Putamen
CRH-1	++	++	++	+++++	+++++	+++
CRH-2	++	+	+++++	++++	+++++	+++++
CRH-3	+++	+	+++++	+	+++++	+++++
CRH-4	+++	++	+	+++++	+++++	+
CRH-5	+++	+++	+++++	+++++	++++	+++++
CON-1	+					
CON-2	+					
CON-3	+					
CON-4	+					
CON-5	+					

ABmc, accessory basal nucleus, magnocellular subdivision; Astr, amygdalostriatal transition zone; Bmc, basal nucleus, magnocellular subdivision; CeLcn/pc, central nucleus, lateral central subdivision/parapupsular subdivision; CeM, central nucleus, medial subdivision; CON, control; CRH, corticotropin-releasing hormone.

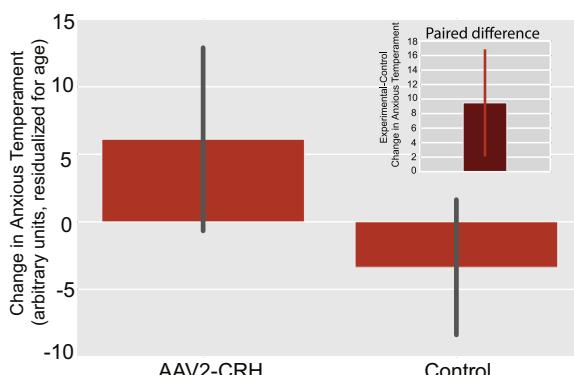
studying human psychopathology. The marked difference in distribution of brain CRH receptors between rodents and primates further supports the use of primates for studies of stress-related psychopathology. For example, primates have both CRH receptor 1 (CRHR1) and CRH receptor 2 (CRHR2) in the Ce, whereas the rodent Ce is only populated with CRHR1 (31,62). Early rodent studies site-specifically administering CRH or CRH antagonists established a key role for the amygdala, including the Ce, as being important in mediating the effects of CRH on anxiety and fear responses (63–69). Other studies suggest that within the rodent Ce, it is likely that CRH acts via activation of CRHR1 (70–72). Additionally, mouse transgenic and knockout studies manipulating expression of CRH or CRHR1 demonstrated an important role for CRH systems in mediating adaptive and maladaptive behavioral and physiological responses to stress [for review, see (73)]. Because of the presence of CRHR2 receptors in the

primate Ce, it is possible that the anxiogenic effects of CRH are mediated by activation of CRHR2 receptors.

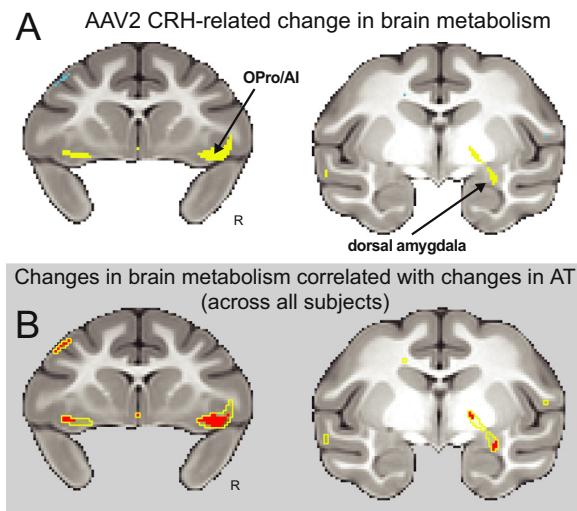
Our early work in rhesus monkeys is consistent with results from the rodent studies. For example, we demonstrated that intraventricularly administered CRH increased anxiety when administered at low doses and at higher doses resulted in depressive-like behaviors (74). Because of the wide distribution of CRH receptors throughout the brain (31,62,75,76), it is likely that these effects were mediated by activation of CRH receptors in diverse brain regions—a finding that is supported by brain-wide metabolic brain changes seen following very high doses of intraventricular CRH (77). In another study, we also reported a relation between CSF levels of CRH and threat-induced behavioral inhibition (78); however, this finding has not been replicated (Ned Kalin, M.D., unpublished data, 2015). It is important to note that some, but not all, human studies demonstrate increased CSF CRH concentrations associated with depression and/or suicide (79) and postmortem analyses of suicide victims have revealed increased activity in brain CRH systems (80–84). Neurotoxic lesion studies in primates demonstrated that Ce lesions not only reduced anxiety but also decreased concentrations of CSF CRH (26). Thus, in the Ce lesion study, the reduction in anxiety could be accounted for by the global reduction in CSF CRH. In the current study, in which we used a viral vector strategy to overexpress CRH in the dorsal amygdala region, anxiety and brain function were affected in the absence of a detectable increase in CSF CRH levels. Taken together, these findings suggest that while CRH can have profound impacts throughout the brain, the Ce is a site that is critically involved in mediating these effects.

Recent viral vector studies in rodents have implicated the chronic overexpression of Ce CRH in inducing anxiety- and depression-related behaviors (46,48,49,85,86). In general, but not always, overexpression of Ce CRH is reported to affect physiological parameters such as the startle response and hypothalamic-pituitary-adrenal activity (48,49,85,86). Our current findings in nonhuman primates are consistent as we demonstrate that overexpression of CRH increases AT.

With the functional and structural brain imaging measures used in our study, we were able to extend the rodent studies by examining the impact of chronically increased dorsal amygdala CRH on brain metabolism, as well as functional and structural connectivity. These analyses provide potential insights into mechanisms underlying the neural circuit alterations associated with human anxiety and other stress-related psychopathologies. Our data are the first to demonstrate that an overactive amygdala CRH system has local effects on brain metabolic activity as well as on other components of the neural circuit associated with anxiety and AT. Specifically, we found that CRH overexpression resulted in increased metabolism in posterior regions of the orbitofrontal cortex (OPro), anterior insula, and hippocampus. Since the areas of overexpression encompassed Ce, other dorsal regions of the amygdala, and neighboring structures (e.g., putamen), it is possible that CRH expression in these different regions contributed to the observed effects. Additionally, we found evidence that in some cases the virus was anterogradely and/or retrogradely transported to other brain regions that are monosynaptically connected to the site of infusion.



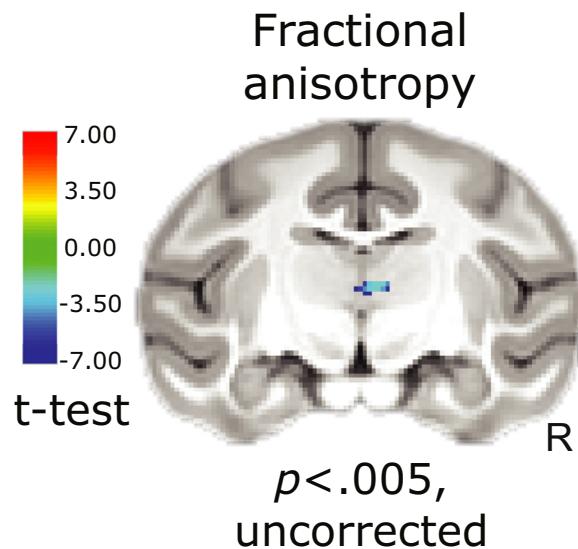
**Figure 5.** Corticotropin-releasing hormone (CRH) overexpressing animals demonstrated a significant increase in anxious temperament. Compared with their matched control animals, the CRH overexpressing animals demonstrated increased postsurgical levels of anxious temperament (mean  $\pm$  SEM). Significance was determined using a paired-samples  $t$  test comparing dorsal amygdala CRH animals and their cage-mate control animals (CRH group  $[post-pre]$  – control group  $[post-pre]$ ) ( $p < .05$ , one-tailed; see inset and Methods and Materials for details). AAV2, adeno-associated virus type 2.



**Figure 6.** Corticotropin-releasing hormone (CRH) overexpressing animals demonstrated significant increases in brain metabolism. **(A)** Compared with their matched control animals, the CRH overexpressing animals demonstrated increased post-pre change in metabolism within the dorsal amygdala, orbital preisocortex/anterior insular cortices (OPro/AI), and hippocampus (yellow,  $p < .01$ , two-tailed, uncorrected; *Supplemental Table S1*). All of these regions have been implicated in anxious temperament (AT). **(B)** Within regions that were affected by CRH overexpression (yellow outline), we identified areas in which the post-pre change in metabolism correlated with the post-pre change in AT (red;  $p < .05$ , one-tailed, uncorrected). Across all 10 animals, changes in AT were associated with changes in brain metabolism in the dorsal amygdala and in bilateral regions of the posterior OPro/AI. AAV2, adeno-associated virus type 2; R, right.

We also found that dorsal amygdala CRH overexpression increased functional connectivity between this area and the OPro/AI region of posterior orbital cortex. The findings regarding the OPro/AI region may be particularly relevant, as this region of the prefrontal cortex is highly connected with the amygdala (87). We recently demonstrated in a sample of 592 young rhesus monkeys that NEC-related glucose metabolism in the OPro/AI region (along with the bed nucleus of the stria terminalis and the periaqueductal gray) correlated with AT and was heritable (10). Importantly, brain metabolism in these regions was also genetically correlated with AT, which implies the involvement of similar genes in mediating AT and altered brain function in OPro/AI, bed nucleus of the stria terminalis, and periaqueductal gray.

Perhaps even more interesting are the structural brain changes that were associated with long-term increased CRH overexpression. Using measures of white matter integrity, we found evidence for decreases in FA in the medial thalamus encompassing portions of the central medial thalamic nucleus, paracentral thalamic nucleus, and the magnocellular division of the ventral anterior thalamic nucleus. Primate studies demonstrate that these regions contain CRH-immunoreactive cell bodies and fibers, as well as relatively high densities of CRHR1 (31,88,89). It is therefore possible that overexpression of CRH in the dorsal amygdala could lead to increased activation of medial thalamic CRHR1. Tractography methods demonstrated that white matter fibers link the thalamic region of significant FA change with other components of the neural network important in the expression and regulation of anxiety



**Figure 7.** Diffusion tensor imaging demonstrated altered thalamic structural integrity as assessed with fractional anisotropy. Whole-brain voxelwise analyses indicated corticotropin-releasing hormone-induced reductions in fractional anisotropy in a region overlapping with the medial dorsal/midline thalamus (corticotropin-releasing hormone group  $[post-pre] - control group [post-pre]$ ) ( $p < .005$ , uncorrected). Deterministic tractography demonstrates that this thalamic region is connected to the prefrontal cortex and medial temporal lobe (*Supplemental Figure S4*). R, right.

(*Supplemental Figure S4*). This is consistent with ex vivo tract tracing studies in macaques that demonstrate projections from the dorsal amygdala to midline thalamic nuclei (90,91). Also, medial thalamic nuclei are reciprocally connected to posterior orbitofrontal cortex regions that include the OPro/AI (92). Thus, the medial/midline thalamus may link dorsal amygdala CRH to metabolic changes in the OPro/AI.

Early excitement related to developing new treatments for human anxiety and depression resulted from numerous mechanistic studies, mostly performed in rodents, directly manipulating brain CRH systems [for reviews, see (93,94)]. Although findings from these studies failed to be translated to positive outcomes in human CRHR1 antagonist clinical trials (95–97), our current findings suggest that continued pursuit of mechanisms directed at altering Ce CRH function in primates might be useful for providing insights into optimizing CRH-altering treatments for human disorders. For example, it may be worth performing human studies targeting CRHR2 because of the possibility that CRHR2 could mediate the effects we observed. Our study also underscores the potential for gene delivery in primate models to elucidate the mechanisms of regional gene expression on distributed brain function, as well as to explore novel treatment strategies for refractory psychiatric illnesses. Taken together, these results indicate that chronically increased dorsal amygdala CRH expression influences AT, metabolic activity within AT's neural substrates, as well as long-range functional connectivity and white-matter microstructure. This work, aimed at understanding the effects of increased CRH in the dorsal amygdala, will help motivate the design of novel interventions to prevent the development of anxiety disorders and other stress-related psychopathology.

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## ARTICLE INFORMATION

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