The lateral habenula (LHb) is a key brain region involved in the pathophysiology of depression. It is activated by stimuli associated with negative experiences and is involved in encoding aversive signals. Hyperactivity of LHb is found in both rodent models of depression and human patients with depression. However, little is known about the underlying molecular mechanisms. Here we show that in LHb neurons, p11, a multifunctional protein implicated in depression, is significantly upregulated by chronic restraint stress. Knockdown of p11 expression in LHb alleviates the stress-induced depression-like behaviors. Moreover, chronic restraint stress induces bursting action potentials in LHb neurons, which are abolished by p11 knockdown. Overexpression of p11 in dopamine D2 receptor-containing LHb neurons of control mice induces depression-like behaviors. These results have identified p11 in LHb as a key molecular determinant regulating negative emotions, which may help to understand the molecular and cellular basis of depression.
Depression-like behavioral tests were performed with 473 nm laser light (10 mW, 60 Hz, 5 ms, Agilent Technologies, California, CA, USA). See methods in Supplementary Materials and Methods for details.

Behavioral assessments

Behavioral studies including tail suspension test (TST), forced swim test, sucrose preference test and social interaction test, novelty suppressed feeding test, fecal boli test and two-way active avoidance test were performed to examine the depression-like phenotypes, and locomotor activity. Details are included in Supplementary Materials and Methods.

Immunohistochemistry

Immunostaining was carried out using the standard free-floating method as previously described. Detailed description of antibody preparation, antigen retrieval, image acquisition and quantification is presented in Supplementary Materials and Methods.

Figure 1. Effects of chronic stress and p11 expression on the activity of LHb neurons. (a) Immunofluorescence image illustrating p11-positive cells (EGFP+, green) in habenula (Hb) of p11-EGFP mice. Scale bar, 100 μm. DG, dentate gyrus; EP, ependymal cells; IML, inner molecular layer of DG; LHb, lateral habenula; MHB, medial habenula; PF, parafascicular thalamic nucleus; PV, paraventricular thalamic nucleus; SM, stria medullaris; 3v, third ventricle. (b, c) Western blot analysis (b) and quantification (c) showing the expression of c-fos in control (CON) and chronically restraint stressed (RST, 2 h per day, 14 days) mice (n = 6 per group). *P < 0.05, two-tailed t-test. (d, e) Immunofluorescence image (d) and quantification (e) illustrating c-fos-positive cells (red), p11-positive cells (EGFP+, green) and c-fos-/p11+ cells in LHb from control (CON) versus restraint stressed (RST) p11-EGFP mice. Scale bar, 20 μm. (n = 6, CON; n = 12, RST). *P < 0.05, two-tailed t-test. (f–h) Western blots and quantification of p11 protein (f, g) and quantitative PCR of p11 mRNA (h) in LHb from CON and RST mice with or without antidepressant treatments (f and g, n = 10, CON; n = 12 for each group of RST, RST+imipramine (Imi), RST+fluoxetine (Flu) and RST+escitalopram (Esci); h, n = 6 per group). *P < 0.05, **P < 0.01, compared to CON; *P < 0.05 and **P < 0.01, compared to RST, one-way analysis of variance (ANOVA). (i, j) Western blots (i) and quantification (j) showing the expression of p11 in control versus stressed mice at 14, 30, 60 and 90 days after stress cessation (n = 6 per group). *P < 0.05, **P < 0.01, two-way ANOVA. Data presented as means ± s.e.m.
Western blot analysis and quantitative reverse transcription-PCR
All biochemical analysis were performed as previously described. See Supplementary Materials and Methods for details.

Electrophysiological recordings
Whole-cell patch-clamp recording technique was used to measure spontaneous action potentials and synaptic currents in LHb neurons. See Supplementary Materials and Methods for details.

Statistics
Two-sample comparisons were performed using the Student’s t-test, while multiple comparisons were made using one-way analysis of variance followed by a Newman–Keuls post hoc test or two-way analysis of variance by a Bonferroni post hoc test. PRISM software (GraphPad Software, La Jolla, CA, USA) was used to perform statistical analyses. All data are presented as means ± s.e.m.

RESULTS
Effects of chronic stress and p11 expression on the activity of LHb neurons
To investigate the functional role of p11 in LHb, we examined p11 expression in a mouse model of depression, in which mice were exposed to chronic restraint stress (RST, 2 h per day, 14 days).28,30,33 Immunofluorescence studies of the anatomical distribution of p11 in habenula with anti-GFP antibody using p11 promoter-driven EGFP (p11-EGFP) mice indicated that p11 was present in the LHb, especially in the medial part of the LHb, but not in the medial habenula (Figure 1a). Western blotting results revealed that the protein level of c-fos, a marker of cell activity, was significantly elevated in Hb from chronically stressed mice (Figures 1b and c). Double immunofluorescence staining with GFP and c-fos antibodies in p11-EGFP mice showed that stressed mice exhibited a significantly increased p11 (green) expression in LHb, which was co-localized with the c-fos (red) expression (Figures 1d and e). These data indicate that the LHb neurons activated by chronic stress have elevated p11 expression.

Next, we investigated the impact of antidepressant treatment on the stress-induced regulation of p11 expression in LHb. Western blotting and quantitative PCR revealed that the level of p11 protein and mRNA in LHb were significantly increased in chronically stressed mice, which were reversed by 2-week treatment with three distinct antidepressants; imipramine (tricylic class of antidepressant), fluoxetine (selective serotonin reuptake inhibitor) and escitalopram (Figures 1f–h).

We further examined how long the stress-induced upregulation of p11 in LHb was sustained. At 14 and 30 days post stress, the level of p11 in LHb was significantly higher than that in control animals, but at 60 and 90 days post stress, the level of p11 in LHb had returned to the control level (Figures 1i and j), which was...
consistent with the time frame of depression-like behaviors in these stressed animals. To confirm that depressive behaviors can be induced by direct activation of LHb via optogenetic stimulation, we injected AAV-channelrhodopsin (ChR2; AAV-CaMKIIa-ChR2-eYFP) into LHb. Three weeks later, we optically stimulated ChR2-expressing axons in LHb, and performed the two-way active avoidance test and the TST for aversion, helplessness and hopelessness behaviors. ChR2-injected mice with optogenetic stimulation (ChR2+ON) exhibited a significantly decreased avoidance response and increased latency to escape the shock in active avoidance test compared to eYFP+ON and eYFP+OFF or non-stimulated ChR2-injected mice (ChR2+OFF) (Supplementary Figures S1a and b). ChR2+ON mice also had an increased immobility in TST (Supplementary Figure S1c), while locomotor activity was not altered (Supplementary Figure S1d). These data suggest that LHb activation induces depression-like behaviors, consistent with prior findings.

Next, we tested the role of p11 in depression-like behaviors induced by direct activation of LHb. Western blotting results revealed that the protein level of c-fos was upregulated in optically stimulated ChR2-injected mice (Supplementary Figures S1e and f), compared to the three control groups. Immunofluorescence staining using c-fos, ChR2 and p11 antibodies revealed that ~ 98% of ChR2-expressing LHb neurons were c-fos-positive (c-fos+), and ~ 73% of p11-expressing neurons were c-fos+/ChR2+ (Supplementary Figure S1g), suggesting that most of the p11-expressing LHb neurons are activated by optogenetic stimulation.

Knockdown of p11 in LHb ameliorates stress-induced depression-like behavior

To determine whether the stress-induced upregulation of p11 in LHb is responsible for depression-like behaviors, we knocked down p11 expression by injecting p11 shRNA (sh-p11) lentivirus into LHb (Figure 2a). The p11 knockdown efficiency in vivo was demonstrated by the significantly diminished p11 expression in LHb from mice with viral expression of p11 shRNA (CON+sh-p11), compared to those injected with GFP-shRNA (CON+sh-GFP) or non-injected (CON). In stressed mice with viral injection of p11 shRNA (RST+sh-p11), the stress-induced p11 upregulation was potently suppressed, compared to those either injected with GFP-shRNA (RST+sh-GFP) or non-injected (RST) (Figures 2b and c).

We next investigated the effect of p11 knockdown in the LHb on depression-like behaviors. Compared to the unstressed control groups (CON and CON+sh-GFP), stressed mice injected with or without GFP-shRNA to LHb (RST and RST+sh-GFP) exhibited significantly decreased time in the interaction zone in the social interaction test (Figure 2d), a measurement of social avoidance and withdrawal behavior. RST and RST+sh-GFP mice also had an increased immobility in TST (Figure 2e) and forced swim test (Figure 2f), two measurements of helplessness and hopelessness, as well as anxiety in the novelty suppressed feeding test (Figure 2g) and fecal boli test (Figure 2h). Locomotor activity (Figure 2i) and food consumption (data not shown) were not altered in these mice. Interestingly, stressed mice with LHb expression of p11 shRNA (RST+sh-p11) did not exhibit depression-like behaviors in social interaction test, TST and forced swim test (Figures 2d–f) or anxiety-like behaviors in novelty suppressed feeding test and fecal boli test (Figures 2g and h). No behavioral
changes were found in control animals with LHb knockdown of p11 (CON+sh-p11, Figures 2d–i). These data indicate that the stress-induced p11 upregulation in LHb contributes to the manifestation of depression-like behaviors.

To determine the physiological basis underlying the role of LHb p11 in stress-induced depression-like behaviors, we examined the membrane excitability of LHb neurons in control versus stressed animals with or without p11 knockdown. As shown in Figures 3a–c, the resting membrane potentials (~ −45 mV) and the spontaneous action potential frequencies of LHb neurons from stressed animals (RST) were similar to those from control animals (CON), which were not significantly affected by p11 shRNA injection (RST+sh-p11). When cells were hyperpolarized to −65 mV (Figures 3d and e), most of the LHb neurons from control animals (CON) stopped firing APs, while most of the LHb neurons from stressed animals (RST) exhibited the rhythmic bursting of APs. The bursting APs were abolished in stressed animals injected with p11 shRNA (RST+sh-p11), but not with GFP-shRNA (RST+sh-GFP).

To test the possibility that the altered excitability of LHb neurons in stressed animals could be caused by changes in synaptic inputs, we examined spontaneous excitatory and inhibitory postsynaptic currents (sEPSC and sIPSC) in LHb neurons. As shown in Supplementary Figures S2a and b, sIPSC frequency, but not amplitude, was significantly reduced in LHb neurons from stressed animals. No changes in sEPSC amplitude or frequency were found (Supplementary Figure S2c). Knockdown of p11 in LHb from stressed animals significantly elevated sIPSC frequency without changing sEPSC amplitude, while injecting the GFP-shRNA was ineffective (Supplementary Figures S3a and b). The amplitude or frequency of sEPSC was not altered by p11 or GFP-shRNA (Supplementary Figure S3c). Taken together, these data suggest that the stress-induced hyperexcitability of LHb neurons may be attributable to the loss of inhibitory inputs, and p11 may normalize LHb neuronal activity via restoring synaptic inhibition.

p11 overexpression in dopamine D2 receptor-containing glutamatergic LHb neurons of control mice induces depression-like behavior

It has been found that LHb D2⁺ neurons regulate emotional behaviors in response to negative reward by altering LHb

Figure 4. Overexpression of p11 in D2R-containing lateral habenula (LHb) neurons of control animals induces depression-like behavior. (a) Images showing the co-localization of p11 (green) with dopamine D2 receptor (D2; D2-tdT, red) and CaMKII (blue, a marker of glutamatergic neurons) in medial part of the LHb neurons from D2-tdT (D2-Cre×tdTomato) mice. Scale bar, 10 μm. (b) Immunofluorescence image illustrating the localization of D2⁺ neurons with the viral injection of Cre-dependent eYFP (AAV-DIO-eYFP) into LHb of D2-Cre mice. Scale bar, 100 μm. (c) Immunofluorescence image illustrating the co-localization of Cre-dependent p11 (AAV-DIO-p11) (green) with D2 (red) in LHb neurons. Scale bar, 20 μm. (d–g) Depression-like behaviors as measured by sucrose preference test (d), tail suspension test (TST; e), forced swim test (FST; f) and novelty suppressed feeding test (g) in D2-Cre mice with the viral expression of p11 (AAV-DIO-p11) or eYFP (AAV-DIO-eYFP) in D2⁺ LHb neurons (n=13, AAV-DIO-eYFP; n=17, AAV-DIO-p11). *P<0.05, **P<0.01, two-tailed t-test. Data presented as means ± s.e.m.
neuronal activity. Therefore, we sought to determine whether p11 in D2+ LHb neurons was involved in stress-induced depression-like behaviors. Immunofluorescence staining (Supplementary Figure S4; Supplementary Tables S1 and 2) revealed that p11+ neurons in LHb express CaMKII or calmodulin-dependent kinase II (CaMKII) or vesicular GABA transporter, suggesting that they are glutamatergic or GABAergic. In the medial part of LHb, most (~79%) of D2+ neurons, but not D1+ neurons, co-expressed p11 (Figure 4a; Supplementary Figure S5a; Supplementary Table 3), and most (~88%) of p11+/D2+ neurons expressed CaMKII (Figure 4a; Supplementary Table S1), but not vesicular GABA transporter (Supplementary Figure S5; Supplementary Table 2), indicating that they are glutamatergic neurons.

To specifically express p11 in D2+ LHb neurons, we injected Cre-dependent viruses into LHb of D2-Cre mice. As shown in Figure 4b, D2-Cre mice injected with AAV-Dio-eYFP exhibited the distribution of D2+ neurons in the medial part of LHb. D2-Cre mice injected with AAV-Dio-p11 exhibited the co-expression of p11 and D2 in LHb (Figure 4c). Depression-like phenotypes emerged in control mice with viral expression of p11 in D2+ LHb neurons, as reflected in sucrose preference test (Figure 4d), TST (Figure 4e), forced swim test (Figure 4f) and novelty suppressed feeding test (Figure 4g) assays. Collectively, these results show that p11 overexpression in D2+ LHb neurons of control animals is sufficient to induce negative emotional behaviors.

DISCUSSION

The LHb has emerged as a key brain region for the study of aversive behaviors and the pathophysiology of depression. LHb neurons, especially in the medial part of the LHb, are activated by aversive emotional cues including stress, disappointment, fear, aggression or anticipation of a negative reward in monkeys, as well as rodents. Increased activation of the LHb nucleus leads to the inhibition of the connected serotonergic, noradrenergic, dopaminergic systems and stimulation of the hypothalamic–pituitary–adrenal axis. Consistently, depression-like symptoms in neuropsychiatric disorders occur along with hyperactivity of LHb neurons in humans. Furthermore, in animal models of depression, synaptic activity, spike output and metabolic activity of LHb neurons were enhanced, which is in contrast with the decreased activity in prefrontal cortex (PFC), and ventral tegmental area.

In this study, using complementary molecular, electrophysiological and behavioral approaches, we have identified the stress-induced upregulation of p11 in the LHb neurons as a key molecular mechanism underlying the role of LHb in depression-like behaviors. Knockdown of p11 in LHb neurons ameliorated depressive phenotypes and LHb hyperexcitability in stressed animals. Moreover, overexpression of p11 in LHb neurons of control animals induced depression-like behaviors.

p11 is present in various neuronal circuits in distinct neuronal types, such as cholinergic neurons in nucleus accumbens, mossy cells and basket cells in dentate gyrus, and layer 5 corticostriatal projection neurons, involved in emotional control. Lower levels of p11 mRNA and protein have been found in the brains of depressed humans and suicide subjects. Recent knockout mice exhibit depression-like behavior, and p11-overexpressing mice show antidepressant-like behaviors. Recently, we have found that chronic stress induces the downregulation of p11 expression in layer 2/3 PrL cortical neurons and the attenuation of PrL glutamatergic synaptic transmission, which are causally linked to depressive symptoms in stressed animals. LHb relays information from the limbic forebrain regions, including PrL, to multiple monoamine centers. Reciprocal interaction between LHb and medial PFC (mPFC) via dopaminergic neurons in ventral tegmental area has been found to regulate emotional and cognitive function in rodent, monkeys and humans. LHB neurons synapse primarily on dopaminergic neurons in the ventral tegmental area projecting to the mPFC. Pharmacological and optogenetic manipulation of the LHb activity could alter dopaminergic regulation of mPFC neuronal activity, which controls executive processes. In stressed animals, the decreased p11 expression and diminished synaptic output of PrL neurons, as well as the increased p11 expression and elevated excitability of LHb neurons, may cooperatively contribute to the emergence of depressive phenotypes.

In both LHb and PrL, increased p11 levels are associated with increased neuronal excitability. It will be important to identify the underlying molecular mechanisms. Our data indicate that p11 upregulation in the LHb-stressed animals may interfere with synaptic inhibition, leading to disinhibition and hyperactivation of the LHb neurons. In addition to the stress-induced upregulation of p11 in LHb, increased βCaMKII expression and PP2A activity in the LHb have been found to act as powerful regulators of the LHb neuronal activity and depression-like phenotypes in stressed animals, suggesting that parallel cellular processes converge in the LHb to dictate the response to aversive signals.

The LHb has two major types of neurons, a majority of glutamatergic and a minority of GABAergic, both of which express p11. Dopamine signals from the ventral tegmental area innervate the whole LHb, influencing LHb neuronal activity and LHb-dependent functions. D2 receptors are functionally expressed in the LHb, especially medial part of the LHb, which regulate aversive behaviors in response to negative rewards. Interestingly, we have found that overexpression of p11 in this subpopulation of LHb neurons is sufficient to induce depressive phenotypes in control animals, confirming that p11 in D2+ LHb neurons is an important molecular and cellular determinant for depression.

Finally, it will be important to elucidate the molecular basis for the opposing action of p11 in LHb where it is a depressant agent versus p11 in other brain regions examined where it has an antidepressant action, including the cholinergic neurons in nucleus accumbens, mossy cells and basket cells in dentate gyrus, layer 5 corticostriatal projection neurons and D2+ glutamatergic neurons in layer 2/3 PrL cortex.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We are grateful to Drs Angus C Nairn, Marc Flajolet, Yotam Sagi and Lucian Medrihan for their helpful advice and discussion; to Dr Jodi Gresack for setting up the two-way active avoidance test; and to Drs Jung-Hyuck Ahn and Yong Kim for generating the AAV construct. We thank Drs Daesoo Kim and Karl Deisseroth for helpful advice on optogenetic stimulation. We thank the Rockefeller University Comparative Bioscience Center, Transgenics Services Laboratory. This work was supported by NIDA grant P01DA010044 (PG), NIMH grant P50MH090963 (PG), DOD/USAMRAA grant W81XWH-09-1-0402 (PG), the JPB Foundation (PG), the Fisher Center For Alzheimer’s Research Foundation (PG), the Leon Black Family Foundation (PG), NIH grants R01 MH108842 (ZY) and R01 DA037618 (ZY), and the donation from E. F. Trachtmann (ZY).

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