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Blockade of protein phosphatase 2B activity in the amygdala increases anxiety- and depression-like behaviors in mice

Amine Bahi, **Yann S. Mineur**, 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—Organ transplant patients receive chronic administration of the calcineurin inhibitor Cyclosporin-A (CsA) and demonstrate increased incidence of mood disorders. Significant calcineurin expression can be observed using immunohistochemistry in the amygdala, a brain area important for behaviors related to mood disorders and anxiety. 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 globally and selectively in the amygdala of the mouse brain to determine the role of calcineurin in behaviors related to depression and anxiety.

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 A increased behavioral measures of anxiety in both the elevated plus maze and light/dark tests with no changes in locomotor activity. In the forced swim and tail suspension models of depression-like behavior, calcineurin blockade in the amygdala increased immobility similar to manipulations that lead to a depression-like phenotype.

Conclusions—Taken together, these data demonstrate that decreasing calcineurin activity in the amygdala increases anxiety- and 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 clinical consequence of treatment to prevent transplant rejection.

Keywords

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

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

Introduction

Cyclosporin-A (CsA) is an 11 amino acid cyclic peptide inhibitor of calcineurin first used to prevent rejection following kidney transplantation (1,2). Since the introduction of CsA for immunosuppression in organ transplantation, the rate of allograft rejection has decreased dramatically; however, treatment with CsA is correlated with neuropsychological complications in patients, including anxiety and depression (3,4), although it is unclear whether CsA can alter mood on its own. Major depression impairs the recovery from a number of somatic illnesses (5-8). It is therefore critical to identify neurobiological mechanisms underlying mood-altering effects of calcineurin blockade.

The amygdala is critical for regulation of emotion and mood (9). Hypertrophy of the amygdala correlates with increased anxiety, fear and aggression (10). Accordingly, brain imaging of depressed patients has shown increased glucose metabolism, decreased volume and changes in resting cerebral blood flow in the amygdala (11-13). In addition, exposure to environmental stress, resulting in a high risk for anxiety and depression, was correlated with robust, bilateral amygdala hyperactivity (14).

Changes in amygdala activity can also be achieved by altering intracellular signaling in amygdala neurons. For example, in animal studies, viral-mediated expression of cAMP-response element binding protein (CREB) in the basolateral amygdala of rats alters depression- and anxiety-like behaviors (15). In addition, reduction of amygdala c-fos expression has been observed after chronic (16) or acute (17) treatment with antidepressants. Finally, the antidepressant effect of sleep deprivation also results in a reduction of amygdalar perfusion that may contribute to its antidepressant efficacy (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.

Calcineurin is a calcium-regulated serine/threonine-specific protein phosphatase comprising two different subunits. The catalytic subunit, or calcineurin A, contains the active site (19, 20), the calmodulin-binding domain (21), and the autoinhibitory (AID) domain (22), which binds to the active site in the absence of calmodulin (23) to inhibit the enzyme. The regulatory subunit, or calcineurin B, contains Ca^{++} binding sites (24). The highest levels of calcineurin are found in the hippocampus, striatum, substantia nigra, and amygdala (25,26). Calcineurin can regulate basic neuronal functions including excitability (27), G protein-mediated inhibition of calcium channels (28) and glutamatergic neurotransmission (29). Several calcineurin targets have been identified that are likely 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) (30).

In the current study we investigated the role of calcineurin inhibition on depression- and anxiety-like behaviors in mice following chronic, systemic treatment with CsA, confirming that peripheral administration of the drug results in a significant increase in behaviors related to anxiety and depression. We then used adeno-associated virus (AAV) carrying short hairpin RNAs targeting calcineurin A (shCnAs) to knock down calcineurin selectively in the amygdala. The results demonstrate that calcineurin activity in the amygdala is critical for behavior in tests related to anxiety- and depression-like behaviors. These studies also suggest a molecular mechanism and an anatomical locus for the ability of CsA to increase symptoms of anxiety and depression, and imply that CsA analogues with less penetration into the brain would be a significant advance in treatment for transplant patients. In addition, these data demonstrate a role for calcineurin signaling in maintaining appropriate activity in neurons of the amygdala, suggesting that altered calcium signaling in these neurons could contribute to development of anxiety and depressive disorders.

Materials and Methods

Animals

Male C57BL/6J mice (10-12 weeks old; Jackson Laboratories, Bar Harbor, ME, USA) 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*. Three groups of mice were used for these studies. Group 1 received pharmacological challenge with systemic CsA (N=12-15/group), Group 2 was infused with AAV-GFP or AAV-shCnA (N=12-15/group) and Group 3 was infused with AAV-GFP or AAV-shCnA and then subjected to an acute (15 min) swim stress 90 min before sacrifice (N=5-8/group). Behavioral tests for Groups 1 and 2 were performed as shown in table 1.

Mice began testing 2 weeks after arrival and were habituated to handling for at least 3 days before testing. 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.

Drug dosing and administration

For systemic administration studies, mice received daily (2 weeks) intraperitoneal injections (10 ml/kg) of 15 mg/kg CsA (Sandimmune® oral injection; Novartis, East Hanover, NJ) or olive oil vehicle (Sigma, St. Louis, MO).

Construction of shRNAs and viral production and purification

Three short hairpin RNAs (bp 158-182; bp 466-490; bp 1452-1476) were designed to target the mRNA encoding calcineurin A (NM_008915) (31). Targets were ligated into pAAV-shRNA as described previously (32,33) and sequenced. Viral production was accomplished by triple-transfection in HEK 293 cells. Cells were cultured in five 150×25 mm cell culture dishes and transfected with 135 μg each of pAAV-shCnA, pHelper and pAAV-RC plasmids using calcium phosphate (32,34).. After 72h, cells pelleted and resuspended in freezing buffer (0.15 M NaCl and 50 mM Tris, pH 8.0). After two freeze/thaw cycles, in dry ice and a 42°C water bath, to lyse the cells, benzoase was added (50 U/ml, final) and the mixture was incubated at 37°C for 30 min. The lysate was added to a centrifuge tube containing a 15%, 25%, 40% and 60% iodixanol gradient, spun at 200,000 g for 3h at 10 °C, and the 40% fraction was collected. Iodixanol was exchanged with PBS using Amicon BioMax 100K NMWL concentrators. The final purified virus was stored at 4°C (32,34).

Stereotaxic injection of viral vectors

Using a stereotaxic apparatus (Kopf Instruments, Tijunga, CA) AAV-shCnA and AAV-EGFP were infused into the basolateral nucleus of the amygdala (BLA) under ketamine/xylazine (100/10 mg/kg, i.p.) anesthesia (the nucleus accumbens (NAc) and the ventral tegmental area (VTA) were used as control regions). 1 μl AAV-shCnA or AAV-EGFP was infused bilaterally into the BLA over 5 min using a 26s/2"/2-gauge, blunt-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, and also on pilot studies of c-fos immunoreactivity in response to stress; however, since viral diffusion could occur, the central amygdala would likely also be infected. After infusion, the syringe was held in place for an additional 10 min before being removed. Coordinates for the stereotaxic injections (relative to bregma) were: BLA: -2.1 anteroposterior, ±3.2 lateral, and -4.5 mm dorsoventral (DV); NAc: +1.5 AP, ±1.5 lateral, and -4.4 mm DV and VTA: -3.3 AP, ±1.0 lateral, and -4.6 mm DV (Paxinos & Watson, 1997). The placement of the injections was determined by cryostat sectioning through the infusion site.

Elevated plus maze

The elevated plus maze was made of black Plexiglas and had four 30 × 5 cm arms and was elevated 50 cm above the floor as has been described previously (35). Two arms were enclosed by 15-cm walls, the other 2 arms had a 3-mm edge to prevent slipping and all arms were illuminated equally. A 5×5 cm center platform at the center was considered a neutral area. One hour prior to the experiment, the animals were placed in the test room. At the beginning of the test, mice were placed in the center of the maze facing an open arm and were allowed to explore the maze 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 and number of entries into each arm was recorded.

Locomotor activity in an open field

Each mouse was placed into the center of a brightly lit, novel cage (48 × 22 × 18 cm) with no bedding for 20 min. Beam breaks were used as an index of distance traveled.

Light/dark test

The light/dark test was performed similarly to what has been previously described (36-38). The apparatus consisted of two opaque Plexiglas compartments of the same size connected by a central opening (18 × 10 × 13 cm dimensions: light compartment illuminated by a 60 W desk lamp through a transparent Plexiglas cover). 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.

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 (38). After completion of the test, mice were returned to a holding cage until all cage-mates were tested.

Forced swim test

Mice were placed in clear glass beakers filled with 15 cm water (~25°C) for 15 min with care taken not to put the nose of the mouse below water level. Mice were scored for time spent immobile (immobility was defined as a minimal amount of movement made by the mouse excluding respiratory and whisker movements). After testing, each mouse was placed in a heated holding cage (30-35°C) with bedding covered by a paper towel. After testing, animals were returned to the holding room (17,38).

Calcineurin activity

Immediately after the completion of behavioral testing, brains were removed following rapid decapitation. The amygdala, NAc and VTA were punched from 1 mm sections using an 18 gauge syringe and placed immediately in lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 100 μM EDTA, 100 μM EGTA and 0.2% Nonidet P-40). Twenty μg of protein from each sample was incubated with a specific calcineurin substrate, RII phosphopeptide, according to the manufacturer's protocol. Reactions were stopped by adding GREEN™ reagent (Calbiochem, La Jolla, CA) and colorimetric intensity was measured at 620 nm in a microplate spectrophotometer. Data are values obtained from three independent experiments performed in triplicate.

Real-time (RT)-PCR and mRNA quantitation

RNA quantitation was performed as described previously (39). Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) including an RNase-free DNase step. RNA was

quantified by spectrophotometry and integrity was verified by agarose gel electrophoresis as visualized with ethidium bromide staining. First-strand cDNA was generated from 2 μg of total RNA using Oligo(dT_{12-18}) primer with the SuperScript III reverse transcription kit (Invitrogen) in a total volume of 20 μL according to the manufacturer's instructions. Primers for RT-PCR of the calcineurin A mRNA were designed using Primer3 (http://frodo.wi.mit.edu/). Settings in this program were chosen to avoid hairpin secondary structures and self- and cross-dimers. The reaction product was used for quantitative RT-PCR using the StepOnePlus™ Real-Time PCR System (Applied Biosystems Inc, Foster City, CA). cDNA (5 μL), 20 μM primers and 10 μL of QuantiTect™ SYBR Green PCR Kit (Invitrogen) were combined in a total volume of 20 μL and PCR was performed for 40 cycles as follows: 3 min at 95°C (initial denaturation), 20°C/s temperature transition rate up to 95 °C for 45s then 45s at 62°C (amplification). Specificity of the primers and the PCR reaction were verified by melt-curve analysis and by checking the PCR products on 2% agarose gel, confirming that all PCR protocols were highly specific and that only one PCR fragment was amplified in every PCR reaction. Each PCR experiment was performed three times and variability was less than 10%. Negative controls (samples without cDNA) were included in all experiments.

Immunohistochemistry

For GFP and c-fos studies, a separate group of mice was anesthetized by an overdose of chloral hydrate and were then quickly perfused intra-cardially with chilled PBS (0.1 M, pH 7.3) followed by chilled 4% paraformaldehyde (PFA) for 10 min each. Brains were subsequently removed from the skull and post-fixed for 24 h in PFA at 4°C. After fixation, samples were placed in PBS (0.1 M, pH 7.3) with 30% sucrose for cryoprotection. Brains were then stored in sucrose at 4 °C until slicing. Forty-micrometer sections were cut with a microtome. Sections were labeled with a polyclonal rabbit c-fos primary antibody (1:2500; SC-52 Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight, followed by a Cy3-conjugated peroxidase-conjugated anti-rabbit secondary antibody (1:2500; C2306, Sigma-Aldrich, St. Louis, MO, USA for 2 h at room temperature as free-floating sections in mesh wells. Three sections from the amygdala region (spaced out by 240 μm) were then mounted onto gelatin-coated slides, covered with mounting media (Vectashield, Vector Laboratories), and sealed with a cover slip (38). c-fos positive cells were counted in both amygdalae of each of the best two slices per mouse with a Nikon Apotome microscope. Results are expressed as the number of c-fos positive in each amygdala on each slice ± SEM.

Statistical analysis

For comparison of the mean values between groups, statistical evaluation was performed using analysis of variance (ANOVA) using SPSS 16 (SPSS Inc., Chicago, IL, USA). The factor used for analysis of chronic CsA injections was drug vs. vehicle and for viral-mediated shRNA expression the factor used was AAV-GFP vs. AAV-shCnA. P values < 0.05 were considered statistically significant. All data are presented as means ± standard error of the mean (SEM). N refers to the number of statistical cells in each group.

Results

Systemic CsA exposure induces anxiety- and depression-like behaviors but does not affect locomotor activity

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 (anxiety-like behavior), forced swim test and tail suspension test (depression-like behavior).

In the elevated plus maze, systemic injection of CsA significantly decreased the time spent in the open arms ($F(1,28) = 253.943$; $p < 0.001$; Fig. 1A) and the number of entries ($F(1,28) = 165.526$; $p < 0.001$; Fig. 1B) while increasing the time spent in closed arms (data not shown). In the light/dark test, CsA-treated mice spent more time in the dark compartment compared to vehicle-treated mice ($F(1, 28) = 180.015$; $p < 0.001$; Fig. 1C). The number of entries into the light compartment was also decreased in CsA-treated mice compared to vehicle injection ($F(1,28) = 419.154$; $p < 0.001$; Fig. 1D). The latency time was not significantly different between control and CsA-injected mice (Fig. S1A) ($F(1,28) = 0.049$; $p = 0.826$).

In the forced swim and tail suspension tests, CsA-injected mice showed a significant increase in immobility time (forced swim: $F(1,28) = 328.031$; $p < 0.001$; tail suspension: $F(1,28) = 116.876$; $p < 0.001$; Fig. 2A,B). No differences were seen in overall locomotor activity in a novel environment ($F(1,28) = 0.524$; $p = 0.475$; Fig. 2C), suggesting that peripheral CsA administration did not result in locomotor activity changes that would account for the results observed in tests of anxiety- and depression-like behavior.

Peripheral CsA administration inhibits brain calcineurin phosphatase activity but not mRNA expression

Following behavioral testing, calcineurin A mRNA levels were measured in the brain. Consistent with a purely pharmacological effect on calcineurin activity, calcineurin A, GAPDH and beta-actin mRNAs were unchanged as a result of chronic CsA infusion (calcineurin A: $F(1,10) = 0.769$, $P = 0.414$; GAPDH: $F(1,10) = 0.275$, $P = 0.619$; beta-actin: $F(1,10) = 0.031$, $P = 0.866$; Fig. 3A). Although there were no effects on calcineurin mRNA levels, chronic, systemic CsA injection inhibited calcineurin activity in all brain regions examined as measured by the dephosphorylation of the RII peptide substrate (BLA: $F(1,10) = 571.914$; $P < 0.001$, VTA: $F(1,10) = 320.761$; $P < 0.001$; NAc $F(1,10) = 765.445$; $P < 0.001$; Fig. 3B).

Local knockdown of calcineurin A levels in the brain

In order to determine whether the changes in anxiety- and depression like behavior following chronic, systemic CsA treatment resulted from a decrease in calcineurin activity in a specific brain area, we used adeno-associated virus (AAV) to express short hairpin RNAs (shCnAs) targeting calcineurin A in the amygdala, VTA and NAc. RT-PCR revealed a significant reduction of calcineurin A mRNA levels in the amygdala of AAV-shCnA-injected mice as compared to AAV-GFP-injected animals ($F(1,10) = 72.516$, $P < 0.001$; Fig. 4A) with no change in GAPDH and beta-actin transcripts ($F(1,10) = 0.377$, $P = 0.556$; $F(1,10) = 0.081$, $P = 0.783$, respectively). Consistent with a very local knockdown, following AAV-shCnA injection into the amygdala, calcineurin activity was also significantly reduced only in the amygdala ($F(1,10) = 562.888$; $P < 0.001$) (Fig. 4B) but not the VTA ($F(1,10) = 1.114$; $P = 0.351$) or the NAc ($F(1,10) = 0.038$; $P = 0.855$) in the same animals.

AAV-shCnA-mediated knockdown of calcineurin A in the amygdala increases anxiety- and depression-related behaviors in mice

Mice that had received AAV-GFP or AAV-shCnA infusions into the amygdala, VTA or NAc were tested in the same behavioral paradigms used to assess anxiety- and depression-like behavior in CsA-treated mice. A significant difference in the time spent in the open arms and the total number of arm entries ($F(1,24) = 27.82$, $P < 0.001$; $F(1,24) = 121.882$, $P < 0.001$ respectively; Fig. 5A,B) and the number of open arm entries ($F(1,24) = 9.965$, $P = 0.006$; data not shown) was observed in the mice that received AAV-shCnA infusions in the amygdala compared to mice with AAV-GFP infusion into the same brain area. In contrast, no difference was observed when mice were injected either into the VTA or the NAc (VTA: $F(1,22) = 0.532$; $P = 0.473$; $F(1,22) = 0.043$; $P = 0.838$ in the VTA; NAc: $F(1,22) = 2.140$; $P = 0.158$; $F(1,22) = 0.800$; $P = 0.381$).

In the light/dark test, amygdala AAV-shCnA-injected mice had fewer entries into the light chamber ($F(1,24) = 68.727$, $P < 0.001$; Fig. 5B) and greater time spent in the dark chamber ($F(1,24) = 91.424$, $P < 0.001$; Fig. 5B) compared to GFP mice. Injection of AAV-shCnAs or AAV-GFP into the VTA or the NAc had no effect on behavior in the light/dark test (VTA: $F(1,22) = 0.161$; $P = 0.692$; $F(1,22) = 0.255$; $P = 0.523$; NAc: $F(1,22) = 0.192$; $P = 0.366$; $F(1,22) = 0.482$; $P = 0.217$). Viral expression of shCnA in the 3 different brain regions had no effect on the latency time (Fig. S1B) (amygdala: $F(1,24) = 1.693$; $P = 0.210$; VTA: $F(1,22) = 1.516$; $P = 0.231$; NAc: $F(1,22) = 0.536$; $P = 0.472$).

In the forced swim test, stereotaxic injection of AAV-shCnA significantly increased the duration of immobility ($F(1,24) = 62.350$, $P < 0.001$; Fig. 6A) as compared to amygdala AAV-GFP injected mice. In contrast, when calcineurin expression was knocked down in the VTA or the NAc no significant differences were observed between GFP and shCnA expressing animals (VTA: $F(1,22) = 2.381$; $P = 0.116$; NAc: $F(1,22) = 0.223$; $P = 0.642$).

In the tail suspension test, knockdown of calcineurin A in the amygdala increased immobility compared to AAV-GFP injected mice ($F(1,24) = 43.124$, $P < 0.001$). Injection of AAV-shCnAs or AAV-GFP into either the VTA or the NAc did not alter immobility in the tail suspension test (VTA: $F(1,22) = 1.474$; $P = 0.251$; NAc: $F(1,22) = 0.639$; $P = 0.433$)

No change in overall locomotor activity was observed following knockdown of calcineurin A in the amygdala ($F(1,24) = 2.260$; $P = 0.150$), VTA ($F(1,22) = 0.961$) or NAc $P = 0.338$ and $F(1,22) = 0.092$; $P = 0.764$, Fig. 6C), suggesting that changes in behavior in the anxiety- and depression-related tests were not related to a non-specific change in overall locomotor activity.

The localization of CnA knockdown was verified following behavioral testing using either real-time RT PCR or measurement of calcineurin activity in punches of the BLA. Two mice injected with AAV-shCnA showed no decrease in calcineurin activity in amygdala punches as measured by RT-PCR compared to AAV-GFP-injected animals (Fig. S2A). As a result, we removed the behavioral data for these animals and analyzed their behavior separately. In accord with the lack of change in amygdala calcineurin A there were no behavioral changes observed in these 2 mice as compared to AAV-GFP injected mice in the elevated plus maze (Fig. S2B), the light/dark test (Fig. S2C,D), the forced swim and tail suspension tests (Fig. S2E,F) or the open field (Fig. S2G). These data are further demonstration of the specific role of amygdala calcineurin activity in the increase in anxiety- and depression-related behaviors.

Local calcineurin knock-down in amygdala increases stress-induced c-fos expression

In order to determine whether there were likely to be changes in neuronal activity in the amygdala following calcineurin knockdown in the amygdala, c-fos was measured as an index of neuronal activation in mice that had been injected with either AAV-shCnAs or AAV-GFP. Acute 15 min swim stress significantly increased c-fos expression in the amygdala of mice previously infused with AAV-shRNAs targeting calcineurin A into this brain area (~ 30%; $F(1, 42) = 13.3$, $p = 0.0007$; Fig. 7). Conversely, only a small increase in c-fos expression was observed in the amygdala of mice infused with AAV-GFP ($F(1, 30) = 3.19$, $p = 0.083$). No gross morphological differences were observed between neurons infected with AAV-GFP or AAV-shCnA.

Discussion

These studies demonstrate that chronic, peripheral administration of CsA decreases calcineurin activity in the brain resulting in increased anxiety- and depression-like behaviors in a battery of behavioral tests in mice. Similar phenotypes are seen following local knockdown of calcineurin activity in the amygdala, but not the VTA or NAc, by viral-mediated delivery of

shRNAs targeting calcineurin A. These behavioral changes could be mediated through increased neuronal firing, since acute swim stress resulted in a significant increase in c-fos activity in the amygdala of AAV-shCnA infused mice as compared to control mice. Systemic injection of CsA also decreased calcineurin activity in the prefrontal cortex (Fig. S3); however, this change in prefrontal calcineurin activity did not appear to alter behavioral changes mediated through decreased calcineurin activity in the amygdala. Taken together, these results suggest that calcineurin is necessary for maintaining normal activation patterns in amygdala neurons, and that decreased calcineurin activity results in amygdalar hyperactivity that is not compensated by prefrontal hyperactivity, leading to increased emotional behaviors.

Patients treated with CsA to prevent rejection after organ transplantation display increased emotional problems including depression and anxiety (3,4,40-42). While invasive surgery and homeostatic perturbation, as well as the stress of serious illness, are likely to play a pivotal role in mood, treatment with the calcineurin inhibitor CsA may also alter mood. The current study suggests that both enzymatic blockade of calcineurin, and interfering with calcineurin mRNA expression in the amygdala genetically, promote anxiety- and depression-like behaviors, strongly suggesting that CsA treatment in transplant patients contributes to increased mood disorder symptoms seen in these patients. Previous studies have suggested that increased calcineurin activity results in greater sensitivity to classical antidepressants (43). In addition, polymorphisms in the gene encoding the catalytic subunit of calcineurin have been associated with a greater risk of bipolar disorder (44). Furthermore, calcineurin inhibition with either CsA or specific shRNA-expressing AAVs increase ERK phosphorylation (Fig. 4S), suggesting that CnA plays a pivotal role in the balance between phosphatase and kinase activities in the amygdala. Taken together, these studies suggest that calcineurin activity in the amygdala is essential for normal mood regulation.

In summary, calcineurin activity likely regulates neuronal firing and overall activity of the amygdala. Blockade of calcineurin, either pharmacologically in transplant patients, or genetically, appears to result in an increase in the symptoms of affective disorders. Therefore, 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 mood disorders in organ transplant patients. In addition, recognition of the increased risk of affective disorders in patients treated with CsA and increased psychiatric support is likely to improve health outcomes in these patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AAV, adeno-associated virus
AID, auto inhibitory domain
BLA, basolateral amygdala
CaM, calmodulin
CnA, calcineurin A

CREB, cAMP response element binding protein
CsA, Cyclosporin-A
HEK, human embryonic kidney
NAc, nucleus accumbens
PP2B, protein phosphatase 2B
shCnA, short hairpin RNA targeting calcineurin A
VTA, ventral tegmental area

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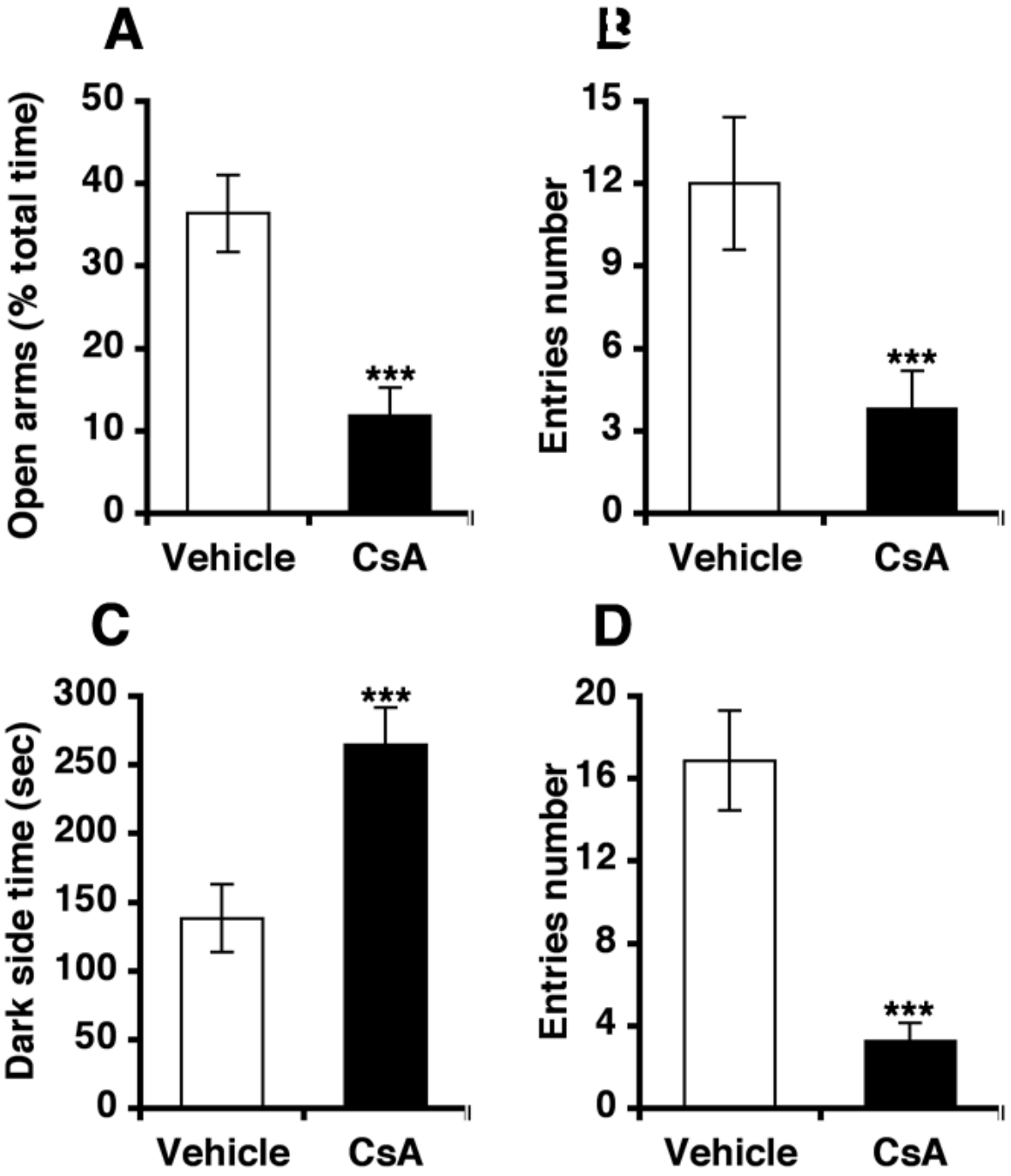

Figure 1. Anxiety-like behavior in mice treated chronically with CsA

A) Percent time spent in and B) number of entries into open arms in the elevated plus maze test. C) Time spent in dark side and D) number of entries into the dark side in the light/dark test. Data are expressed as mean ± SEM, N = 12-15 per group. *** $p<0.001$.

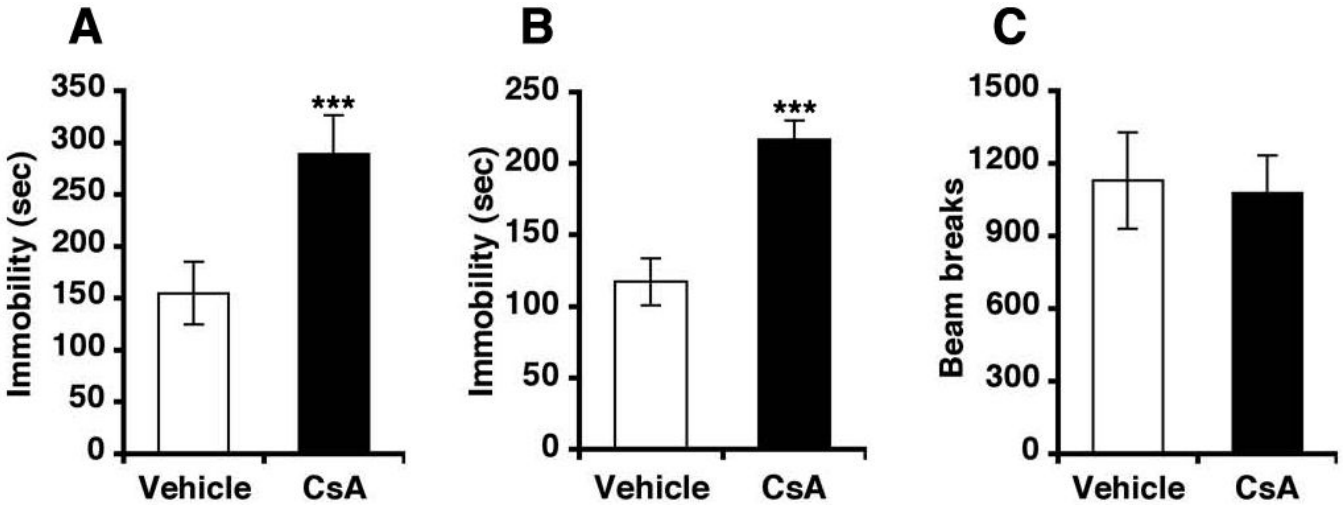

Figure 2. Depression-like behavior in mice treated chronically with CsA

Immobility time in the A) forced swim and B) tail suspension tests. C) Distance traveled in a novel environment as measured by beam breaks. Data are expressed as mean ± SEM, N = 12-15 per group. *** $p<0.001$

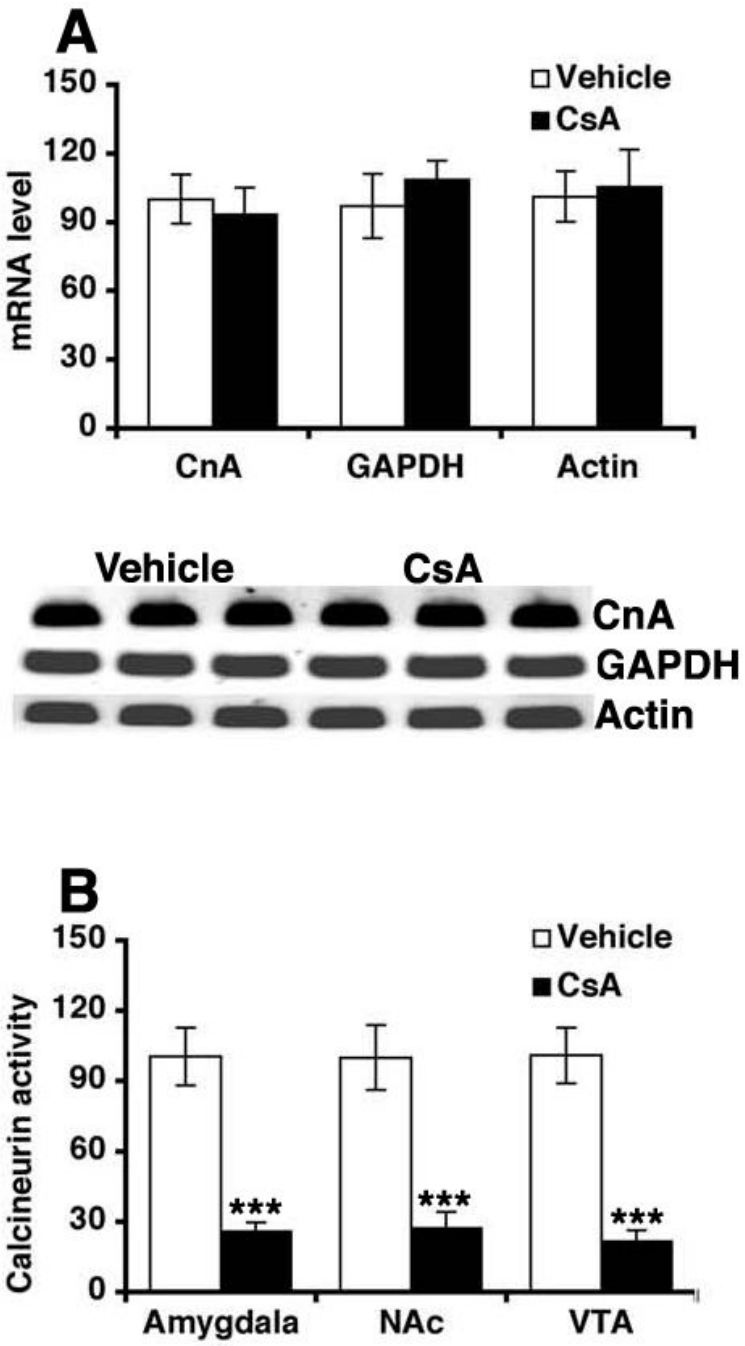

Figure 3. Calcineurin A mRNA levels and activity in the amygdala, nucleus accumbens (17) and ventral tegmental area (VTA) of mice treated chronically with CsA

A) RT-PCR analysis of the mRNA encoding calcineurin, GAPDH and β-actin in the amygdala. B) Calcineurin activity in brain extracts from amygdala, VTA and NAc. Data are expressed as mean ± SEM, N = 9 per group. *** $p<0.001$.

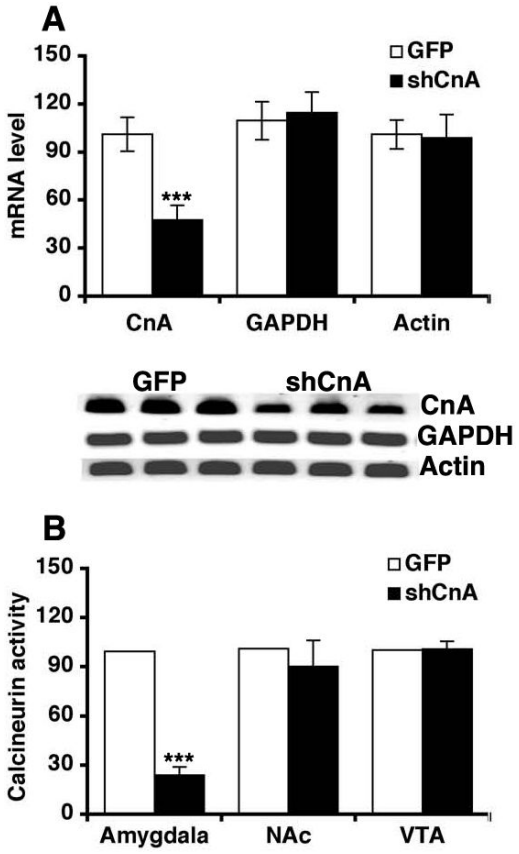

Figure 4. Calcineurin A mRNA levels and activity in the amygdala following local knockdown of calcineurin in the amygdala, nucleus accumbens (17) or ventral tegmental area (VTA)

A) RT-PCR analysis of calcineurin, GAPDH and β-actin transcripts in the amygdala after stereotaxic injection of AAV-shCnA or AAV-GFP into amygdala, NAc or VTA. B) Calcineurin activity in brain extracts from amygdala, NAc and VTA following stereotaxic injection with AAV-shRNAs or AAV-GFP into the amygdala. Data are expressed as mean ± SEM, N = 9 per group. *** $p<0.001$.

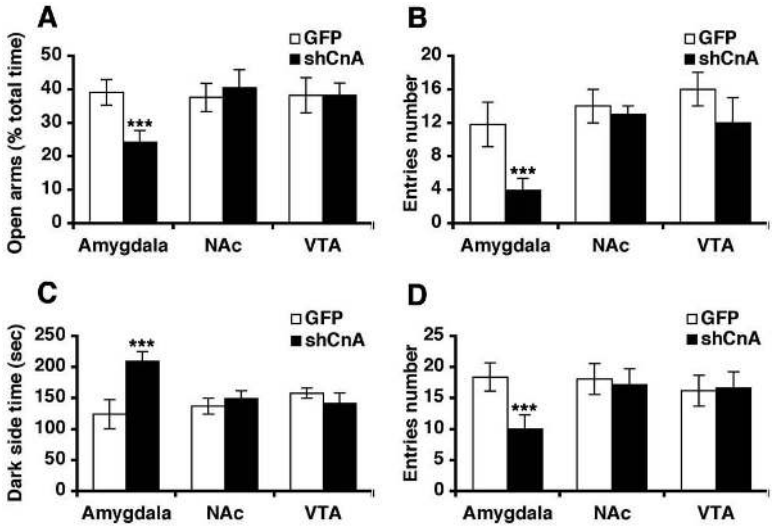

Figure 5. Anxiety-like behavior following local knockdown of calcineurin A in the amygdala, nucleus accumbens (17) or ventral tegmental area (VTA)

A) Time spent and B) number of entries into open arms in the elevated plus maze test. C) Time spent in dark side and D) number of entries into the dark side in the light/dark test for groups of mice with local knockdown of calcineurin in the amygdala, NAc or VTA. Data are expressed as mean ± SEM, N = 12-15 per group. *** $p<0.001$.

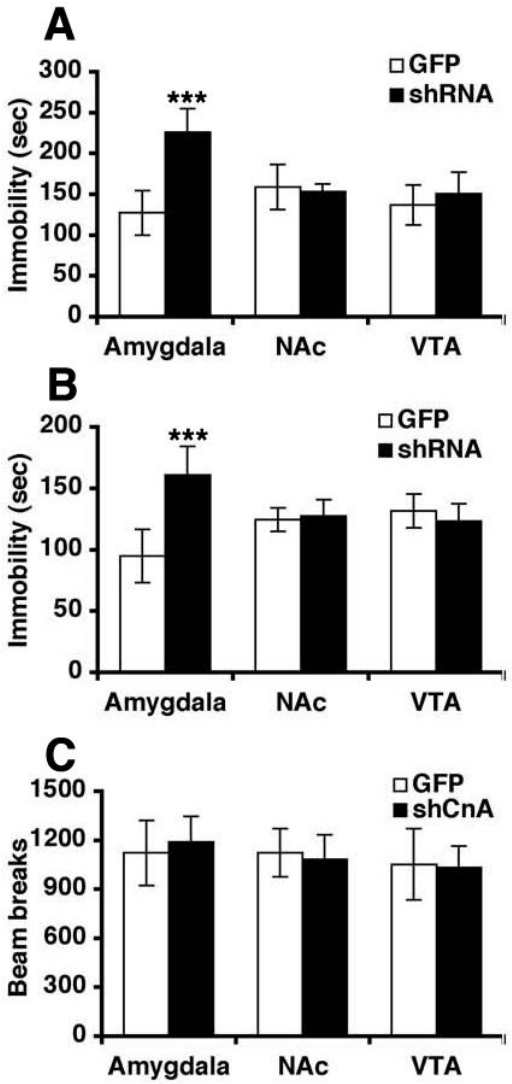

Figure 6. Depression-like behavior following local knockdown of calcineurin A in the amygdala, nucleus accumbens (17) or ventral tegmental area (VTA)

Immobility time in the A) forced swim and B) tail suspension tests for groups of mice with local knockdown of calcineurin in the amygdala, NAc or VTA. C) Distance traveled in a novel environment as measured by beam breaks in each group. Data are expressed as mean ± SEM, N = 12-15 per group. *** $p<0.001$.

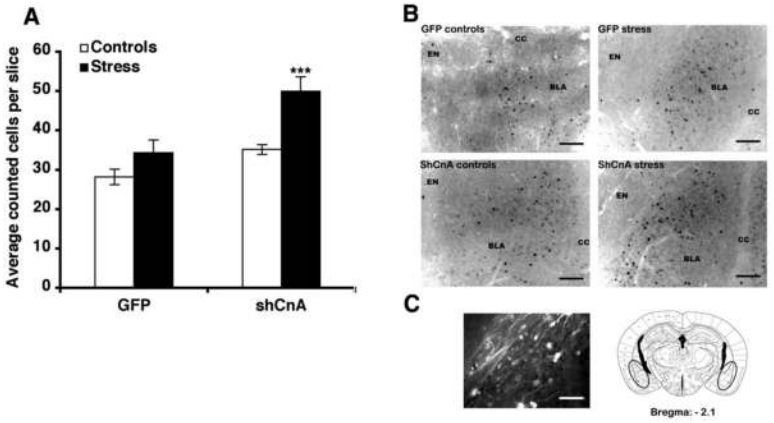

Figure 7. c-fos immunoreactivity in the BLA in response to an acute swim stress following local knockdown of calcineurin A in the amygdala

A) Average number of c-fos-positive cells per amygdala in mice injected with AAV-GFP or AAV-shCnA following an acute swim stress or no stress (Stress or Controls). Data are expressed as mean ± SEM. N = 12 to 24 per group. *** $p<0.001$. B) Representative photomicrographs of amygdalar sections stained for c-fos. CC: corpus callosum; BLA: basolateral amygdala; EN: endopiriform nucleus. C) Left: Microphotograph of GFP-positive neurons after viral infection in the amygdala; Right: mouse brain diagram from Franklin and Paxinos (45) indicating sites of viral injections (black ovals). Scale bar = 50 μm.

Table 1

Time-table of the experiments

Days	CsA/vehicle groups	AAV-shCnA/GFP groups	Days
1-15	**Injection of CsA or vehicle**	**Stereotaxic surgery and recovery**	**1-16***
16	Elevated plus maze		17
18	Light/dark test		19
20	Open field locomotor activity		21
22	Tail suspension test		23
24	Forced swim test		25
24	Mice were sacrificed by rapid decapitation 90 min after the last forced swim test for RT-PCR, enzymatic activity and immunohistochemistry		25

* stereotaxic injections were performed over 2 days and mice recovered for at least 15 days.