

the model. B.C.C. performed the cross-linking studies and prepared all the protein for functional assays. J. Z. and R.A.G. performed the translocase assays (J.Z. performed all the mutational studies, and R.A.G. performed capuramycin and divalent metal experiments) under the guidance of P.Z. D.-Y.K. synthesized capuramycin under the guidance of

J.H. Z.G. performed mass spectrometry characterization. B.C.C. and S.-Y.L. wrote the paper.

#### Supplementary Materials

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Materials and Methods

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# $\beta$ CaMKII in Lateral Habenula Mediates Core Symptoms of Depression

Kun Li,<sup>1,2\*</sup> Tao Zhou,<sup>1,2\*</sup> Lujian Liao,<sup>3†</sup> Zhongfei Yang,<sup>1</sup> Catherine Wong,<sup>3†</sup> Fritz Henn,<sup>4</sup> Roberto Malinow,<sup>5</sup> John R. Yates III,<sup>3</sup> Hailan Hu<sup>1‡</sup>

The lateral habenula (LHb) has recently emerged as a key brain region in the pathophysiology of depression. However, the molecular mechanism by which LHb becomes hyperactive in depression remains unknown. Through a quantitative proteomic screen, we found that expression of the  $\beta$  form of calcium/calmodulin-dependent protein kinase type II ( $\beta$ CaMKII) was significantly up-regulated in the LHb of animal models of depression and down-regulated by antidepressants. Increasing  $\beta$ -, but not  $\alpha$ -, CaMKII in the LHb strongly enhanced the synaptic efficacy and spike output of LHb neurons and was sufficient to produce profound depressive symptoms, including anhedonia and behavioral despair. Down-regulation of  $\beta$ CaMKII levels, blocking its activity or its target molecule the glutamate receptor GluR1 reversed the depressive symptoms. These results identify  $\beta$ CaMKII as a powerful regulator of LHb neuron function and a key molecular determinant of depression.

Major depressive disorder (MDD), one of the most prevalent and disabling mental disorders, is characterized by low mood, loss of motivation, feelings of despair, and an inability to feel pleasure, also known as anhedonia (1). Modern views on the cause of MDD suggest that the neural activities of specific brain circuits are altered in response to external stimuli, such as stress, as a result of maladaptive molecular and cellular changes (2, 3). Recently, the lateral habenula (LHb), a nucleus that relays information from the limbic forebrain to multiple monoamine centers, has emerged as a key brain region in aversive behaviors and the pathophysiology of depression (4–10). LHb neurons are activated by aversive emotional cues, including stress, disappointment, fear, or anticipation of a negative reward (4–6). Consistently, neuroimaging studies have identified heightened habenula activity in the depressed state (11–13). Furthermore, synaptic activity and spike output of LHb neurons were enhanced in animal models of depression (14). However, what molecular mechanisms

underlie these aberrant cellular processes in LHb and how depression-inducing stimuli lead to these changes are yet to be determined.

We conducted an unbiased, mass spectrometry-based, quantitative proteomic screening to compare habenular protein expression of wild-type controls with that of congenitally learned helpless (cLH) rats, a well-accepted model of depression (15). cLH rats were selectively bred for the phenotype of learned helplessness (16). They displayed significantly reduced lever pressing to escape foot shocks, which was reversed by chronic antidepressant treatment (imipramine, injected intraperitoneally 10 mg per kg of body weight, for 14 days) (Fig. 1A). cLH rats also showed increased (longer) immobility in the forced swim test (Fig. 1A), another animal model of depression that reflects behavioral despair (17), although basic motor and cognitive functions are normal (15).

We microdissected the habenula of cLH and wild-type control rats and extracted protein for quantitative proteomic analysis based on <sup>15</sup>N stable isotope labeling (Fig. 1B) (18). To reduce sample complexity, we extracted the membrane fraction and analyzed three independent sets of samples (figs. S1 to S3 and table S1). The  $\beta$  form of calcium/calmodulin-dependent protein kinase type II ( $\beta$ CaMKII) was significantly up-regulated in the habenula of cLH rats (1.9 times the  $\beta$ CaMKII of wild-type control,  $P = 0.01$ ) (Fig. 1C). Other CaMKII family isoforms were also examined:  $\alpha$ CaMKII levels varied widely across samples, although an increasing trend was observed;  $\delta$ CaMKII remained unchanged; and  $\gamma$ CaMKII showed a 1.3-fold increase ( $P = 0.0013$ ) (fig. S4). As  $\beta$ CaMKII is more enriched in the brain than  $\gamma$ CaMKII (19, 20), we focused on this CaMKII isoform. Secondary

validation by Western blot analysis confirmed that  $\beta$ CaMKII in the membrane fraction of cLH habenular protein samples increased to 1.86 times those of the controls ( $P = 0.03$ ) (Fig. 1D). In contrast, the  $\beta$ CaMKII protein level in cLH hippocampal samples decreased (63% of control,  $P = 0.048$ ) (Fig. 1E), probably because of neural atrophy and spine loss in the hippocampus associated with depression (21, 22).  $\beta$ CaMKII mRNA in cLH habenula increased to 1.37 times the amount in controls ( $P = 0.04$ ), as measured by quantitative real-time PCR (QPCR) (Fig. 1F), which suggested that transcriptional regulation contributed to at least part of the change in the amount of protein. Immunohistochemical staining of habenular brain slices revealed that the CaMKII protein increased in the lateral part of the habenula (LHb) (fig. S5).

We further examined  $\beta$ CaMKII in two additional depression models, acute learned helplessness, induced by repeated inescapable and uncontrollable foot shocks (16), and chronic mild stress, induced by prolonged exposure to unpredictable mild stressors (23). The amounts of  $\beta$ CaMKII were also significantly increased in these two stress paradigms (Fig. 1G). Furthermore, chronic antidepressant treatment with imipramine, which reversed the depressive phenotypes of cLH rats (Fig. 1A), caused significant down-regulation of  $\beta$ CaMKII protein in the habenula of cLH rats (Fig. 1H).

We next investigated to what extent the change in  $\beta$ CaMKII in LHb is necessary or sufficient to cause depressive behaviors or whether it is merely a biological marker of the depressive state. We first constructed viral vectors [adeno-associated virus 2 (AAV2)] to overexpress  $\beta$ - or  $\alpha$ CaMKII in the LHb of wild-type rats and mice and tested the effects on various depression models (Fig. 2, A to C). We used the ubiquitin promoter to drive ubiquitous, high-level gene expression, as LHb neurons are almost uniformly glutamatergic (24). To estimate the level of overexpression, we injected CaMKII-expressing viruses into one side of the habenula. Fourteen days after injection, CaMKII levels in the injected side were 4.4 ( $\pm 0.6$ )-fold for  $\beta$ CaMKII and 4.6 ( $\pm 0.9$ )-fold for  $\alpha$ CaMKII of the noninjected side, respectively (fig. S6). We then injected virus into both sides of LHb and allowed expression for 10 days before behavioral testing of depression (Fig. 2, B and C, and fig. S7).

Overexpression of  $\beta$ CaMKII in the LHb of unstressed mice produced significantly increased immobility time and decreased latency to immobility onset in the forced swim test, compared with the injected control (Fig. 2D). Locomotor activities of these mice were not significantly different (fig. S8), which indicated that the immobility

<sup>1</sup>Institute of Neuroscience and State Key Laboratory of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, P. R. China. <sup>2</sup>Graduate School of Chinese Academy of Sciences, University of Chinese Academy of Sciences, Shanghai 200031, P. R. China. <sup>3</sup>The Scripps Research Institute, Department of Molecular and Cellular Neurobiology, La Jolla, CA 92037, USA. <sup>4</sup>Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA. <sup>5</sup>University of California at San Diego, La Jolla, CA, 92093, USA.

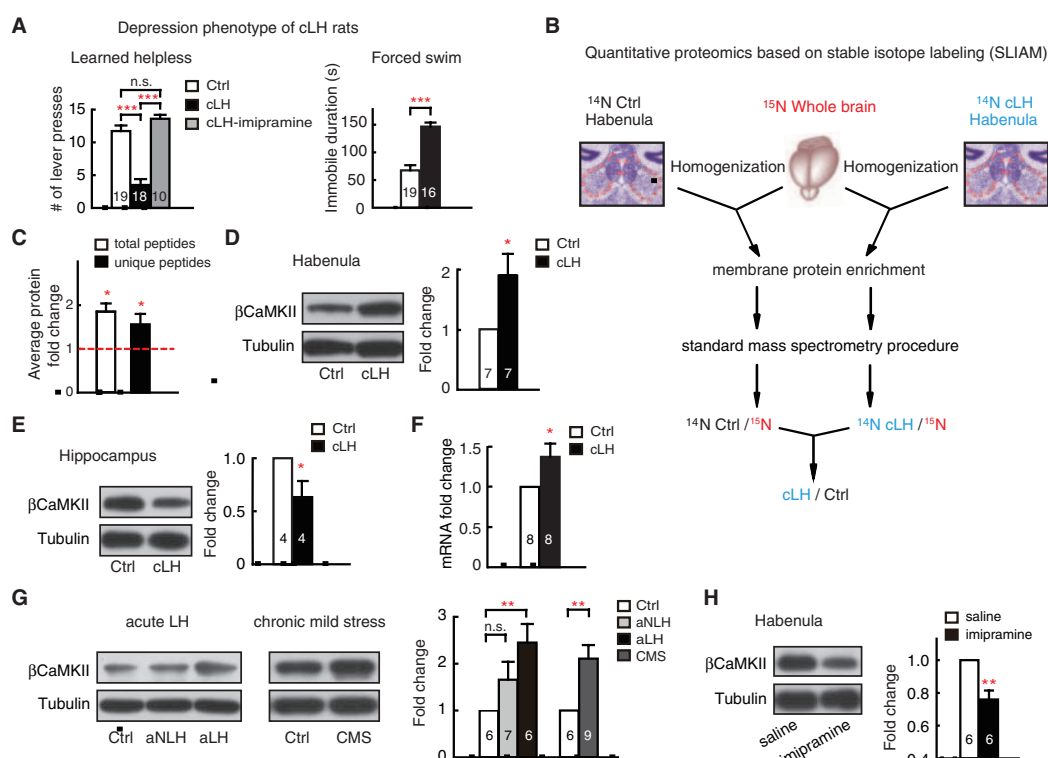
\*These authors contributed equally to this work.

†Present address: Institute of Biochemistry and Cell Biology, National Center for Protein Science, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, P. R. China.

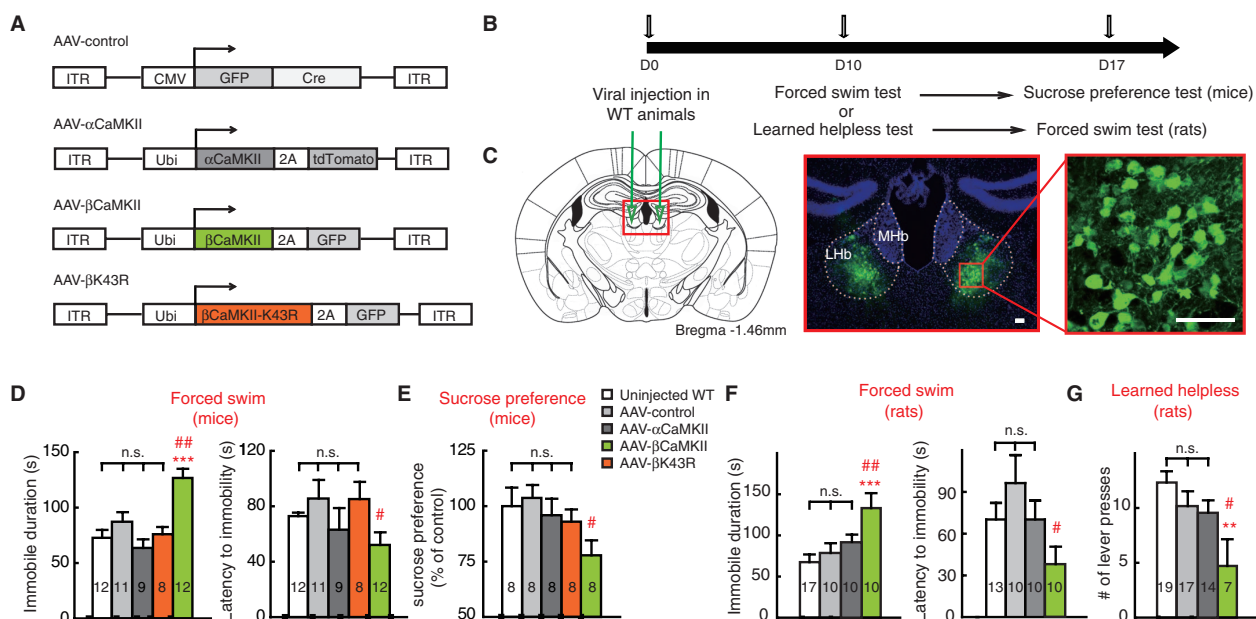
‡Corresponding author. E-mail: hailan@ion.ac.cn

### Fig. 1. $\beta$ CaMKII is up-regulated in the LHb of animal models of depression.

(A) Depression phenotypes of cLH rats. Numbers in the bars indicate the number of animals used. Note that, in the LH test, the maximal number of bar presses is 15. (B) Experimental outline of the high-throughput quantitative proteomics based on stable isotope labeling. Briefly, habenula of (red dotted area) unlabeled ( $^{14}\text{N}$ ) wild-type control (WT) or cLH rats were dissected, homogenized, and mixed in a 1:1 ratio with total brain homogenate from a  $^{15}\text{N}$ -labeled rat. Membrane fraction was enriched, and a 100- $\mu\text{g}$  protein sample was used for standard mass spectra analysis. We calculated the  $^{14}\text{N}/^{15}\text{N}$  ratio for each identified peptide. Peptide ratios for each protein were then compared between cLH and control sample. For details, see methods. (C) Proteomic analysis of  $\beta$ CaMKII, based either on total peptides or unique peptides (peptides not shared by other CaMKII family members) identified in three independent proteomic runs. (D and E) Western blot analysis showing change of  $\beta$ CaMKII in membrane fraction of habenula (D) or hippocampus (E) of cLH rats. Tissue amounts of tubulin were used as loading control. Protein expression was normalized by control amount. (F) QPCR analysis of  $\beta$ CaMKII mRNA in habenula. (G)  $\beta$ CaMKII increase in acute learned helpless and chronic mild stress (CMS) depression models. aLH and aNLH were rat groups subjected to LH stress that did (aLH), or did not (aNLH), display LH symptom. (H) Western



blot analysis showing amount of  $\beta$ CaMKII in the membrane fraction of habenula of cLH rats treated with saline or antidepressant imipramine. Data are means  $\pm$  SEM. We used two-tailed Student's *t* tests for two-group comparison, one-way analysis of variance (ANOVA) with Bonferroni post hoc analysis for multiple-group comparison. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 compared with the control group; n.s., not significant.



**Fig. 2. Overexpression of  $\beta$ CaMKII but not  $\alpha$ CaMKII in LHb caused depressive-like behaviors in both mice and rats.** (A) Schematics of AAV vectors engineered to overexpress a control construct,  $\beta$ CaMKII,  $\alpha$ CaMKII, or a kinase-dead mutant of  $\beta$ CaMKII. ITR, inverted terminal repeats; CMV, cytomegalovirus promoter; Ubi: ubiquitin promoter; 2A: viral 2A linker peptide allowing translation of multiple unfused proteins. (B) Experimental paradigm for behavioral testing of WT mice or rats. (C) Illustration of

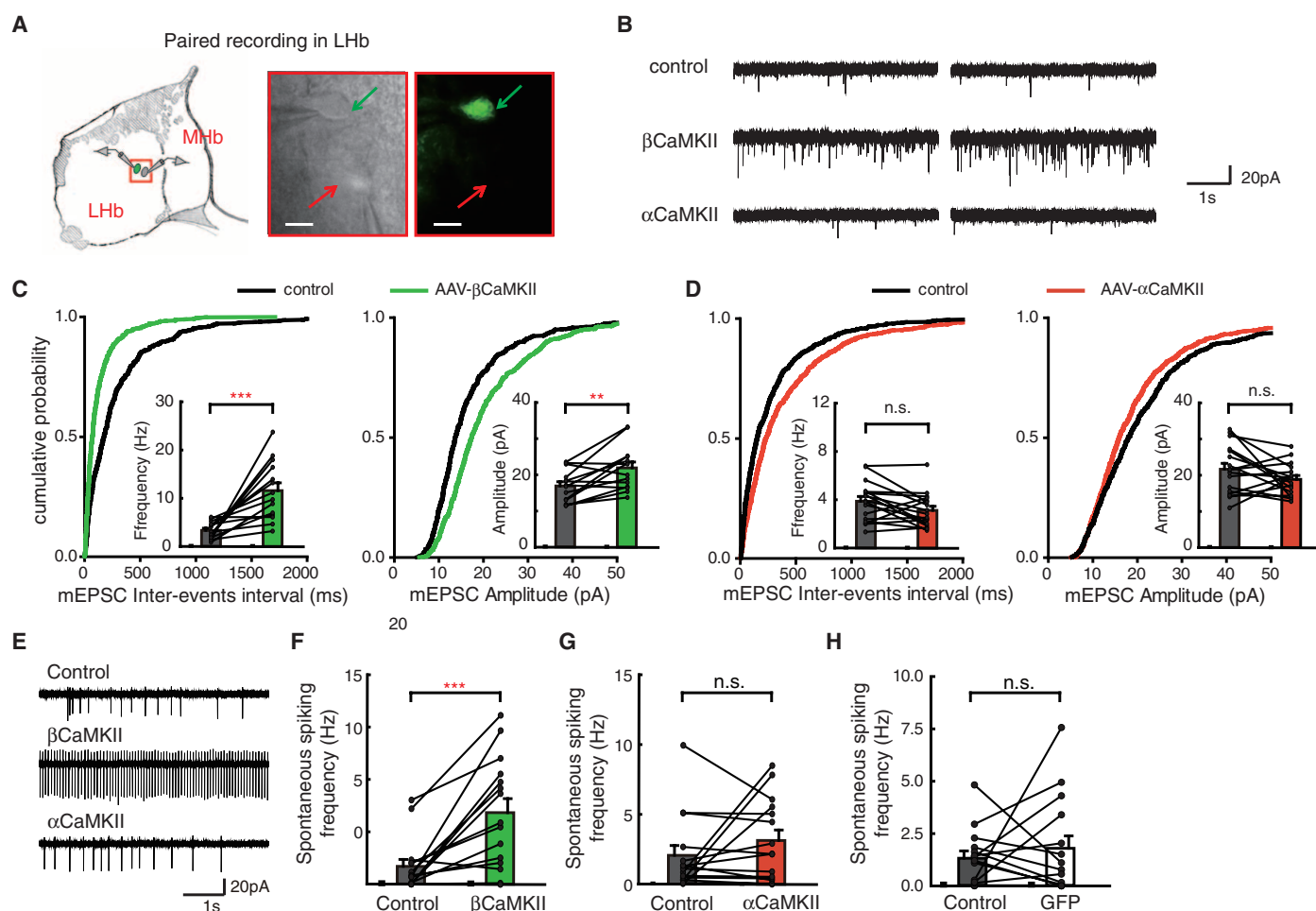
bilateral viral injection of AAV- $\beta$ CaMKII in mouse LHb (counterstained with antibody against GFP and nuclear marker Hoechst). Scale bars, 50  $\mu\text{m}$ . (D to G) Behavioral effects of expressing various viral constructs in LHb in animal models of depression in mice (D to E) or rats (F and G). \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 compared with noninjected WT, #*P* < 0.05, ##*P* < 0.01 compared with AAV-control, one-way ANOVA with Bonferroni post hoc analysis.

was not likely due to motor defects. In addition,  $\beta$ CaMKII overexpression also caused anhedonia, evident from a significant reduction in the preference for the sucrose solution (Fig. 2E). To estimate the minimal infection rate required to produce the depression phenotype, we bilaterally injected an additional group of mice with 1:10 diluted AAV- $\beta$ CaMKII virus. Unlike the normal injection group (having an infection rate of  $38 \pm 3\%$ ), this sparse injection group (having an infection rate of  $5 \pm 0.6\%$ ) did not exhibit depressive-like phenotypes (fig. S9). Overexpression of  $\alpha$ CaMKII, or a control green fluorescent protein (GFP)-Cre construct, at a similar infection rate ( $36 \pm 2.5\%$  for  $\alpha$ CaMKII,  $36 \pm 3\%$  for GFP-Cre) did not cause similar depressive-like effects (Fig. 2, D and E).  $\beta$ CaMKII can act both as a kinase and a structural scaffolding protein at the synapses (25). A kinase-dead version of  $\beta$ CaMKII [in which lysine 43 is replaced by arginine ( $\beta$ K43R)] (26), even when overexpressed similarly to wild-

type  $\beta$ CaMKII ( $4.6 \pm 0.5$ -fold, infection rate of  $40 \pm 3\%$ ) (fig. S6), did not cause depressive-like phenotypes (Fig. 2, D and E), which suggested that the kinase function of  $\beta$ CaMKII was required to produce depression. Furthermore, in rats, overexpression of  $\beta$ CaMKII in the LHb significantly increased immobility in the forced swim test and reduced the escape behavior, as indicated by the number of times the bar was pressed to terminate foot shocks in the learned helplessness test (Fig. 2, F and G).

To investigate the cellular mechanism by which  $\beta$ CaMKII overexpression alters LHb neuron activity and function, we performed paired whole-cell patch-clamp recordings on viral-infected and neighboring uninfected LHb neurons in acute brain slices of wild-type rats (Fig. 3A). First, to examine the synaptic property of LHb neurons, we measured the miniature excitatory postsynaptic currents (mEPSCs), which are mediated by the AMPA subtype of glutamate receptors and reflect

individual synaptic responses onto recorded neurons. In noninfected brain slices, neighboring LHb neuron pairs showed highly similar mEPSC frequency, despite heterogeneity across different subregions of the LHb (fig. S10). In neurons infected by AAV- $\beta$ CaMKII, mEPSC frequency was greatly increased ( $351 \pm 51\%$  of neighboring controls,  $P = 0.0001$ ), as was mEPSC amplitude ( $130 \pm 10\%$  of controls,  $P = 0.007$ ) (Fig. 3, B and C). In contrast, AAV- $\alpha$ CaMKII infection caused a slight decrease in mEPSC frequency ( $81 \pm 9\%$  of controls,  $P = 0.08$ ) and no change in mEPSC amplitude ( $87 \pm 5\%$  of controls,  $P = 0.16$ ) (Fig. 3, B and D). The effects on mEPSC frequency largely resembled what has been shown in hippocampal neurons for these two CaMKII isoforms (27). Further, to examine the output of these LHb neurons, we measured the spontaneous spiking rate in a cell-attached configuration. The spiking rate of AAV- $\beta$ CaMKII-infected neurons increased 3.0-fold compared with neighboring uninfected neurons ( $P = 0.0004$ )



**Fig. 3. Overexpression of  $\beta$ CaMKII increased synaptic activity and spike output of LHb neurons.** (A) Schematics of paired recording configuration in LHb (left). (Right) Paired patching of a  $\beta$ CaMKII-infected LHb neuron (green arrow) and a neighboring uninfected neuron (red arrow) under transmitted and fluorescent light microscopy. Scale bars, 10  $\mu$ m. (B) Example mEPSC traces, measured in a whole-cell configuration, from LHb neurons of control neurons or neurons infected by AAV- $\beta$ CaMKII or AAV- $\alpha$ CaMKII. (C and D) Cumulative distribution of mEPSC interevents interval and aver-

age frequency (left), or mEPSC amplitude (right) of neurons infected by AAV- $\beta$ CaMKII (C) or AAV- $\alpha$ CaMKII (D). Each line represents values from a pair of control and neighboring viral-infected neurons. (E) Example traces of spontaneous spiking, measured in a cell-attached configuration, from LHb neurons of control neurons, or neurons infected by AAV- $\beta$ CaMKII or AAV- $\alpha$ CaMKII. (F to H) Average spontaneous spiking frequency of neurons infected by AAV- $\beta$ CaMKII (F), AAV- $\alpha$ CaMKII (G), or AAV-GFP (H).  $**P < 0.01$ ,  $***P < 0.001$ , Wilcoxon signed-rank test.



(Fig. 3, E and F), whereas no such change was detected for AAV- $\alpha$ CaMKII-infected ( $P = 0.7$ ) (Fig. 3G), or AAV-GFP-infected neurons ( $P = 0.95$ ) (Fig. 3H).

To determine whether down-regulation of  $\beta$ CaMKII or blockade of  $\beta$ CaMKII functions in LHB reversed depressive phenotypes, we first used RNA interference (RNAi) to reduce (knock down) the amount of  $\beta$ CaMKII protein (Fig. 4A). A previously reported short hairpin RNA specifically targeting the  $\beta$ CaMKII transcript can effectively reduce  $\beta$ CaMKII protein without affecting  $\alpha$ CaMKII (28) (Fig. 4B). We then expressed this RNAi form of  $\beta$ CaMKII in AAV virus (AAV- $\beta$ RNAi) and targeted its expression to the LHB of cLH rats by bilateral stereotactic injection (Fig. 4C). In cLH rats infected by AAV- $\beta$ RNAi, the immobility time in the forced swim test was markedly reduced ( $P < 0.001$ ), and the escape behavior in the learned help-

less test significantly increased ( $P < 0.05$ ) (Fig. 4, D and E). The percentage of learned helpless animals (defined as those that pressed the bar less than five times) dropped from 83.3 to 25% (Fig. 4F).

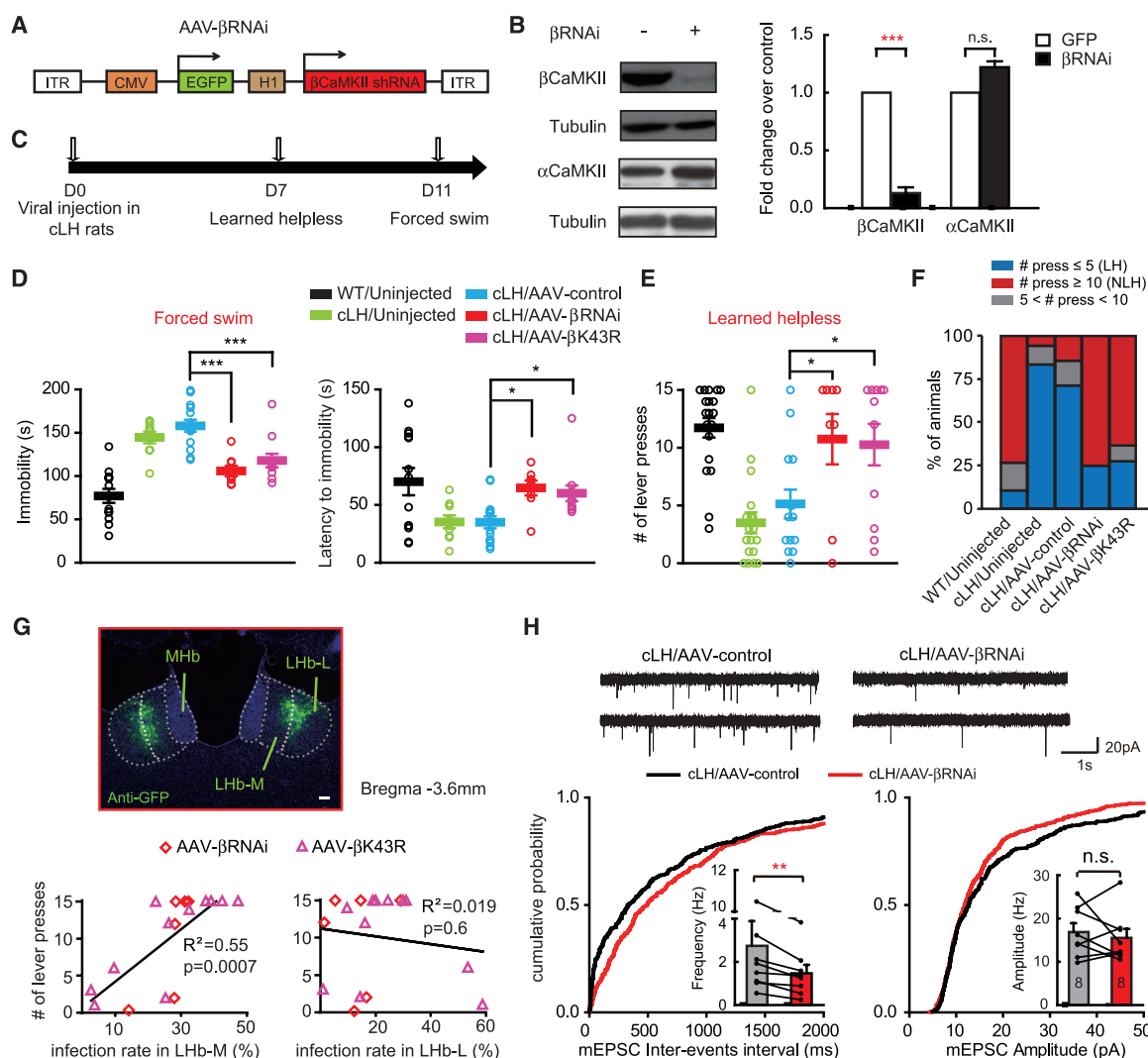
To rule out potential off-target effects by RNAi, we tested a kinase-dead version of  $\beta$ CaMKII,  $\beta$ K43R, which acts as a dominant-negative protein to block endogenous  $\beta$ CaMKII function when overexpressed (26) (Fig. 2A). When we injected AAV- $\beta$ K43R virus into the LHB of cLH rats, we observed antidepressive effects similar to those seen with AAV- $\beta$ RNAi in both the forced swim and learned helplessness tests (Fig. 4, D and E). It was noteworthy that an infection rate as low as  $17 \pm 3\%$  for AAV- $\beta$ RNAi or  $24 \pm 3\%$  for AAV- $\beta$ K43R was sufficient to achieve these strong antidepressive effects, which suggested that the LHB neural network has little redundancy and that altering the activity of a relatively small percentage

of LHB neurons is sufficient to ameliorate depression. Thus, we further analyzed infection within subregions of LHB, and found that infection rates in the medial, but not lateral, part of LHB were strongly correlated with the rescuing effects on learned helplessness (Fig. 4G). In the LHB brain slices of cLH rats, neurons infected by the AAV- $\beta$ RNAi showed significantly reduced frequency of mEPSCs ( $53.5 \pm 15.3\%$  of neighboring uninfected controls,  $P = 0.008$ ) (Fig. 4H).

What could be the downstream molecular targets of  $\beta$ CaMKII in mediating the LHB hyperactivity in depression? Up-regulation of  $\beta$ CaMKII increases the synaptic expression and delivery of glutamate receptor GluR1-type AMPAR in cultured hippocampal neurons (29). Thus, we examined the amount of GluR1 in the LHB of cLH rats and found that the membrane fraction of GluR1 was up-regulated ( $222 \pm 41.7\%$  of controls,

**Fig. 4. Knocking-down of  $\beta$ CaMKII in LHB rescued depression-like phenotypes of cLH rats and reduced synaptic activity of LHB neurons.** (A) Schematic of the AAV vector engineered to overexpress an RNAi form of  $\beta$ CaMKII. H1, human H1 promoter. (B) Specific knocking down of  $\beta$ CaMKII but not  $\alpha$ CaMKII by the  $\beta$ CaMKII RNAi construct.

pSuper- $\beta$ CaMKII-RNAi construct was cotransfected with AAV- $\beta$ CaMKII or AAV- $\alpha$ CaMKII plasmid in 293TN cells and expressed for 48 hours before Western analysis. (Left) Representative Western blot. (Right) quantification of knock-down efficiency. (C) Experimental paradigm for behavioral testing of cLH rats infected by virus. (D to F) Behavioral effects of expressing AAV- $\beta$ RNAi and AAV- $\beta$ K43R in the LHB of cLH rats in forced swim (D) or learned helplessness test (E and F). \* $P < 0.05$ , \*\*\* $P < 0.001$ , one-way ANOVA with Bonferroni post hoc test. (F) Percentage of animals in each category. LH, learned helpless rats with  $\leq 5$  lever presses; NLH, non-learned helpless rats with  $\geq 10$  lever presses. (G) Subregional characterization of viral infection. (Top) The division of lateral LHB (LHB-L) and medial LHB (LHB-M) in an AAV- $\beta$ RNAi virus-infected cLH rat brain slice. Scale bar, 100  $\mu$ m. (Bottom) Behavioral response in the learned helplessness test plotted against infection rates in the LHB-L and LHB-M of cLH rats. (H) mEPSCs of AAV- $\beta$ RNAi-infected LHB neurons



from cLH rats were altered. (Top) Examples of mEPSC traces. Cumulative distribution of mEPSC interevents interval and average frequency (bottom left) or mEPSC amplitude (bottom right) of cLH LHB neurons infected by AAV- $\beta$ RNAi. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared with cLH/AAV-control, Wilcoxon signed-rank test.

$P < 0.01$ ) (fig. S11A). Conversely, in cLH rats treated with the antidepressant imipramine, the membrane GluR1 level was decreased ( $72 \pm 8\%$  of controls,  $P = 0.003$ ) (fig. S11B). To test the role of GluR1 in  $\beta$ CaMKII-mediated depression, we coexpressed  $\beta$ CaMKII with a dominant-negative form of GluR1, GluR1Ct, which blocks the synaptic trafficking of GluR1 (30, 31), using an AAV- $\beta$ CaMKII-2A-GluR1Ct viral construct. Tests after unilateral injection revealed that  $\beta$ CaMKII exhibited a 3.8-fold and GluR1Ct a 3.7-fold overexpression of endogenous levels (fig. S11C). When this virus was bilaterally injected into the LHb of wild-type mice (infection rate of  $37 \pm 2\%$ ), mice performed normally in both the forced swim and sucrose preference tests (fig. S11, D and E). Therefore, coexpression of GluR1Ct prevented the depressive effects of  $\beta$ CaMKII overexpression.

Here, we identified  $\beta$ CaMKII as a key molecular determinant of habenular hyperactivity and behavioral depression, using a combination of molecular, behavioral, and electrophysiological approaches. Our results point to a model in which stress-induced up-regulation of  $\beta$ CaMKII in the LHb causes more GluR1 insertion into synapses, resulting in increased synaptic efficacy. In addition,  $\beta$ CaMKII may regulate other channels and membrane properties of LHb neurons to enhance spike output, all of which work in concert to cause LHb hyperactivity and lead to enhanced suppression of downstream monoamine centers (fig. S12A). Previous studies have implicated changes in CaMKIIs related to stress and antidepressant response (32–35). However, it was unclear whether these changes are necessary or sufficient for depression etiology, and which brain region or isoform is crucial (36).  $\beta$ CaMKII is about eight times as sensitive to calcium/calmodulin as  $\alpha$ CaMKII (37) and has an actin-binding motif that is absent in  $\alpha$ CaMKII (38). It will be interesting to pinpoint in the future which feature of  $\beta$ CaMKII renders this isoform-specific function in depression.

An important future question is how depressive stimuli and antidepressants lead to bidirectional changes in  $\beta$ CaMKII levels in the LHb. Aversive emotional stimuli activate LHb neurons (4–6), whereas serotonin boosted by antidepressants suppresses excitatory inputs onto the LHb (7). Thus, it is tempting to speculate that  $\beta$ CaMKII levels are regulated by LHb neural activity. During the onset of depression, prolonged activation of LHb neurons may cause the up-regulation of  $\beta$ CaMKII to reach a threshold level, which can lead to further LHb neural activation. Conversely, antidepressant-caused sup-

pression of the LHb may down-regulate  $\beta$ CaMKII, which can further lower LHb activity. These self-reinforcing feedback processes may eventually drive long-term adaptive changes in emotional states. Whether and how neuronal activity regulates  $\beta$ CaMKII expression awaits further investigation.

In the context of our finding that the medial part of the LHb (LHb-M) is critical for the rescue of learned helplessness behavior (Fig. 4G), it is relevant to note that stress-induced c-Fos activation is relatively confined to the LHb-M (4) and that there were more neurons with higher mEPSC frequency in LHb-M than those in LHb-L (fig. S10A). Further, LHb-M and LHb-L have different circuit wiring, which could contribute to their differential involvement in aversive behaviors and depressive symptoms (fig. S12B). Molecular manipulations in the nucleus accumbens (NAc) specifically mediate anhedonia but not behavioral despair (39). We found manipulation of  $\beta$ CaMKII levels in the LHb affected both of these symptoms, which suggests that LHb may function upstream of NAc in the depression-related circuitry to control multiple aspects of depressive symptoms. Hence, the molecular targets identified in the LHb in this study should provide new insights for therapies that treat both of these core symptoms of depression.

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#### Supplementary Materials

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Materials and Methods  
Figs. S1 to S12  
Table S1  
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