Reversal of Depressed Behaviors in Mice by p11 Gene Therapy in the Nucleus Accumbens

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The etiology of major depression remains unknown, but dysfunction of serotonergic signaling has long been implicated in the pathophysiology of this disorder. p11 is an S100 family member recently identified as a serotonin 1B (5-HT1B) receptor-binding protein. Mutant mice in which p11 is deleted show depression-like behaviors, suggesting that p11 may be a mediator of affective disorder pathophysiology. Using somatic gene transfer, we have now identified the nucleus accumbens as a key site of p11 action. Reduction of p11 with adeno-associated virus (AAV)–mediated RNA interference in the nucleus accumbens, but not in the anterior cingulate, of normal adult mice resulted in depression-like behaviors nearly identical to those seen in p11 knockout mice. Restoration of p11 expression specifically in the nucleus accumbens of p11 knockout mice normalized depression-like behaviors. Human nucleus accumbens tissue shows a significant reduction of p11 protein in depressed patients when compared to matched healthy controls. These results suggest that p11 loss in rodent and human nucleus accumbens may contribute to the pathophysiology of depression. Normalization of p11 expression within this brain region with AAV-mediated gene therapy may be of therapeutic value.

INTRODUCTION

The neuroanatomical and molecular substrates underlying major depressive disorder remain poorly understood. Serotonin is considered to be a key neurotransmitter in depression-like states, and the most commonly prescribed antidepressant therapies increase extracellular concentrations of serotonin. Abnormalities of the serotonergic system underlie an array of psychiatric pathologies, including depression, anxiety, alcoholism, and impulsive behavior (1). Recently, the serotonin receptors 5-HT1B (5-hydroxytryptamine 1B) and 5-HT4 (serotonin 4) were reported to interact with p11, a member of the S100 family of cytoplasmic, small acidic EF-hand-type helix-loop-helix proteins (2, 3). p11 facilitates cell membrane localization and ligand-mediated activation of these receptors, a function analogous to those served by p11 for several other proteins and ion channels, such as the NaV1.8 sodium and TASK-1 potassium channels (4). p11 knockout (KO) mice exhibit depression-like phenotypes, such as increased immobility times in the tail suspension test (TST) and forced swim test (FST), whereas transgenic overexpression of p11 induces antidepressant-like effects (2).

To better understand the involvement of p11 action in depression-like states and to inform possible p11-based antidepressant therapies, we have used viral vector–mediated gene transfer to focally alter p11 expression in normal adult mouse brain. Several brain regions have been implicated in the pathogenesis of depression from animal and human imaging studies and therapeutic interventions such as stereotactic surgery. These areas include the ventral striatum/nucleus accumbens (NAcc), subgenual cingulate (area 25), hippocampus, amygdala, habenula, and anterior cingulate cortex (5–7). We chose two of these regions, NAcc and anterior cingulate cortex, for further study. NAcc was chosen on the basis of both human functional imaging and results from therapeutic deep brain stimulation, which corroborate animal studies suggesting that reduced activity within this region may promote human depression (5, 7, 8). In addition, the ability of serotonin to inhibit glutamatergic synaptic transmission in NAcc is blunted in p11 KO mice. p11 KO mice are also relatively resistant to the effects of 5-HT1B agonists on abnormal movements induced by L-dopa after dopaminergic denervation of the dorsal striatum (9). Given the anatomic and physiological similarities between the dorsal and the ventral striatum, these results further support a potential role for p11 in the function of the ventral striatum (2, 9). The anterior cingulate was chosen as the second region to be studied on the basis of findings in our original report of reduced p11 expression in the anterior cingulate cortex of depressed human patients compared with controls and up-regulated p11 expression in this region after various antidepressant therapies in rodents (2).

RESULTS

To alter p11 concentrations focally within the adult brain, we used adeno-associated virus (AAV)–mediated gene transfer as described (10, 11). A small interfering RNA (siRNA) was created that efficiently blocked expression of a p11-GFP (green fluorescent protein) fusion protein in human embryonic kidney 293 (HEK293) cells. A control siRNA against the luciferase gene had no effect (Fig. 1A). A GFP expression cassette contained within both siRNA plasmid constructs yielded equivalent expression of unmodified GFP. These siRNA sequences were
then cloned into AAV2 vectors as small hairpin RNAs (shRNAs), which also expressed yellow fluorescent protein (YFP) cassettes (AAV.sip11.YFP or AAV.siLuc.YFP), to monitor the location of AAV transduction within the brain. AAV.sip11.YFP efficiently blocked endogenous p11 expression in primary NAcc neurons in culture (Fig. 1B), whereas control AAV.siLuc.YFP had no effect. Infusion of the AAV vectors into NAcc in vivo after optimization of infusion parameters confirmed their efficient focal transduction (Fig. 1C) and consequent reduction in neuronal p11 expression (Fig. 1D). For our overexpression experiments, we used a vector containing a p11 complementary DNA (cDNA), which also harbored a control luciferase siRNA expression cassette (AAV.siLuc.p11), so that it would match the AAV.sip11.YFP vector. This approach permitted the use of a third vector encoding both luciferase siRNA and YFP (AAV.siLuc.YFP) as a single negative control for both the AAV.sip11.YFP and the AAV.siLuc.p11 vectors.

We examined the behavioral effects of localized p11 knockdown and overexpression in C57BL/6 mice after AAV-mediated gene transfer with an automated, unbiased video monitoring system to measure immobility times on TST and FST. These are commonly used as depression-related behavioral tests, because time of immobility in each assay is reduced by treatment with several antidepressants used in current clinical practice, whereas factors associated with human depression, such as chronic or early life stress exposure, postpartum state, or genetic predisposition, all increase TST and/or FST immobility times (12, 13). Focal reduction of p11 in NAcc of otherwise normal adult mice with AAV-mediated RNA interference resulted in a significant increase in immobility in TST (Fig. 1E). The time spent immobile and percent change from control were comparable to previous observations in p11 KO mice (2). A similar increase in immobility was observed in FST (Fig. 1F). The increased immobility seen in TST and FST was not due to a defect in locomotor activity, because horizontal movement in an open field did not differ between control and p11 knockdown groups (fig. S1). Infusion of the p11 knockdown vector into the anterior cingulate (fig. S2A) did not yield any significant change in these behaviors (fig. S2B).

For evaluation of the possible involvement of NAcc p11 in the behavioral effects of antidepressant medications, a separate group of mice received p11 knockdown or control viruses or no surgery, and then acute treatment with the tricyclic antidepressant imipramine or saline before behavioral testing. Imipramine blocks reuptake of both serotonin and norepinephrine, but was chosen for this study to directly compare our results with previous data obtained with imipramine in p11 KO mice (2). When mice were treated with saline, the previously observed effect of focal NAcc p11 knockdown on increasing TST immobility times was replicated. As expected, imipramine treatment of mice in both the no-surgery and the control vector groups resulted in significant reductions
in TST immobility times (Fig. 1G). The p11 knockdown mice also responded to imipramine with a reduction in TST immobility (Fig. 1G). However, the imipramine-treated knockdown mice still showed a significant increase in immobility time compared with control mice treated with imipramine (Fig. 1G). These results are similar to data obtained with the p11 KO mice, which remained responsive to imipramine, but retained higher immobility compared to wild-type littermates (2).

To determine whether the behavioral consequences of congenital loss of p11 expression can be reversed, we injected a virus containing p11 cDNA into NAcc of p11 KO mice, which restored p11 expression selectively in this brain region (Fig. 2A).

We further examined the effect of p11 overexpression on 5-HT1B receptor function using calcium imaging of primary ventral striatal neuronal cultures (Fig. S3, A and B). Although AAV can transduce neurons in primary culture, this is inefficient, and because gene expression requires several days after transduction, glial overgrowth can often complicate studies. Therefore, we developed a methodology of direct intrastriatal injection of AAV vectors in postnatal day 2 living rat pups (Fig. S3C) followed by harvest 1 week later. This resulted in efficient gene transfer into cultured ventral striatal neurons with no overgrowth of glial cells because the neurons could be studied almost immediately (Fig. S3D). As an assay for 5-HT1B function, we used calcium imaging because previous studies have indicated that activation of this receptor can activate pathways that increase intracellular calcium concentrations (16, 17). Treatment of cultured ventral striatal neurons with the 5-HT1B agonist anpirtoline resulted in a reproducible, dose-dependent increase in the calcium signal expressed as the area under the curve (Fig. 2D and fig. S5). Increased 5-HT1B receptor binding was found in the terminal regions of NAcc neurons in the hemisphere injected with p11-overexpressing vector compared with the hemisphere injected with the control AAV vector expressing the YFP marker gene. The increased ligand binding in the axon terminal fields is consistent with the known distribution of 5-HT1B receptors (14, 15). The magnitude of this effect was similar to that seen when comparing p11 KO mice with wild-type littermates (2).

**Fig. 2.** Overexpression of p11 in NAcc neurons potentiates 5-HT1B agonist binding and signaling. (A) Immunohistochemistry (left, low-power magnification; right, high-power magnification) demonstrates focal restoration of p11 expression in NAcc of p11 KO mice after AAV-mediated gene therapy with a p11 cDNA but not with control YFP, which demonstrates only light background staining. Scale bars, 80 μm. (B and C) 5-HT1B receptor-binding levels in p11 KO mice after intracerebral injection of p11 overexpression (OE) vector (AAV:Siluc.p11) or the control vector (AAV:Siluc.YFP) using 10 pM [125I]cyanopindolol. (B) Autoradiography revealed increased binding in the pallidum terminal regions of accumbens neurons in the hemisphere overexpressing p11 after AAV-mediated gene transfer (p11 OE) compared with the control hemisphere expressing the YFP marker gene (Ctrl). (C) Quantification confirmed the significant increase in 5-HT1B binding in the p11-overexpressing hemisphere. **P < 0.01, two-tailed t test. (D) Treatment of primary striatal neurons with the 5-HT1B agonist anpirtoline (10 μM) resulted in a repeated increase in fluorescence as measured by the area under the curve with the calcium-sensitive dye Fluor4. Black bars, exposure to anpirtoline; green bar, exposure to glutamate as a positive control for calcium fluorescence. (E) Effect of anpirtoline (25 mM; black bars) was completely blocked by exposure to the 5-HT1B antagonist GR127935 (10 μM; red bar). Green bar, exposure to glutamate as a positive control for calcium fluorescence. (F) Calcium fluorescence was significantly increased by AAV-mediated overexpression of p11 (black bars) in ventral striatal neurons compared with control RFP (gray bars) expression after exposure to 10, 25, and 50 μM anpirtoline. ****P < 0.0001.
transduced with red fluorescent protein (RFP) (Fig. 2F). This confirmed not only that AAV.p11 increases 5-HT1B binding in vivo but also that it increases the functional response of ventral striatal neurons to a 5-HT1B agonist as well.

Having confirmed the functional efficacy of our AAV.p11 vector, we then examined the behavioral effects of restoring p11 expression exclusively in the ventral striatum of p11 KO mice. Focal replacement of p11 expression in NAcc bilaterally with AAV-mediated gene therapy normalized TST immobility times of p11 KO animals (Fig. 3A); the performance of these mice on TST was indistinguishable from all wild-type littermates, including those with no surgery, as well as those receiving control or p11-overexpressing virus. As expected, p11 KO mice receiving no surgery or control AAV had significantly increased TST immobility times compared with wild-type littermates. Results similar to those observed with TST were also obtained on FST, confirming the normalization of depressed phenotypes in these mice (Fig. 3B). Anhedonia is also a key component of human depression that can be tested in mice with a sucrose preference test. p11 KO mice consume less sucrose, consistent with an anhedonic phenotype (2).

Reintroduction of p11 into NAcc of p11 KO mice resulted in a significant increase in sucrose preference compared with mice receiving the control vector (Fig. 3C). Overall activity and feeding behavior were all unaffected by transgenic knockdown of p11 and by overexpression of p11 in NAcc of p11 KO and wild-type mice (fig. S4). Thus, several depression-like behaviors resulting from the absence of p11 during development can be fully overcome through reintroduction of p11 into the adult NAcc without influencing nonspecific activities.

In view of these results implicating NAcc in the behavioral effects of p11 in mice, we also examined p11 expression in postmortem NAcc of depressed human patients compared with controls matched for age, sex,
and postmortem interval using methodology described previously (18). Blinded Western blot analysis of extracts from NAcc tissue (n = 17 per group) revealed a significant reduction in p11 protein in depressed patients compared with controls (Fig. 4). The original report linking p11 to depression noted a similar reduction in p11 expression in the anterior cingulate of depressed patients (2), whereas another study noted reductions in p11 messenger RNA (mRNA) in the hippocampus, frontal cortex, and amygdala of depressed patients (2, 19). The present data support these earlier observations implicating reduced p11 expression as a feature of clinical depression.

DISCUSSION

Our data identify NAcc as a key brain region mediating the ability of p11 to prevent the development of depression-like phenotypes in mice. Some regional specificity is suggested by the lack of behavioral effect of p11 knockdown in the anterior cingulate cortex (20). Furthermore, because AAV2 is highly selective for neurons, these results indicate that p11 loss within NAcc induces depression-like behaviors (10, 21). These results also demonstrate that the effects of p11 deletion in p11 KO mice (2) are not likely to be attributable to developmental consequences of embryonic p11 loss, because we observed similar behavioral effects from somatic blockade of p11 in normally developed adult mice.

The effect of p11 loss specifically in NAcc neurons in the current study provides support for the importance of this region as a mediator influencing the development of depression-like behaviors. Other brain structures, such as the hippocampus and frontal cortex, have received the majority of attention in experimental studies of the neurobiology of depression (22). NAcc is a key component of the mesolimbic dopamine reward circuitry, receiving dopamine inputs from the ventral tegmental area (VTA), and as such has been most frequently studied in drug addiction and reward research (7). Although data regarding the relationship between this area and depression are limited, several studies have identified protein alterations within NAcc that correlate with depression-like behaviors in rodents (7). Viral vectors have also been used to focally overexpress genes, such as the transcription factors CREB (cyclic adenosine monophosphate response element–binding protein) and ATF3 (activating transcription factor 3), to induce depression-like behaviors, whereas expression of antagonizing proteins such as dominant-negative CREB or ATF2 resulted in antidepressant responses (23). Here, we have demonstrated depression-like behaviors that mimic whole-animal, embryonic deletion of a gene by somatic blockade of gene expression exclusively within a specific brain region. We have also reversed depression-like behaviors in a genetic mouse model with gene therapy by restoring gene expression within a focal, restricted region. Because p11 is expressed in other areas of the brain that have been linked to depression, including the hippocampus and infralimbic cortex, it is likely that there is a role for p11 in the functioning of these other key regions as well (24). Furthermore, because NAcc is anatomically linked to these and other brain regions relevant to depression, altered NAcc p11 could indirectly influence the activity of these other areas (25, 26). Our results not only advance understanding of the mechanism of p11 action within the brain but also suggest that NAcc may be a target for development of antidepressant therapies.

Modeling complex behaviors such as depression can be challenging in rodents, and results of our current and previous studies suggest that both the p11 KO mouse and AAV-mediated inhibition of p11 expression may be useful as new genetic models for depression research. The tests used here to quantify depression-like behaviors (TST and FST) use immobility as a measure of lack of motivation, and they are the most widely used assays for depression-like behaviors in rodents. A number of interventions known to be involved in the susceptibility to or induction of major depression in humans increase immobility times on TST and FST, which further supports the relevance of these tests to human depression (12). A variety of other depression models have been described in rodents, including chronic mild stress and social defeat. However, these models use environmental stresses to induce behaviors that have been suggested to reflect human depression. Here, we have used TST and FST as assays to quantify depression-like behaviors that occurred spontaneously after genetic manipulation of p11 concentrations (either transgenic or somatic) and reversed by restoration of p11 expression. Similarly, sucrose intake is generally used as a measure of anhedonia in mice, because intake of sweets is generally pleasurable and preferred by rodents (12). Although other substances (for example, sweet milk) or novel environments have also been used as alternatives, sucrose preference remains the most widely used measure of anhedonic responses in rodents. Both environmental factors and genetic predisposition are likely to influence the risk of developing depression in humans, and therefore, it will be of interest to determine whether NAcc p11 concentrations alter sensitivity to developing depression-like behaviors in various models of chronic stress.

Serotonin signaling has long been of interest in depression studies, in part due to the mechanism of action of effective human antidepressant medications (1). This was in fact the basis for originally studying factors that could influence 5-HT1B receptor function, which resulted in the identification of p11 (2). Our studies demonstrate that p11 improves the response of NAcc neurons to 5-HT1B agonists, because overexpression of p11 increased the response of NAcc neurons to 5-HT1B agonists in primary culture and increased 5-HT1B ligand binding in NAcc terminal fields in live mouse brain. These observations support previous suggestions of a role for 5-HT1B in depression and also confirm that the p11 overexpressed by AAV vectors in these studies was functional in NAcc neurons. Nonetheless, these data do not conclusively demonstrate that the mechanism of p11 action in NAcc is through modulation of 5-HT1B receptor activity. p11 can also influence 5-HT4 receptor binding, although the density of 5-HT4 receptors in NAcc is far lower than that of 5-HT1B receptors (3, 24). There are also a variety of other ion channels and proteins that are modulated by p11 binding, although the relevance of most of these to NAcc function remains unclear (4). It is also possible that p11 interacts with one or more as yet unidentified factors that are critical to NAcc regulation of mood. Therefore, although our data demonstrate that NAcc is a key site of p11 action to prevent the development of depression-like behaviors in rodents, further studies will be necessary to determine whether p11 functions within these neurons through interactions with 5-HT1B receptors or other gene products.

The reduction in p11 expression in NAcc from depressed humans suggests that this may be a pathophysiological feature of major depression in at least some patients. The behavioral consequences of re-
duced p11 expression throughout the brain of p11 KO mice were reversed by reintroducing p11 expression focally in NAcc. These combined data suggest that gene therapy to normalize p11 expression in the human NAcc may be useful as a treatment for select patients with major depression who prove refractory to other types of antidepressant treatment. Together with recent reports of the same AAV2 vector being used for human gene therapy in the brain for Parkinson’s disease and pediatric genetic disorders, our data highlight the therapeutic potential of normalizing p11 expression in NAcc (10, 27).

MATERIALS AND METHODS

Western blotting

HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) for 24 to 48 hours, followed by 2-hour incubation with serum-free DMEM. Cells were washed with ice-cold phosphate-buffered saline (PBS), and then lysed in lysis buffer [20 mM tris (pH 8.0), 1% Triton X-100, 150 mM NaCl, protease and phosphatase inhibitors] (Sigma). Samples were cleared by centrifugation, and equal amounts of protein (15 to 30 μg) were denatured in a sample buffer and resolved on a NuPAGE 4 to 12% gradient gel (Invitrogen). Blots were incubated with various amounts of antibody to mouse S100A10 from R&D Systems followed by secondary antibodies and enhanced chemiluminescent detection (Pierce Thermo Scientific).

AAV vectors

AAV vectors were constructed as described (10, 11). The following oligonucleotides were annealed and cloned immediately downstream from the H1 promoter of AAV.H1.YFP into Bam H1 and Hind III sites to generate AAV.H1.siLuc.YFP, AAV.H1.si.p11.YFP, and AAV.H1.siLuc.p11, respectively: siLuc (5’-CATCTTCCTGTCAATGCAGTTGCTCTCCAGCGGTTTTTGAA-3’), sip11 (5’-GATCCCCCCGTGAGAGCAACTGCTATCTCCCTGTCAATGCAGTTGCTCTCCAGCGGTTTTTGAA-3’), and sip11 (5’-GTAGTCCCTGGTGGAATGGAGAGCAACTGCTATCTCCCTGTCAATGCAGTTGCTCTCCAGCGGTTTTTGAA-3’), sip11 (5’-GTAGTCCCTGGTGGAATGGAGAGCAACTGCTATCTCCCTGTCAATGCAGTTGCTCTCCAGCGGTTTTTGAA-3’), and p11 (5’-ATGCCATGGAAACCATGATGCTTACGTTTCACAGGTTTG-CAGGGCAGAAGACCTCATCTGACAAAGGAGAGGACCTGAGGATGTGCTCATGGAAGCGGATCTCCCTGTGAGTTTTTGGAATCCAAAGGATCTCTGCCTGTTGGAACAAATAATGGAAGACCTGAGGACAGTGGAGATGGCAAAATGTCTCCAGCTTCTTCTTCTATCTACTGGGCGGGGCTGCAATGCTGATGAGGAAAGCT-3’). All siRNA expression cassettes were verified by sequencing. Virus stocks were prepared by packaging the vector plasmids into AAV serotype 2 particles with a helper-free plasmid transfection system. The vectors were purified with heparin affinity chromatography and dialyzed against PBS. AAV titers were determined by quantitative polymerase chain reaction (PCR) with primers to a fragment of the AAV backbone and deoxyribonuclease 1 (DNase 1) (1 U/ml), and incubated for 30 min with a helper-free plasmid transfection system. The vectors were purified with heparin affinity chromatography and dialyzed against PBS. AAV titers were determined by quantitative polymerase chain reaction (PCR) with primers to a fragment of the AAV backbone and deoxyribonuclease 1 (DNase 1) (1 U/ml), and incubated for 30 min.

Animals and histology

C57BL/6 male mice (11 weeks old) were used in this study. All animal studies were approved by the Institutional Animal Care and Use Committee at Weill Cornell Medical College and followed all institutional guidelines. Stereotactic surgical procedures were performed under ketamine-xylazine anesthesia. After each mouse was placed in a stereotactic frame, 1 μl of each virus (1 × 1012 genomic particles) in PBS was injected into the NAcc (anteroposterior +1.3, mediolateral ±0.9, dorsoventral −4.7 from bregma) over 10 min with a 10-ml Hamilton syringe and an infusion pump (World Precision Instruments). These coordinates, and this volume and rate of infusion, were determined empirically to adequately cover the NAcc without significant spread to other regions in pilot studies with a variety of volumes, flow rates, and minor coordinate adjustments. The needle was left for an additional 5 min and then slowly withdrawn. Animals received bilateral injections of AAV.siLuc.YFP (control), AAV.sip11.YFP (knockdown), or AAV.siLuc.p11 (overexpression). Mice were killed by perfusion with 4% paraformaldehyde and PBS. The brains were analyzed by immunohistochemistry with a free-floating section method. Coronal slices were blocked with 2% donkey serum (Vector Laboratories) before being incubated overnight with primary antibody (1:100; goat antibody to S100A10; R&D Systems). Sections were incubated in secondary antibody (1:200; Alexa Fluor 546–conjugated donkey antibody to goat; Molecular Probes/Invitrogen) for 1 hour before being mounted with antifade media (Molecular Probes/Invitrogen).

5-HT1B receptor autoradiography

Cryostat sections (12-μm thick) were made from p11 KO mice injected with p11-overexpressing vector (AAV.siLuc.p11) or control vector (AAV.siLuc.YFP) in the right and left hemispheres, respectively, as described above. 5-HT1B receptors were detected by incubating the sections in 170 mM tris–150 mM NaCl (pH 7.4) (25°C) containing the antagonist [125I]cyanopindolol (10 pM; 2200 Ci/mmol), 100 nM 8-OH-DPAT as a 5-HT1A blocker, and 30 μM isoproterenol as a β-adrenergic receptor blocker for 2 hours. Nonspecific binding was determined by measurements in the presence of 100 μM serotonin. Sections were then rinsed two times for 5 min in cold binding buffer, dipped in distilled water at 4°C, and dried under cold air. The sections were apposed to Biomax MR film for 3 to 5 days. Absorbance measurements were obtained with the National Institutes of Health (NIH) Image 1.63 image analysis system. Specific binding was calculated by digital subtraction of nonspecific labeling from total binding.

Rat pup intracranial injection

At postnatal day 2, male rat pups were anesthetized by isoflurane inhalation and kept on ice for the time of the procedure. A hole was made with a 26-gauge needle through skin and bone at the injection point. The NAcc injection site was assessed relative to bregma with a 0.05% PBS wash in Hibernate A (BrainBits). NAcc was dissected, minced, and returned to the home cage.

Primary NAcc cultures

NAcc neurons were isolated from postnatal male rats (7 to 10 days old). Briefly, the brains were quickly removed and dissected at room temperature in Hibernate A (BrainBits). NAcc was dissected, minced, transferred to a solution containing papain (2 mg/ml; Worthington) and deoxyribonuclease 1 (DNase 1) (1 U/ml), and incubated for 30
min at 37°C with gentle rocking. The tissue was then washed twice with warm Hibernate A containing 1% bovine serum albumin (BSA) and dissociated by trituration in Hibernate A media containing DNase I (1 U/ml). The suspension was placed on top of a solution of 10% BSA in Hibernate A and centrifuged for 5 min at 200g. After centrifugation, the cells were resuspended in 250 µl of Hibernate A and the debris was allowed to settle for 10 min. The top 200 µl of cell suspension was cleared again through 10% BSA in Hibernate. The cell pellet was then washed once with 1 ml of Hibernate A. The pellet was re-suspended in 20 µl of Hibernate A, and 2 µl was plated onto poly-D-lysine–coated gridded coverslips, which were then placed in a six-well plate with 2 ml of astrocyte-conditioned Neurobasal A/10% FBS medium (Invitrogen) at 37°C. The cells were allowed to attach for 3 to 4 hours, and then the medium was replaced with astrocyte-conditioned Neurobasal A/2% B-27 medium (Invitrogen).

Calcium imaging
Measurements of intracellular Ca2+ fluctuations were performed by digital imaging fluorescence microscopy 24 to 48 hours after plating. The coverslips were rinsed with Hanks’ balanced salt solution (HBSS) buffer (Invitrogen) and 1.25 mM probenecid (pH 7.4) and incubated with 1 µM Fluoro4 (Molecular Probes) for 25 min at room temperature. The cells were then washed twice with the loading buffer and incubated for an additional 25 min before mounting in a recording chamber. The cells were continuously perfused with HBSS at a rate of 1 ml/min and allowed to equilibrate in the chamber for another 15 min before recording. All assays were performed at room temperature (20°C to 25°C). Fluorescence images were acquired every 6 s with an Olympus BX61 inverted microscope with a 20×/0.75 objective. At the end of the experiment, the cells were fixed with 4% paraformaldehyde for 2 min directly in the recording chamber and then immunolabeled for neuronal β-tubulin (Tuj1, 1:2000; Covance), P11 (AF2377, 1:200; R&D Systems), orDsRed (C-20, 1:500; Santa Cruz Biotechnology). Image stacks were created and analyzed with ImageJ. Results are presented as ΔF/F = [(Fx − B) − (Fbaseline − B)]/(Fbaseline − B), where B is background intensity, Fx is intensity at any given time, and Fbaseline is the average baseline intensity (before any treatment).

Sucrose preference test
The sucrose preference test was performed with a two-bottle procedure, during which mice had free access to both water and a sucrose solution. Group-housed animals were first habituated for 48 hours both to consume fluids from a small bottle and to adjust to the 2% sucrose solution. After habituation, the sucrose preference was measured over 3 days. Each day, group-housed mice were individually caged and two bottles were presented to them for 24 hours: one with tap water and one with a 2% sucrose solution. Consumption of water or sucrose solution was measured by weighing the bottles before and after the sessions. Bottles were counterbalanced across the left and the right sides of the cage, and their position was alternated from test to test. Animals were weighed after the last day of test. Sucrose preference was calculated in two ways: Preference = [average sucrose solution intake (ml)/animal weight (g)], and Preference (%) = [average sucrose solution intake (ml)/average total fluid intake (ml)] × 100.

Open-field activity
Mice were tested for 30 min in an open-field apparatus (27.9 cm by 27.9 cm; Med Associates Inc.) under dim red light during the dark phase of the cycle. Distance traveled (horizontal activity) based on light beam breaks within the open-field device was recorded for each mouse. Twenty-four hours before the initiation of recording, each mouse was single-caged and allowed to acclimate to the cage, novel food hopper, and large cabinet in which testing was performed. Lighting conditions were maintained according to the mouse’s established circadian rhythm. Each cage contained a water spout, bedding, nesting pads, and a food hopper that prevented scattering of crumbs. Additionally, food intake was measured during the 24-hour acclimation period and was comparable to intake during the subsequent 24-hour recorded period. Rodent activity was analyzed with automated software from Clever Systems, as described above.

Characteristics of human subjects
Human brain specimens were obtained from the Dallas Brain Collection (18). Briefly, after obtaining next-of kin permission, brain tissue was collected from cases at the Dallas County Medical Examiners Office, Transplant Service Center, and Willed Body Program at the University of Texas Southwestern Medical Center. Blood toxicology screens were conducted in each case. Subjects with known history of neurological disorders or head injury were excluded. Clinical records and collateral information from direct telephone interviews with a primary caregiver were obtained for each case. Two board-certified psychiatrists carried out an extensive review of the clinical information and made independent diagnoses followed by a consensus diagnosis with the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria. Demographic characteristics associated with the tissue are presented in tabular form in table S1. The collection of human brain specimens was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center. Seventeen normal controls and 17 cases of major depression were included in this study. The two groups were matched as closely as possible for race, gender, age, pH, postmortem interval, and RIN (RNA integrity number) (detailed in table S1).

Human tissue preparation
In each case, cerebral hemispheres were cut coronally into 1- to 2-cm blocks. The NAcc was dissected from the appropriate coronal section...
and immediately placed in a mixture of dry ice and isopentane (1:1, v/v). The frozen tissue was then pulverized on dry ice and stored at −80°C. For determination of pH and RIN, tissue weighing −150 mg was punched from the cerebellum, homogenized in 5 ml of double-distilled water (pH adjusted to 7.0), and centrifuged for 3 min at 8000g at 4°C. pH of the supernatant was measured in duplicate (Thermo-Electron Corp.). RIN determination was performed by isolating total RNA with Trizol (Invitrogen) followed by analysis with an Agilent 2100 Bioanalyzer. The samples were sonicated in 1% SDS and boiled for 10 min. Small aliquots of the homogenate were retained for protein determination by the bicinchoninic acid protein assay method. Equal amounts of protein were processed with 16% acrylamide gels. Immunoblotting was carried out with monoclonal antisera against p11 (1:1000) and polyclonal antisera against actin (1:1000). Antibody binding was detected by enhanced chemiluminescence and quantified by densitometry with NIH Image 1.63 software. The concentration of p11 was normalized to the concentration of actin. All data are presented as normalized levels. The investigator completing the Western blots was blinded as to sample group assignment until the data were obtained and analysis was complete.

Statistical analyses
Two-tailed t test was used for statistical comparison of all animal groups, quantitative histological measures, and human patient samples, with statistical significance set as less than a P value of 0.05.
award (P.G.); NARSAD Young Investigator awards (M.F. and P.S.); Vetenskapsrådet (P.S.); and Swedish Royal Academy of Sciences (P.S.). **Author contributions:** M.G.K. and P.G. were responsible for the overall design of the study, data interpretation, and primary writing of the manuscript, with the assistance of B.A. and J.W.-S. The animal surgeries and most behavioral studies were completed by B.A. with the assistance of M.V. M.S. provided advice and technical assistance regarding behavioral studies. S.M. provided reagents and technical advice regarding viral vector preparation. J.W.-S. completed most of the histological studies. M.A.-L. performed the sucrose preference test and developed and performed the primary cell culture assays for gene transfer. M.F. provided p11 reagents and technical advice, as well as assistance with data interpretation. P.S. and T.E. completed the 5-HT1B binding and autoradiography, as well as Western blot and blinded analysis of the human tissue extracts, whereas C.T. and T.E. obtained and processed the human tissue and maintained the blinded code. **Competing interests:** M.G.K. is a founder of and paid consultant for Neurologix Inc., which has licensed intellectual property rights to p11 gene therapy for behavioral disorders, and P.G. is a founder of and equity holder in Intracellular Therapies Inc., which has licensed intellectual property rights to p11. M.G.K. and B.A. are inventors on a patent application assigned to Cornell University related to p11 gene therapy for behavioral disorders, and P.G. and P.S. are inventors on a patent application assigned to the Rockefeller University related to the p11 gene and behavioral disorders. The other authors declare that they have no competing interests.

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Abstracts

One-sentence summary: Reduction of the 5-HT1B receptor–binding protein p11 in the mouse nucleus accumbens induces depression-like behaviors, and gene therapy to restore p11 expression in this region reverses depression-like behaviors.

Editor's Summary:
Dialing Down Depression

Despite much progress in the study and treatment of depression, the mechanisms underlying this debilitating disease are still unclear. Altered activity of several major neurotransmitters in the brain including serotonin is involved, but pinpointing the parts of the brain affected in depression has proved challenging. Alexander and colleagues now implicate a brain region called the nucleus accumbens and a protein called p11 expressed in this region as important mediators of depression in humans and mice.

The authors were alerted to the potential importance of p11 in depression because mice that lack this protein show depressive-like behavior. This protein is involved in the activation of two receptors for serotonin, 5-HT1B and 5-HT4. Alexander et al. decided to down-regulate expression of p11 specifically in the nucleus accumbens by injecting a viral vector containing a small interfering RNA against p11 directly into this brain region in healthy mice. They then tested the treated mice to see if they exhibited depressive-like behaviors in response to two stress tests (suspension by the tail and swimming in a water tank). In both tests, treated mice showed greater immobility compared with control animals, a sign of depressive-like behavior. To show that these depressive symptoms were indeed caused by loss of p11 in the nucleus accumbens, the investigators overexpressed p11 in the nucleus accumbens of mice that completely lacked this protein. They demonstrated restoration of normal immobility times on the two stress tests and an increased desire to sip sucrose solution (a treat that rodents normally enjoy but depressed animals do not). They also showed increased activity of 5-HT1B serotonin receptors expressed by striatal neurons in the nucleus accumbens of mice overexpressing p11. But do these results have any relevance to depression in humans? Alexander and colleagues tackled this question by comparing postmortem nucleus accumbens brain tissue from individuals with and without depression at the time of death. They discovered that expression of p11 was much lower in the nucleus accumbens of depressed individuals compared with healthy persons. These new findings pinpoint the nucleus accumbens and the p11 protein as important mediators of depression and provide new therapeutic targets for drug development.