# βCaMKII in Lateral Habenula Mediates Core Symptoms of Depression

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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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# βCaMKII in Lateral Habenula Mediates Core Symptoms of Depression

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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 β form of calcium/calmodulin-dependent protein kinase type II (βCaMKII) was significantly up-regulated in the LHb of animal models of depression and down-regulated by antidepressants. Increasing β-, but not α-, 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 βCaMKII levels, blocking its activity or its target molecule the glutamate receptor GluR1 reversed the depressive symptoms. These results identify βCaMKII 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 habenuli of cLH and wild-type control rats and extracted protein for quantitative proteomic analysis based on $^{15}N$ 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 β form of calcium/calmodulin-dependent protein kinase type II (βCaMKII) was significantly up-regulated in the habenula of cLH rats (1.9 times the βCaMKII of wild-type control, $P = 0.01$) (Fig. 1C). Other CaMKII family isoforms were also examined: αCaMKII levels varied widely across samples, although an increasing trend was observed; δCaMKII remained unchanged; and γCaMKII showed a 1.3-fold increase ($P = 0.0013$) (fig. S4). As βCaMKII is more enriched in the brain than γCaMKII (*19*, *20*), we focused on this CaMKII isoform. Secondary validation by Western blot analysis confirmed that βCaMKII 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 βCaMKII 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*). βCaMKII 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 βCaMKII 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 βCaMKII 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 βCaMKII protein in the habenula of cLH rats (Fig. 1H).

We next investigated to what extent the change in βCaMKII 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 β- or αCaMKII 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 (± 0.6)–fold for βCaMKII and 4.6 (± 0.9)–fold for αCaMKII 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 βCaMKII 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

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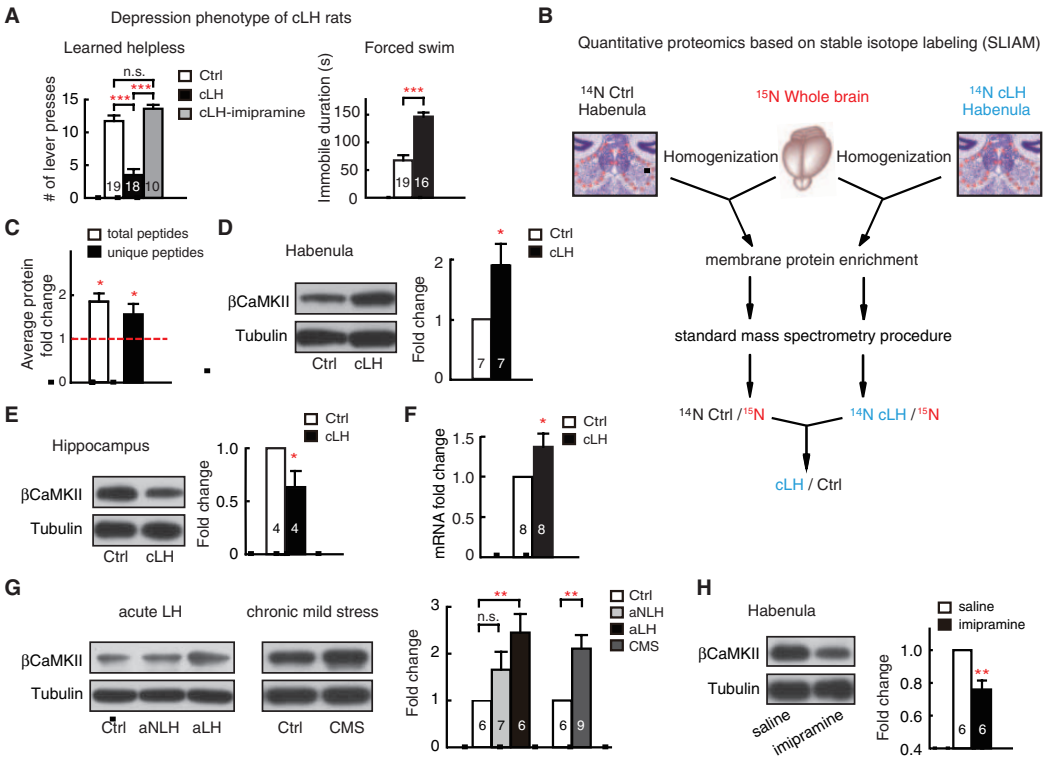


**Fig. 1. βCaMKII is up-regulated in the LHb of animal models of depression.** (**A**) Depression phenotypes of cLH rats. Numbers in the bars indicate the number of animals used. Note that, in the LH test, the maximal number of bar presses is 15. (**B**) Experimental outline of the high-throughput quantitative proteomics based on stable isotope labeling. Briefly, habenula of (red dotted area) unlabeled ($^{14}$N) wild-type control (WT) or cLH rats were dissected, homogenized, and mixed in a 1:1 ratio with total brain homogenate from a $^{15}$N-labeled rat. Membrane fraction was enriched, and a 100-μg protein sample was used for standard mass spectra analysis. We calculated the $^{14}$N /$^{15}$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 βCaMKII, 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 βCaMKII 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 βCaMKII mRNA in habenula. (**G**) βCaMKII increase in acute learned helpless and chronic mild stress (CMS) depression models. aLH and aNLH were rat groups subjecting to LH stress that did (aLH), or did not (aNLH), display LH symptom. (**H**) Western blot analysis showing amount of βCaMKII in the membrane fraction of habenula of cLH rats treated with saline or antidepressant imipramine. Data are means ± 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.

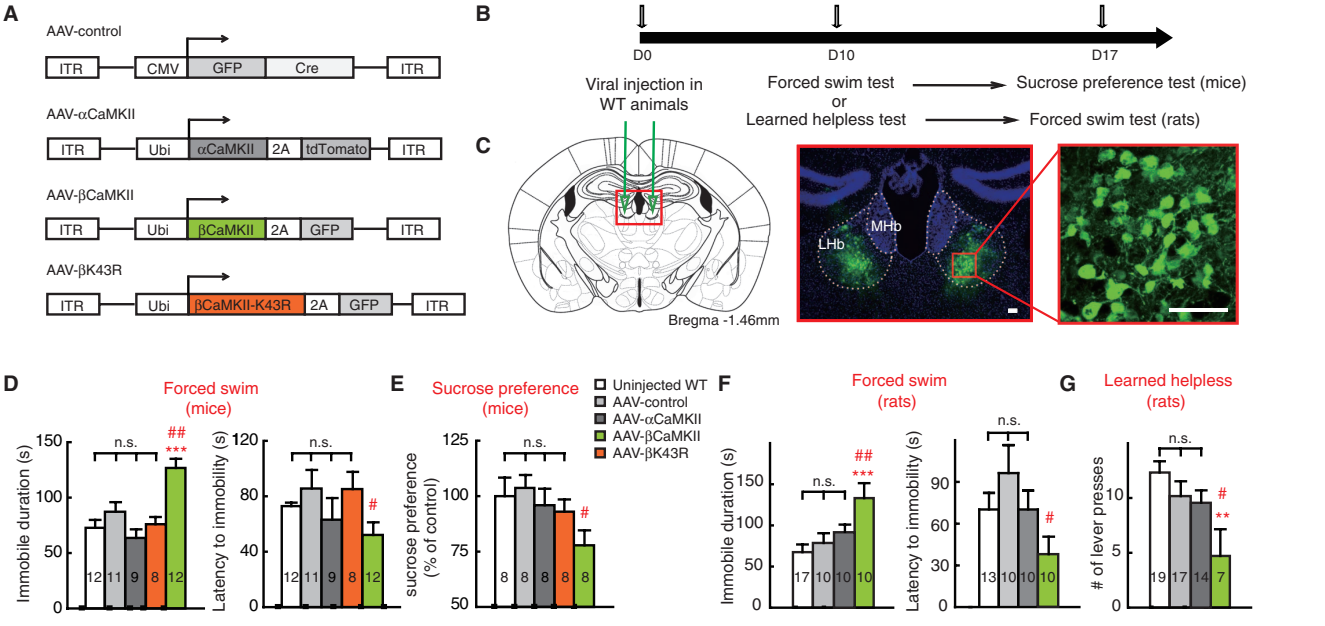


**Fig. 2. Overexpression of βCaMKII but not αCaMKII in LHb caused depressive-like behaviors in both mice and rats.** (**A**) Schematics of AAV vectors engineered to overexpress a control construct, βCaMKII, αCaMKII, or a kinase-dead mutant of βCaMKII. ITR, inverted terminal repeats; CMV, cytomegalovirus 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-βCaMKII in mouse LHb (counterstained with antibody against GFP and nuclear marker Hoechst). Scale bars, 50 μm. (**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, βCaMKII 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-βCaMKII virus. Unlike the normal injection group (having an infection rate of 38 ± 3%), this sparse injection group (having an infection rate of 5 ± 0.6%) did not exhibit depressive-like phenotypes (fig. S9). Overexpression of αCaMKII, or a control green fluorescent protein (GFP)-Cre construct, at a similar infection rate (36 ± 2.5% for αCaMKII, 36 ± 3% for GFP-Cre) did not cause similar depressive-like effects (Fig. 2, D and E). βCaMKII can act both as a kinase and a structural scaffolding protein at the synapses (*25*). A kinase-dead version of βCaMKII [in which lysine 43 is replaced by arginine (βK43R)] (*26*), even when overexpressed similarly to wild-type βCaMKII (4.6 ± 0.5–fold, infection rate of 40 ± 3%) (fig. S6), did not cause depressive-like phenotypes (Fig. 2, D and E), which suggested that the kinase function of βCaMKII was required to produce depression. Furthermore, in rats, overexpression of βCaMKII 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 βCaMKII 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-βCaMKII, mEPSC frequency was greatly increased (351 ± 51% of neighboring controls, $P = 0.0001$), as was mEPSC amplitude (130 ± 10% of controls, $P = 0.007$) (Fig. 3, B and C). In contrast, AAV-αCaMKII infection caused a slight decrease in mEPSC frequency (81 ± 9% of controls, $P = 0.08$) and no change in mEPSC amplitude (87 ± 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-βCaMKII–infected neurons increased 3.0-fold compared with neighboring uninfected neurons ($P = 0.0004$)

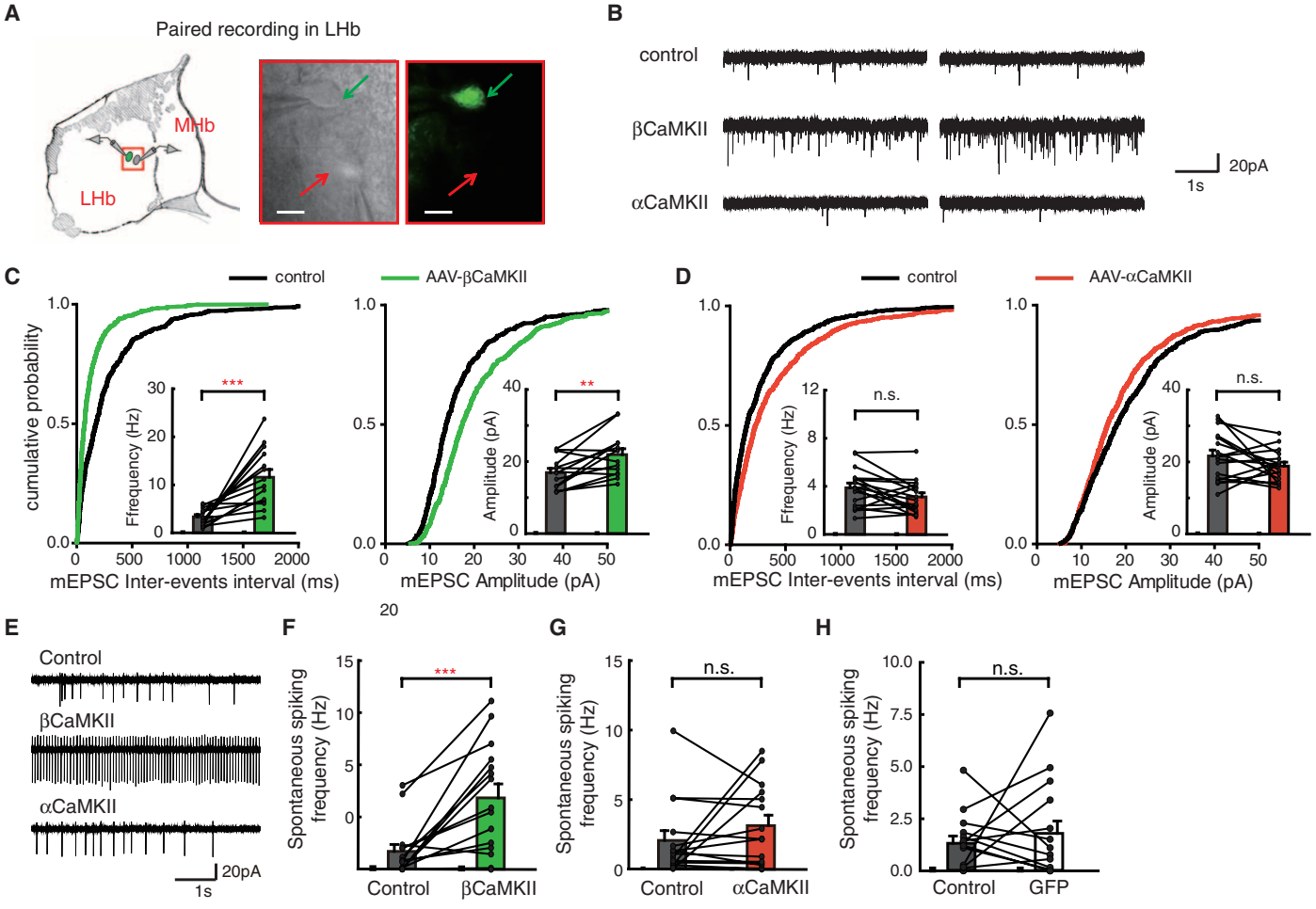


**Fig. 3. Overexpression of βCaMKII increased synaptic activity and spike output of LHb neurons.** (**A**) Schematics of paired recording configuration in LHb (left). (Right) Paired patching of a βCaMKII-infected LHb neuron (green arrow) and a neighboring uninfected neuron (red arrow) under transmitted and fluorescent light microscopy. Scale bars, 10 μm. (**B**) Example mEPSC traces, measured in a whole-cell configuration, from LHb neurons of control neurons or neurons infected by AAV-βCaMKII or AAV-αCaMKII. (**C** and **D**) Cumulative distribution of mEPSC interevents interval and average frequency (left), or mEPSC amplitude (right) of neurons infected by AAV-βCaMKII (C) or AAV-αCaMKII (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-βCaMKII or AAV-αCaMKII. (**F** to **H**) Average spontaneous spiking frequency of neurons infected by AAV-βCaMKII (F), AAV-αCaMKII (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-αCaMKII–infected ($P$ = 0.7) (Fig. 3G), or AAV-GFP–infected neurons ($P$ = 0.95) (Fig. 3H).

To determine whether down-regulation of βCaMKII or blockade of βCaMKII functions in LHb reversed depressive phenotypes, we first used RNA interference (RNAi) to reduce (knock down) the amount of βCaMKII protein (Fig. 4A). A previously reported short hairpin RNA specifically targeting the βCaMKII transcript can effectively reduce βCaMKII protein without affecting αCaMKII (*28*) (Fig. 4B). We then expressed this RNAi form of βCaMKII in AAV virus (AAV-βRNAi) and targeted its expression to the LHb of cLH rats by bilateral stereotactic injection (Fig. 4C). In cLH rats infected by AAV-βRNAi, the immobility time in the forced swim test was markedly reduced ($P$ < 0.001), and the escape behavior in the learned helpless 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 βCaMKII, βK43R, which acts as a dominant-negative protein to block endogenous βCaMKII function when overexpressed (*26*) (Fig. 2A). When we injected AAV-βK43R virus into the LHb of cLH rats, we observed antidepressive effects similar to those seen with AAV-βRNAi in both the forced swim and learned helplessness tests (Fig. 4, D to F). It was noteworthy that an infection rate as low as 17 ± 3% for AAV-βRNAi or 24 ± 3% for AAV-βK43R 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-βRNAi showed significantly reduced frequency of mEPSCs (53.5 ± 15.3% of neighboring uninfected controls, $P$ = 0.008) (Fig. 4H).

What could be the downstream molecular targets of βCaMKII in mediating the LHb hyperactivity in depression? Up-regulation of βCaMKII 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 ± 41.7% of controls,

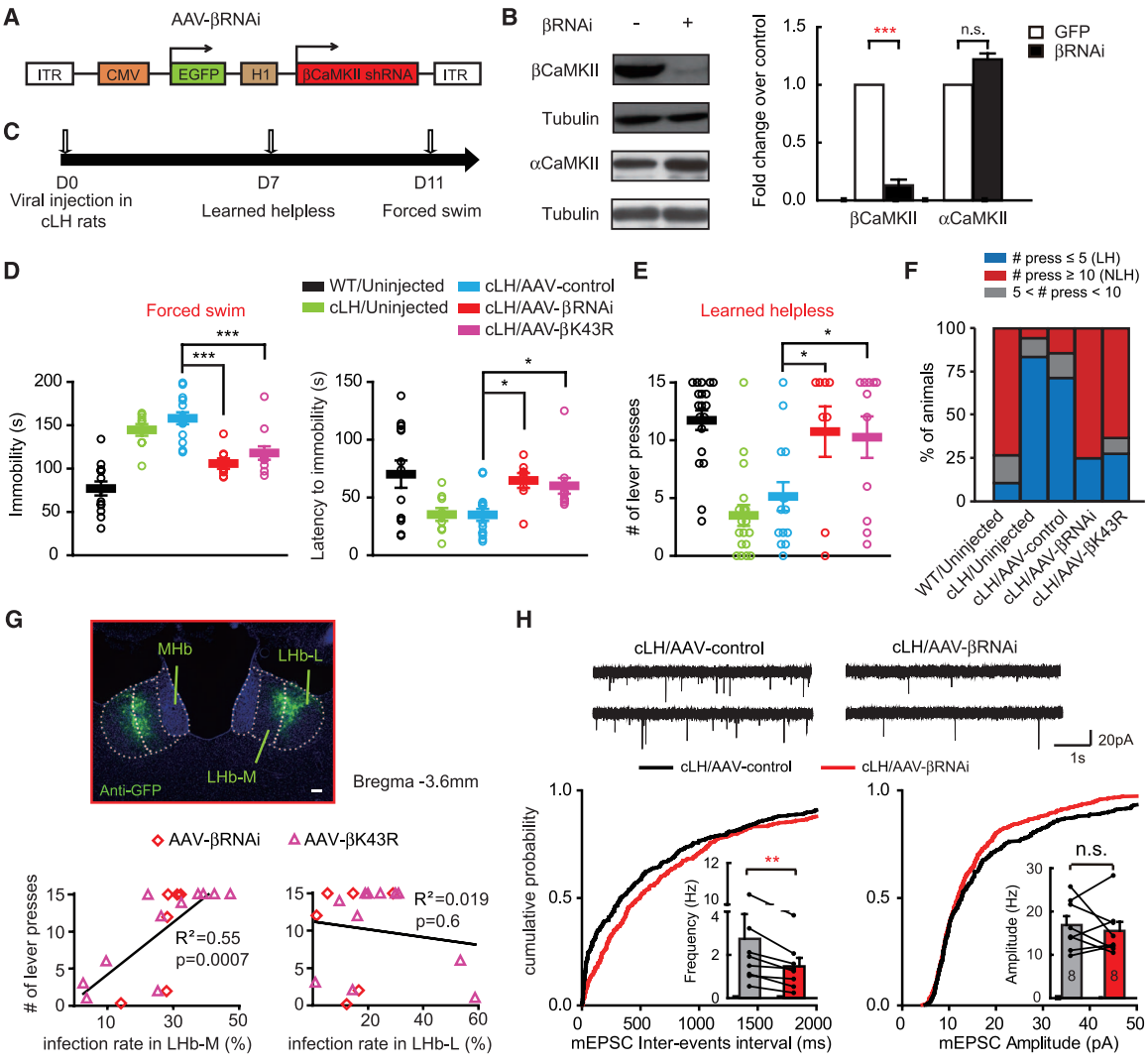


**Fig. 4. Knocking-down of βCaMKII in LHb rescued depression-like phenotypes of cLH rats and reduced synaptic activity of LHb neurons.** (**A**) Schematics of the AAV vector engineered to overexpress an RNAi form of βCaMKII. H1, human H1 promoter. (**B**) Specific knocking down of βCaMKII but not αCaMKII by the βCaMKII RNAi construct. pSuper-βCaMKII-RNAi construct was cotransfected with AAV-βCaMKII or AAV-αCaMKII 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-βRNAi and AAV-βK43R in the LHb of cLH rats in forced swim (D) or learned helpless 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 ≤5 lever presses; NLH, non–learned helpless rats with ≥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-βRNAi virus-infected cLH rat brain slice. Scale bar, 100 μm. (Bottom) Behavioral response in the learned helpless test plotted against infection rates in the LHb-L and LHb-M of cLH rats. (**H**) mEPSCs of AAV-βRNAi-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-βRNAi. *$P$ < 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 ± 8% of controls, $P = 0.003$) (fig. S11B). To test the role of GluR1 in βCaMKII-mediated depression, we coexpressed βCaMKII with a dominant-negative form of GluR1, GluR1Ct, which blocks the synaptic trafficking of GluR1 (*30*, *31*), using an AAV-βCaMKII-2A-GluR1Ct viral construct. Tests after unilateral injection revealed that βCaMKII 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 ± 2%), mice performed normally in both the forced swim and sucrose preference tests (fig. S11, D and E). Therefore, coexpression of GulR1Ct prevented the depressive effects of βCaMKII overexpression.

Here, we identified βCaMKII 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 βCaMKII in the LHb causes more GluR1 insertion into synapses, resulting in increased synaptic efficacy. In addition, βCaMKII 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*). βCaMKII is about eight times as sensitive to calcium/calmodulin as αCaMKII (*37*) and has an actin-binding motif that is absent in αCaMKII (*38*). It will be interesting to pinpoint in the future which feature of βCaMKII renders this isoform-specific function in depression.

An important future question is how depressive stimuli and antidepressants lead to bidirectional changes in βCaMKII 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 βCaMKII levels are regulated by LHb neural activity. During the onset of depression, prolonged activation of LHb neurons may cause the up-regulation of βCaMKII to reach a threshold level, which can lead to further LHb neural activation. Conversely, antidepressant-caused suppression of the LHb may down-regulate βCaMKII, 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 βCaMKII 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 βCaMKII 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
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