Published in final edited form as:

Biol Psychiatry. 2014 June 15; 75(12): 991–998. doi:10.1016/j.biopsych.2014.03.009.

Calcineurin down-regulation in the amygdala is sufficient to induce anxiety-like and depression-like behaviors in C57BL/6J male mice

Yann S. Mineur, Seth R. Taylor, and Marina R. Picciotto

Division of Molecular Psychiatry, Abraham Ribicoff Research Facilities, Connecticut. Mental Health Center, Yale University School of Medicine, New Haven, Connecticut

Abstract

Background—The calcium-dependent phosphatase calcineurin is highly expressed in the amygdala, a brain area important for behaviors related to mood disorders and anxiety. Organ transplant patients are administered the calcineurin inhibitor Cyclosporin-A (CsA) chronically and demonstrate an increased incidence of anxiety and mood disorders. It is therefore important to determine whether chronic blockade of calcineurin may contribute to symptoms of anxiety and depression in these patients.

Methods—Pharmacological (CsA) and viral-mediated gene transfer (adeno-associated viral expression of shRNA (AAV-shRNA)) approaches were used to inhibit calcineurin activity systemically or selectively in the amygdala of the mouse brain to determine the role of calcineurin in behaviors related to anxiety and depression.

Results—Systemic inhibition of calcineurin activity with CsA or local down-regulation of calcineurin levels in the amygdala using AAV-delivered short hairpin RNAs targeting calcineurin B increased measures of anxiety-like behavior in the elevated plus maze, the light/dark box and the open field test. A decrease in locomotor activity was also observed in mice treated systemically with CsA. In the forced swim model of depression-like behavior, both systemic CsA treatment and calcineurin blockade in the amygdala significantly increased immobility.

Conclusions—Taken together, these data demonstrate that decreasing calcineurin activity in the amygdala increases anxiety- and to some extent, depression-like behaviors. These studies suggest that chronic administration of CsA to organ transplant patients could have significant effects on anxiety and mood and that this should be recognized as a potential clinical consequence of treatment to prevent transplant rejection.

© 2014 Society of Biological Psychiatry. Published by Elsevier Inc. All rights reserved.

Corresponding Author: Marina R. Picciotto, 34 Park Street, New Haven, CT 06508, Phone: 203-737-2041; Fax: 203-737-2043; marina.picciotto@yale.edu.

Supplementary material: none

The authors declare no biomedical financial interests or potential conflicts of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

AAV; anxiety; calcineurin; cyclosporin-A; depression; elevated plus maze; forced swim; PP2B; shRNA; tail suspension

INTRODUCTION

Organ transplant is a life-saving procedure that requires life-long immunosuppression treatment to prevent rejection of the allograft. Cyclosporin-A (CsA), an 11 amino acid cyclic peptide inhibitor of calcineurin (Cn) activity, is one of the most commonly used molecules used to prevent tissue rejection. Since first introduced in the late 70's for kidney transplantation (1, 2), CsA has been highly effective in improving the outcome of transplantation; however, treatment is often associated with neuropsychological side effects including anxiety and depressive disorders (3, 4). Because of the traumatic effects of transplant surgery and postoperative care, it is not known whether treatment with CsA is a direct cause of mood disorders. It is therefore important to determine whether there is a biological connection between CsA treatment and anxiety or depressive disorders, since recovery from surgery can be impaired by somatic illnesses related to major depression (5–8).

The amygdala is a critical brain area involved in the regulation of emotions, and in normal and pathological reactivity to stressors (9). Amygdala activity is correlated with anxiety, aggressive behavior and fear, and several studies have reported that hyperactivity of the amygdala is a risk factor in the development of mood disorders and heightened sensitivity to stress (10). Depressed patients can exhibit an increase in glucose metabolism in the amygdala, along with alterations in resting cerebral blood flow in this structure (11–13). Further, functional magnetic resonance imaging (fMRI) has shown that bilateral amygdala hyperactivity in response to environmental stressors is a significant risk factor for both anxiety and depression (14).

Changes in amygdala activity can be achieved through multiple mechanisms, including perturbations of intracellular signaling and gene expression. In rats, viral-mediated overexpression of the cAMP-response element binding protein (CREB) in the basolateral amygdala induces changes in measures of depression- and anxiety-like behavior (15). Interestingly, chronic treatment with several different classes of antidepressant drugs can decrease the expression of c-fos, a marker of neuronal activity in the amygdala (16, 17). Stress also alters amygdala activation in human subjects, as measured by changes in blood flow (18). Thus, molecules involved in signal transduction pathways that alter the dynamics and activity of neuronal function in the amygdala may be involved in neuronal plasticity leading to behaviors related to anxiety and depression.

Cellular calcium dynamics are critical for neuronal activity, and the calcium-regulated serine- and threonine-specific protein phosphatase calcineurin (Cn) is a calcium-dependent enzyme that can alter neuronal function. Cn is composed of a catalytic subunit (CnA) (which contains a calmodulin binding domain, a calcineurin B binding domain and an autoinhibitory domain), and a regulatory subunit (CnB), which binds Ca^{2+} directly (19, 20).

Both subunits are required for enzymatic activity and have multiple isoforms. All CnA isoforms (α , β , and γ) are found in the brain, but only the CnB1 regulatory isoform is expressed in brain (21–23). Cn is expressed throughout the brain (with the highest levels observed in the hippocampus, striatum, substantia nigra and amygdala (24, 25)) and can regulate basic neuronal functions including excitability (26), G protein-mediated inhibition of calcium channels (27) and glutamatergic neurotransmission in cortical neurons (28). A large number of calcineurin targets have been identified that are likely to be involved in the ability of the enzyme to regulate neuronal excitability, including synapsin I, calcium channels, glutamate receptors and the transcription factors CREB and nuclear factor of activated T cells (NFAT) (29).

We hypothesize that CsA-mediated Cn inhibition could induce anxiety- and depression-like phenotypes in an animal model. In addition, decreasing Cn activity specifically in the amygdala may be sufficient to induce these behaviors. To test these hypotheses, we investigated the role of Cn inhibition on depression- and anxiety-like behaviors in mice following chronic systemic treatment with CsA, demonstrating that peripheral administration of the drug results in a significant increase in mouse models of behaviors related to anxiety and depression. We then used an adeno-associated virus (AAV) carrying short hairpin RNAs targeting CnB (shCnBs) to knock-down calcineurin selectively in the amygdala and showed that this was sufficient to recapitulate the behavioral effects of systemic CsA administration.

MATERIALS AND METHODS

Animals

Male C57BL/6J (10–12 weeks old upon arrival) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and were maintained in a temperature-controlled vivarium (21±2°C) under a 12 h light–dark cycle with lights on at 7:00 A.M and housed five per cage. Food and water were available *ad libitum*.

Drug dosing and administration

For systemic administration studies, mice received daily intraperitoneal injections of 15 mg/kg CsA (Sandimmune® oral injection; Novartis, East Hanover, NJ) or olive oil vehicle (Sigma, St. Louis, MO) for 15 days before behavioral testing. Drug administration continued during behavioral testing. All drugs were injected in a volume of 10 ml/kg body weight.

Construction of shRNAs and viral production and purification

As multiple isoforms of CnA are expressed in brain, but only one CnB isoform, we designed an shRNA targeting PPP3r1, the gene encoding CnB (shCnB) (NM_024459.2, targeting bp 451–474 of CDS): GAGGAATTCTGTGCTGTCGTAGGT. A scrambled shRNA (Scr) sequence that did not target any murine mRNAs was used as a control: TTATATGGCGCTTTCGAATGAGC. The shRNA oligos were ligated into pAAV-eGFP-shRNA as described previously (30, 31). Colony PCR and sequencing were used to verify positive clones.

shRNA-containing plasmids were packaged into AAV2 by triple transfection with 135 µg each of pAAV-shRNA, pHelper and pAAV-RC plasmids (Stratagene) using calcium phosphate into HEK293 cells. Five days later, cells were harvested and suspended in freezing buffer (0.15 M NaCl, 50 mM Tris, pH 8.0), then lysed by three freeze-thaw cycles (in ethanol-dry ice and 42°C water bath). Benzonase was added (50 U/ml) and the solution was incubated for 30 min at 37° C. Cellular debris was removed by centrifugation, and the clarified lysate was added to a 15%, 25%, 40%, 60% iodixanol step gradient. This was spun at 50,000 g for 3.5 hours at 10°C, and the 40% fraction was removed and diluted in PBS-MK (1x PBS, 1mM MgCl₂, 2.5 mM KCl), then concentrated, washed, and purified with Amicon 100K filter columns and PBS-MK. Purified, concentrated virus (100–200 µl) was stored at 4° C.

Stereotaxic injection of viral vectors

Using a stereotaxic apparatus (Kopf Instruments, Tijuana, CA) AAV-shCnB or AAV-Scr was infused into the basolateral nucleus of the amygdala (BLA) under isoflurane anesthesia. 0.5 µl of AAV-shCnB or AAV-Scr was infused bilaterally into the BLA over 5 min using a 29G, beveled-tipped Hamilton syringe (Hamilton, Reno, NE). The ventral part of the BLA was targeted based on the observation that interference with calcium-dependent intracellular processes in this brain area can alter behaviors related to anxiety and depression (32); however, since viral diffusion could occur, the central amygdala was also likely infected. After infusion, the syringe was held in place for an additional 5 min before being removed. Coordinates for the stereotaxic injections (relative to bregma) were determined in a stereotaxic atlas (Paxinos & Watson, 1997) and were then adjusted based on the infusion of blue dye in pilot studies. The final coordinates used were: –2.2 anteroposterior, ±3.1 lateral, –4.8 mm dorsoventral. Following infusion, mice recovered for 10 days before undergoing behavioral testing. After all behavioral testing was performed, the placement of the injections was determined by cryostat sectioning through the infusion site and the detection of GFP-positive cells.

Experimental groups

Mice received either pharmacological challenge with systemic CsA for 15 days that continued through behavioral treatment (N=7–10/group) or infusion of AAV-Scr or AAV-shCnB (N=12/group). Behavioral tests were then performed in sequence as follows: elevated plus maze, light/dark box, open field, tail suspension, forced swim test, novelty-suppressed feeding and locomotion (Table 1). To avoid any potential interactions between the behavioral paradigms used, the order of the tests was determined as recommended in (33). Each test was separated by 24 to 48 hours. A separate group of mice was infused with AAV-Scr or AAV-shCnB (N=6/group) for *in vivo* measurement of calcineurin knockdown.

All studies were approved by the Yale University Animal Care and Use Committee and followed the NIH Guide for the Care and Use of Laboratory Animals.

Elevated plus maze

The elevated plus maze was made of black Plexiglas, had four 30 × 5 cm arms and was elevated 50 cm above the floor as has been described previously (34). Two arms were

enclosed by 15-cm walls, while the 2 other arms were open with a 3-mm edge to prevent slipping. The maze was illuminated homogeneously with the regular room light. A 5×5 cm center area at the crossing of the arms was considered to be a neutral area. The animals were placed in the test room 30 to 60 min prior to testing to limit acute stress. A non-test mouse was first placed on the maze to deposit fresh odors. At the beginning of the test, mice were placed in the center of the maze facing an open arm. The subject was then allowed to explore the maze freely for 5 min. The percentage of time spent in the open arms compared to the total time minus time in the center was used as the primary measure of anxiety-like behavior; number of entries into the open and closed arms were recorded, and the total number of entries was also reported.

Light/dark test

The light/dark test was performed similarly to what has been previously described (35–37). The apparatus consisted of two opaque Plexiglas compartments of the same size connected by a central opening ($18 \times 10 \times 13$ cm dimensions: light compartment illuminated by a 60 W desk lamp). Mice were placed into the dark compartment facing away from the opening and tracked for 5 min after the first cross was made. Number of entries into the dark side and time spent in the dark compartment were measured.

Anxiety test in a square open field

Mice were placed in a square open field ($40 \text{ cm} \times 40 \text{ cm}$) composed of a clear floor without bedding, and their behavior was observed for 20 min. Two main endophenotypes relating to anxiety-like behavior were then recorded (38): time spent in the thigmotaxis area (within 5 cm of the walls, with 4 paws in the area) and overall time spent freezing in the open field.

Tail suspension test

As has been described previously, mice were gently suspended by the tail and scored for time spent immobile over the 6 min test (37). After completion of the test, mice were returned to a holding cage until all cage-mates were tested.

Forced swim test

Mice were gently placed in clear glass beakers filled with 15 cm water (room temperature, $\sim 22^\circ\text{C}$) for 15 min. Care was taken not to put the nose of the mouse below water level. Mice were observed and the overall time spent immobile was scored (immobility was defined as a minimal amount of movement made by the mouse excluding respiratory and whisker movements or tetanic movements of the limbs). After testing, each mouse was placed in a heated holding cage ($30\text{--}35^\circ\text{C}$) with bedding covered by a paper towel. Animals were returned to the holding room once dry and placed back in their home cage (17, 37).

Novelty suppressed feeding test

Mice were food-deprived for 24 hours before testing. The day of the test, mice were placed in the behavioral room at least 30 min before testing. The testing arena was dimly lit. Animal were placed in an open field ($54 \text{ cm} \times 28 \text{ cm} \times 12 \text{ cm}$) with a piece of chow on a circular piece of blotting paper. Animals were placed in a corner, facing the center and were

then observed a maximum of 6 min. Time to first feeding episodes was recorded and the test was then halted.

Locomotor activity in an open field

Mice were placed into the center of a brightly lit, novel cage (48 × 22 × 18 cm) with no bedding for 20 min. Three consecutive beam breaks were used as an index of locomotor activity.

Quantitative real-time (q)-PCR and mRNA quantitation of CnB

For *in vitro* testing of shCnB efficacy, N2a cells, mouse neuroblastoma cells that endogenously express calcineurin, were transfected with either pAAV-shCnB or pAAV-Scr using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Twenty four hours later, cells were lysed and RNA was isolated using the Qiagen RNeasy Mini kit, according to the manufacturer's protocol. RNA was stored at -80°C until further processing. 500 ng of RNA from each sample was reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). cDNA was then quantified using real time PCR with SYBR Green in a StepOnePlus Thermal Cycler (Applied Biosystems). Primer sets for CnB and a reference gene (β -glucuronidase, GusB1) were designed using Primer3 to span introns to prevent genomic DNA amplification. CnB primers: Forward, TTTGAGCGTGGAAGAGTTCAT; Reverse, CGCCTTTGACACTGAACTGA. GusB1 primers: Forward, AACCTCTGGTGGCCTTACCT; Reverse, TCCCGATAGGAAGGGTGTAG.

For verification of knockdown *in vivo*, animals infused with AAV-Scr and AAV-shCnB in the amygdala (N=6/group) were sacrificed by rapid decapitation 20 days after surgery. Brains were removed and frozen in isopentane cooled in a dry-ice ethanol bath. Brains were sectioned on a cryostat to the rostral portion of the amygdala. GFP was detected using the NIGHTSEA BlueStar Flashlight and barrier filter glasses (Electron Microscopy Sciences). 200 μ m thick sections containing GFP fluorescence in the amygdala were placed on a glass coverslip, and punches of GFP-enriched amygdala from the frozen tissue sections were taken under LED visualization of GFP. Punches were stored on dry-ice or at -80° C. RNA was extracted from the tissue punches using a Qiagen MicroRNeasy Plus Kit. Reverse transcription (with 28 ng of RNA as starting material) and quantitative PCR were performed as above. For *in vivo* quantitation, GAPDH was used as a reference gene. GAPDH Forward primer: GGTGAAGGTCGGTGTGAACG; Reverse primer: CTCGCTCCTGGAAGATGGTG.

Statistical analysis

For comparison of the mean values between groups, statistical evaluation was performed using analysis of variance (ANOVA) with Cyclosporin A vs. Vehicle or AAV-Scr vs. AAV-shCnB as between subject factors. Knockdown efficacy was quantified using REST 2009 software (Qiagen). P values < 0.05 were considered statistically significant. All data are presented as means $\pm$ standard error of the mean (SEM).

RESULTS

Systemic CsA administration induces a significant anxiety-like phenotype

To evaluate the effect of chronic, systemic inhibition of calcineurin activity on behaviors related to anxiety and depression, we treated mice peripherally with CsA and then tested the animals in the elevated plus maze, light/dark test, the open-field (anxiety-like behaviors), forced swim test and tail suspension test (depression-like behaviors). Locomotion was then assessed.

In the elevated plus maze, systemic injection of CsA significantly decreased the time spent in the open arms ($F(1,18) = 18.12$; $p < 0.01$; Fig. 1A) but the total number of entries was unchanged ($F < 1$; Fig. 1B). In the light/dark test, CsA-treated mice spent more time in the dark compartment compared to vehicle-treated mice ($F(1,17) = 26.47$; $p < 0.001$; Fig. 1C). The number of entries into the light compartment was decreased in CsA-treated mice compared to vehicle injection ($F(1,17) = 5.69$; $p < 0.05$; Fig. 1D), and the animals were also faster to enter the dark compartment $F(1,17) = 6.41$; $p < 0.05$; data not shown). In a square open field, the mice treated with CsA exhibited a greater time engaging in thigmotaxis (time spent next to the walls of the open field: $F(1,22) = 11.59$, $p = 0.01$; Fig. 2A) and the overall freezing time was also increased following treatment ($F(1,22) = 10.57$, $p < 0.05$, Fig. 2B). Finally, the time to initiate a feeding episode was also significantly increased in the novelty suppressed feeding test (Fig. 2C; $F(1,22) = 37.91$, $p < 0.0001$) with no change in body weight following food restriction and no difference in food intake in home cage (Fig. 2 D and E; $F < 1$)).

In the tail suspension test, only subtle effects were detected and the difference in time spent immobile did not reach significance (Fig. 3A). CsA-injected mice showed a significant increase in immobility time in the forced swim test ($F(1,19) = 7.6$; $p < 0.05$; Fig. 3B), however, and a significant difference was only observed after at least 5 min of testing (0 to 5 min: $F < 1$; 5 to 10 min: $F(1,22) = 5.03$, $p < 0.05$; 10 to 15 min: $F(1,22) = 13.51$, $p < 0.01$).

A significant decrease in locomotor activity was measured in mice treated chronically with CsA ($F(1,22) = 50.8$, $p < 0.0001$, Fig. 3C).

Knockdown of calcineurin B

To measure knockdown of CnB *in vitro*, we used a mouse neuroblastoma cell line, Neuro 2a (ATTC CCL-131) which endogenously expresses calcineurin. Transfection with pAAV-shCnB or pAAV-Scr resulted in 30–50% of cells expressing GFP. Quantitation of mRNA levels was done using qRT-PCR. With 6000 iterations of randomized comparisons, CnB mRNA from shCnB-transfected N2a cells was $67 \pm 12\%$ of shScr-transfected levels ($p < 0.01$). To test for knockdown *in vivo*, mice were infused with AAV-Scr or AAV-shCnB into the amygdala. A representative example of the spread of the virus, as determined by GFP expression, is shown in Fig. 4. Quantitative PCR from the amygdala showed that CnB mRNA in shCnB-infused animals was $69 \pm 10\%$ of Scr-infused animals ($p < 0.001$). CnB mRNA in an adjacent cortical area was unchanged in shCnB-infused animals ($100 \pm 11\%$ of Scr-infused animals; Fig 4D).

AAV-shCnB-mediated knockdown of calcineurin B in the amygdala increases anxiety- and depression-like behaviors in mice

In order to determine whether the changes in anxiety- and depression like behavior following chronic, systemic CsA treatment could be caused by a decrease in calcineurin activity in a specific brain area, we used adeno-associated virus (AAV) to express short hairpin RNAs targeting calcineurin in the amygdala, a brain region involved in the control anxiety and depression and with a high level of calcineurin activity. Mice that received AAV-Scr or AAV-shCnB infusions into the amygdala were tested in the same battery of behavioral tests used to assess anxiety- and depression-like behavior in CsA-treated mice. A significant decrease in the time spent in the open arms was observed in the mice that received AAV-shCnB infusion into the amygdala compared to mice with AAV-Scr infusion into the amygdala ($F(1,22) = 19.42, p < 0.001$; Fig. 5A) while the total number of entries was not significantly changed ($F < 1$, Fig. 5B).

In the light/dark test, AAV-shCnB-infused mice spent a greater time in the dark chamber ($F(1,22) = 8.39, p < 0.01$; Fig. 5C) and made fewer entries into the light chamber ($F(1,22) = 9.35, p < 0.01$; Fig. 5D) compared to AAV-Scr-infused mice and were faster to enter the dark compartment ($F(1,22) = 5.98, p < 0.05$ (data not shown)).

In the open field, time spent engaging in thigmotaxis was increased (Fig. 6A; $(1, 22) = 11.31, p < 0.01$), as was freezing time (Fig. 6B; $F(1,22) = 15.38, p < 0.001$). Conversely, in the novelty-suppressed feeding test, animals that received AAV-shCnB infusion into the amygdala were slightly slower to initiate feeding, but this difference did not reach significance (Fig. 6C; $F(1,22) = 3.8, p = 0.06$). No differences were observed in the amount of weight lost after fasting or the amount of food consumed in the home cage (Fig. 6D and E).

In the tail suspension test, knockdown of calcineurin B in the amygdala somewhat increased immobility compared to AAV-Scr infused mice, but this difference did not reach significance ($F(1,22) = 4.07, P = 0.06$; Fig. 7A). In the forced swim test, stereotaxic injection of AAV-shCnB significantly increased the duration of immobility ($F(1,24) = 62.350, p < 0.001$; Fig. 7B).

No significant change in locomotor activity was observed following knockdown of CnB in the amygdala ($F(1,14) = 2.53; p > 0.1$; Fig. 7C).

DISCUSSION

The current set of experiments demonstrates that chronic administration of CsA results in a generalized increase of anxiety-like behavior across several paradigms. Results in tests of depression-like behavior were not as striking, although the change in immobility in the forced swim test suggested that there was a depression-like effect of chronic CsA treatment. Interestingly, very similar phenotypes were seen following local knockdown of calcineurin activity in the amygdala by viral-mediated delivery of shRNAs targeting the calcineurin B subunit. Taken together, these results suggest that inhibition of calcineurin in amygdala alters local activity, is sufficient to recapitulate the effect of systemic cyclosporine

administration and can increase anxiety- and depression-like behaviors. It is worth noting that calcineurin activity in other brain areas may also contribute to anxiety- or depression-like behaviors, but this possibility was not investigated in the current study.

One likely possibility is that antagonism of calcineurin can potentiate neuronal activity in the amygdala, thereby increasing stress-related behaviors. Studies in rats have shown that blockade of calcineurin activity in the amygdala prevents reversal of synaptic potentiation and extinction of fear memory (32). In contrast, the expression of fear memory is correlated with an increase in the activity of calcineurin (32). Thus, CsA may selectively disrupt depotentiation in the amygdala and this may prolong anxiety and fear states, leading to the affective phenotypes measured here.

The current study knocked down CnB in all neuronal subtypes of the amygdala. It is not known whether calcineurin is critical for function of specific classes of amygdala neurons or whether alterations in calcineurin activity change the balance of excitatory and inhibitory signaling in this structure. Future studies targeting specific neuronal subtypes will be necessary to dissect the role of calcineurin activity in GABA and glutamate neurons of the BLA.

Previous studies have reported that patients treated with CsA to prevent rejection after organ transplantation display increased symptoms of depression and anxiety (3, 4, 39–41). While invasive surgery, as well as the stress of serious illness, is likely to play a pivotal role in changes in mood, these reports suggest that treatment with the calcineurin inhibitor CsA may also contribute to affective changes in these subjects. The results of the current study show that both antagonism of calcineurin by peripheral administration of CsA, and genetic downregulation of calcineurin mRNA expression in the amygdala promote anxiety- and depression-like behaviors, strongly suggesting that CsA treatment in transplant patients could contribute to the increase in mood disorders seen in these patients. Previous studies have suggested that increased calcineurin activity results in greater sensitivity to classical antidepressants (42). In addition, polymorphisms in the gene encoding the catalytic subunit of calcineurin have been associated with a greater risk of bipolar disorder (43). Taken together, these studies suggest that calcineurin activity in the amygdala is essential for normal mood regulation.

In summary, blockade of calcineurin activity in the amygdala appears to result in an increase in symptoms related to affective disorders. Therefore, the development of calcineurin antagonists that do not penetrate into the brain or immunosuppressants that do not target calcineurin activity would be a significant advance that would minimize the risk of anxiety and mood symptoms in organ transplant patients and could improve clinical outcomes in these patients.

Acknowledgments

This work was supported by grants DA14241, MH77681 and DA033597 from the National Institutes of Health and the State of Connecticut, Department of Mental Health and Addiction Services.

Abbreviations

AAV	adeno-associated virus
AID	auto inhibitory domain
BLA	basolateral amygdala
CaM	calmodulin
CnB	calcineurin B
CREB	cAMP response element binding protein
CsA	Cyclosporin-A
HEK	human embryonic kidney
NAc	nucleus accumbens
PP2B	protein phosphatase 2B
shCnB	short hairpin RNA targeting calcineurin A
VTA	ventral tegmental area

References

1. Calne RY, Rolles K, White DJ, Thiru S, Evans DB, McMaster P, et al. Cyclosporin A initially as the only immunosuppressant in 34 recipients of cadaveric organs: 32 kidneys, 2 pancreases, and 2 livers. *Lancet*. 1979; 2:1033–1036. [PubMed: 91781]
2. Calne RY, White DJ, Thiru S, Evans DB, McMaster P, Dunn DC, et al. Cyclosporin A in patients receiving renal allografts from cadaver donors. *Lancet*. 1978; 2:1323–1327. [PubMed: 82836]
3. Kahan BD, Flechner SM, Lorber MI, Golden D, Conley S, Van Buren CT. Complications of cyclosporine-prednisone immunosuppression in 402 renal allograft recipients exclusively followed at a single center for from one to five years. *Transplantation*. 1987; 43:197–204. [PubMed: 3544376]
4. de Groen PC, Aksamit AJ, Rakela J, Forbes GS, Krom RA. Central nervous system toxicity after liver transplantation. The role of cyclosporine and cholesterol. *N Engl J Med*. 1987; 317:861–866. [PubMed: 3306386]
5. Katon WJ. Clinical and health services relationships between major depression, depressive symptoms, and general medical illness. *Biol Psychiatry*. 2003; 54:216–226. [PubMed: 12893098]
6. Ormel J, Rijdsdijk FV, Sullivan M, van Sonderen E, Kempen GI. Temporal and reciprocal relationship between IADL/ADL disability and depressive symptoms in late life. *J Gerontol B Psychol Sci Soc Sci*. 2002; 57:P338–347. [PubMed: 12084784]
7. Patten SB. Long-term medical conditions and major depression in a Canadian population study at waves 1 and 2. *J Affect Disord*. 2001; 63:35–41. [PubMed: 11246078]
8. van den Brink RH, van Melle JP, Honig A, Schene AH, Crijns HJ, Lambert FP, et al. Treatment of depression after myocardial infarction and the effects on cardiac prognosis and quality of life: rationale and outline of the Myocardial Infarction and Depression-Intervention Trial (MIND-IT). *Am Heart J*. 2002; 144:219–225. [PubMed: 12177637]
9. Davis M, Whalen PJ. The amygdala: vigilance and emotion. *Mol Psychiatry*. 2001; 6:13–34. [PubMed: 11244481]
10. Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S. Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. *J Neurosci*. 2002; 22:6810–6818. [PubMed: 12151561]

11. Drevets WC, Videen TO, Price JL, Preskorn SH, Carmichael ST, Raichle ME. A functional anatomical study of unipolar depression. *J Neurosci*. 1992; 12:3628–3641. [PubMed: 1527602]
12. Sheline YI, Gado MH, Price JL. Amygdala core nuclei volumes are decreased in recurrent major depression. *Neuroreport*. 1998; 9:2023–2028. [PubMed: 9674587]
13. Drevets WC. Neuroimaging and neuropathological studies of depression: implications for the cognitive-emotional features of mood disorders. *Curr Opin Neurobiol*. 2001; 11:240–249. [PubMed: 11301246]
14. Wolfensberger SP, Veltman DJ, Hoogendijk WJ, Boomsma DI, de Geus EJ. Amygdala responses to emotional faces in twins discordant or concordant for the risk for anxiety and depression. *Neuroimage*. 2008; 41:544–552. [PubMed: 18396414]
15. Wallace TL, Stellitano KE, Neve RL, Duman RS. Effects of cyclic adenosine monophosphate response element binding protein overexpression in the basolateral amygdala on behavioral models of depression and anxiety. *Biol Psychiatry*. 2004; 56:151–160. [PubMed: 15271583]
16. Beck CH, Fibiger HC. Chronic desipramine alters stress-induced behaviors and regional expression of the immediate early gene, c-fos. *Pharmacology, biochemistry, and behavior*. 1995; 51:331–338.
17. Mineur YS, Picciotto MR, Sanacora G. Antidepressant-like effects of ceftriaxone in male C57BL/6J mice. *Biological psychiatry*. 2007; 61:250–252. [PubMed: 16860779]
18. Clark CP, Brown GG, Archibald SL, Fennema-Notestine C, Braun DR, Thomas LS, et al. Does amygdalar perfusion correlate with antidepressant response to partial sleep deprivation in major depression? *Psychiatry research*. 2006; 146:43–51. [PubMed: 16380239]
19. Clipstone NA, Fiorentino DF, Crabtree GR. Molecular analysis of the interaction of calcineurin with drug-immunophilin complexes. *J Biol Chem*. 1994; 269:26431–26437. [PubMed: 7523407]
20. Husi H, Luyten MA, Zurini MG. Mapping of the immunophilin-immunosuppressant site of interaction on calcineurin. *J Biol Chem*. 1994; 269:14199–14204. [PubMed: 7514602]
21. Hashimoto Y, Perrino BA, Soderling TR. Identification of an autoinhibitory domain in calcineurin. *J Biol Chem*. 1990; 265:1924–1927. [PubMed: 2153670]
22. Kissinger CR, Parge HE, Knighton DR, Lewis CT, Pelletier LA, Tempczyk A, et al. Crystal structures of human calcineurin and the human FKBP12-FK506-calcineurin complex. *Nature*. 1995; 378:641–644. [PubMed: 8524402]
23. Rusnak F, Mertz P. Calcineurin: form and function. *Physiol Rev*. 2000; 80:1483–1521. [PubMed: 11015619]
24. Buttini M, Limonta S, Luyten M, Boddeke H. Differential distribution of calcineurin A alpha isoenzyme mRNA's in rat brain. *Naunyn Schmiedebergs Arch Pharmacol*. 1993; 348:679–683. [PubMed: 8133911]
25. Polli JW, Billingsley ML, Kincaid RL. Expression of the calmodulin-dependent protein phosphatase, calcineurin, in rat brain: developmental patterns and the role of nigrostriatal innervation. *Brain Res Dev Brain Res*. 1991; 63:105–119.
26. Yakel JL. Calcineurin regulation of synaptic function: from ion channels to transmitter release and gene transcription. *Trends Pharmacol Sci*. 1997; 18:124–134. [PubMed: 9149541]
27. Zhu Y, Yakel JL. Calcineurin modulates G protein-mediated inhibition of N-type calcium channels in rat sympathetic neurons. *J Neurophysiol*. 1997; 78:1161–1165. [PubMed: 9307144]
28. Victor RG, Thomas GD, Marban E, O'Rourke B. Presynaptic modulation of cortical synaptic activity by calcineurin. *Proc Natl Acad Sci U S A*. 1995; 92:6269–6273. [PubMed: 7541535]
29. Mansuy IM. Calcineurin in memory and bidirectional plasticity. *Biochem Biophys Res Commun*. 2003; 311:1195–1208. [PubMed: 14623305]
30. Mineur YS, Abizaid A, Rao Y, Salas R, DiLeone RJ, Gundisch D, et al. Nicotine decreases food intake through activation of POMC neurons. *Science (New York, NY)*. 2011; 332:1330–1332.
31. Hommel JD, Sears RM, Georgescu D, Simmons DL, DiLeone RJ. Local gene knockdown in the brain using viral-mediated RNA interference. *Nature medicine*. 2003; 9:1539–1544.
32. Lin CH, Lee CC, Gean PW. Involvement of a calcineurin cascade in amygdala depotentiation and quenching of fear memory. *Molecular pharmacology*. 2003; 63:44–52. [PubMed: 12488535]

33. Crawley JN. Behavioral phenotyping strategies for mutant mice. *Neuron*. 2008; 57:809–818. [PubMed: 18367082]
34. Caldarone BJ, King SL, Picciotto MR. Sex differences in anxiety-like behavior and locomotor activity following chronic nicotine exposure in mice. *Neuroscience letters*. 2008; 439:187–191. [PubMed: 18524488]
35. Crawley JN. Exploratory behavior models of anxiety in mice. *Neurosci Biobehav Rev*. 1985; 9:37–44. [PubMed: 2858080]
36. Crawley J, Goodwin FK. Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. *Pharmacology, biochemistry, and behavior*. 1980; 13:167–170.
37. Mineur YS, Somenzi O, Picciotto MR. Cytisine, a partial agonist of high-affinity nicotinic acetylcholine receptors, has antidepressant-like properties in male C57BL/6J mice. *Neuropharmacology*. 2007; 52:1256–1262. [PubMed: 17320916]
38. Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *European journal of pharmacology*. 2003; 463:3–33. [PubMed: 12600700]
39. Jindal RM, Joseph JT, Morris MC, Santella RN, Baines LS. Noncompliance after kidney transplantation: a systematic review. *Transplant Proc*. 2003; 35:2868–2872. [PubMed: 14697924]
40. Sato Y, Onaka T, Kobayashi E, Seo N. The differential effect of cyclosporine on hypnotic response and pain reaction in mice. *Anesth Analg*. 2007; 105:1489–1493. table of contents. [PubMed: 17959987]
41. Telarovic S, Telarovic S, Mihanovic M. Cyclosporine-induced depressive psychosis in a liver transplant patient: a case report. *Lijec Vjesn*. 2007; 129:74–76. [PubMed: 17557548]
42. Crozatier C, Farley S, Mansuy IM, Dumas S, Giros B, Tzavara ET. Calcineurin (protein phosphatase 2B) is involved in the mechanisms of action of antidepressants. *Neuroscience*. 2007; 144:1470–1476. [PubMed: 17207580]
43. Mathieu F, Miot S, Etain B, El Khoury MA, Chevalier F, Bellivier F, et al. Association between the PPP3CC gene, coding for the calcineurin gamma catalytic subunit, and bipolar disorder. *Behav Brain Funct*. 2008; 4:2. [PubMed: 18201382]

Figure 1. Anxiety-like behavior in elevated plus maze and light/dark box in mice treated chronically with CsA

A) Percent time spent in and, B) number of entries into, open arms in the elevated plus maze. C) Time spent in the dark side and, D) number of entries into the dark side, in the light/dark box. Data are expressed as mean $\pm$ SEM, $n = 7-12$ per group. *, $p < 0.05$; ***, $p < 0.001$.

Figure 2. Anxiety-like behavior in the open field and novelty-suppressed feeding in mice treated chronically with CsA

A) Time spent in the thigmotaxis zone (within 5 cm of walls) and, B) amount of time spent freezing, in a square open field. C) Time to initiate the first feeding episode in the novelty-suppressed feeding test. D) Body weight after 24-hour fasting. E) Food consumed in the home cage for 5 min after novelty-suppressed feeding testing. Data are expressed as mean $\pm$ SEM, $n = 7-12$ per group. **, $p < 0.01$.

Figure 3. Depression-like behavior in mice treated chronically with CsA

Immobility time in the A) forced swim, and B) tail suspension, tests. C) Locomotor activity in an open field. Data are expressed as mean $\pm$ SEM, $n = 7-12$ per group. *, $p < 0.05$.

Figure 4. Viral vector-mediated calcineurin B knockdown

A) Low-magnification image of the amygdala after infusion with AAV-shCnB. Scale bar = 500 μ m. B) Higher magnification image of field shown in A. Virally-infused cells are found throughout the amygdala complex. Scale bar = 100 μ m. C) Diagram from Paxinos and Watson demonstrating the spread of viral infusion. D) Quantitative PCR revealed a significant decrease in CnB mRNA levels in the amygdala, but not adjacent cortex, of AAV-shCnB-infused animals compared to AAV-Scr-infused animals. ***, $p < 0.001$.

Figure 5. Anxiety-like behavior following local knockdown of calcineurin in the amygdala
 A) Percent time spent in and, B) number of entries into, open arms in the elevated plus maze. C) Time spent in the dark side and, D) number of entries into the dark side, in the light/dark box for groups of mice with local knockdown of calcineurin in the amygdala. Data are expressed as mean $\pm$ SEM, $n = 12$ per group. *, $p < 0.05$; **, $p < 0.01$.

Figure 6. Anxiety-like behavior in square open field and hyponeophagia in mice following knockdown of calcineurin in the amygdala

A) Time spent in the thigmotaxis zone (within 5 cm of walls) and, B) amount of time spent freezing, in a square open field. C) Time to initiate the first feeding episode in the novelty-suppressed feeding test. D) Body weight after 24-hour fasting. E) Food consumed in the home cage for 5 min after novelty-suppressed feeding testing. Data are expressed as mean ± SEM, n = 12 per group. **, $p < 0.01$.

Figure 7. Depression-like behavior in mice following local knockdown of calcineurin in the amygdala

Immobility time in the A) forced swim, and B) tail suspension, tests. C) Locomotor activity in an open field. Data are expressed as mean $\pm$ SEM, $n = 12$ per group. ***, $p < 0.001$.

TABLE 1

Time table of behavioral experiments.

Day	CsA/vehicle groups	AAV-shCnB/AAV-Scr groups	Days
Injection of CsA or vehicle (15 days pretreatment)		Post-surgery recovery (>2 weeks)	
1	Elevated plus maze		1
2	Light/dark test		2
4	Square open field		4
5	Tail suspension		5
6	Forced swim test		6
9	Novelty-suppressed feeding		9
10	Locomotion Determination of placement for viral vector infusions		10