RCAN1 Mediates Anxiety-related Behaviors in part through Regulation of CREB1 Signaling

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Abstract

cAMP response element binding protein 1 (CREB1) is a transcription factor with known links to anxiety. Previously, we reported that regulator of calcineurin 1 (RCAN1) mediates anxiety-related behaviors through the control of the calcium-dependent phosphatase calcineurin (CaN). CREB1 is a downstream target of CaN activity. In *Rcan1* knockout (KO) mice, expression of anxiety-like behaviors is reduced and associated with increased CREB1 activation across several brain regions involved in anxiety. These findings suggest a signaling pathway from RCAN1 through CaN to CREB activity that mediates anxiety. To test this idea, we reduced CREB1 activity in *Rcan1* KO mice using Cre-mediated removal of *Creb1* from the brain and examined the effect on anxiety-related behaviors. Although the fraction of activated CREB1, indicated by phosphorylation of CREB1 at serine 133 (pCREB1), increased with *Creb1* loss, we found that absolute levels of pCREB1 are reduced. Furthermore, we confirmed that conditional *Creb1* removal reverses the elevated pCREB1 levels observed in the brains of *Rcan1* KO mice. Finally, we found that conditional *Creb1* deletion rescues the anxiolytic-like phenotype of *Rcan1* KO mice in the open field arena test of rodent anxiety-related behavior but not in the elevated plus maze test. These results support the idea that RCAN1 acts through CREB1 activity, but other RCAN1-regulated pathways are also involved in mediating anxiety-related behaviors.

Introduction

Anxiety is characterized as an emotion that reflects intense worry and activates a stress response in the body. Although this stress response is normal, overexpression of this emotion can lead to an inability to cope with daily tasks and living. Human anxiety disorders reflect a constant feeling of worry which can become pathological anxiety, one of the most prevalent comorbidities of psychiatric illnesses (Hoeffer et al. 2013; Duman and Duman, 2005). To study underlying molecular components of anxiety, genetic manipulations have been used to alter and observe the anxiety-related behaviors in rodents (Gould, 2009). Normal mice tend to avoid exposed areas, such as the center of an open field arena (OFA) or the open arms of an elevated plus maze (EPM) and rely on hiding within unexposed areas. This avoidance of exposure in terms of duration and frequency is used as a measure of anxiety in these animals.

Although the molecular mechanisms and pathways of anxiety are not entirely understood, calcineurin (CaN) is a calcium-dependent serine/threonine phosphatase that has been implicated in human anxiety disorders (Manji et al., 2003; Bahi et al., 2009; Baumgartel and Mansuy, 2012). We previously showed that regulator of calcineurin 1 (RCAN1), which functions as a facilitator and inhibitor of CaN activity depending on the cellular context (Kingsbury and Cunningham, 2000; Vega et al., 2002; Hilioti et al., 2004; Sanna et al.,2006; Liu et al., 2009; Rothermel et al., 2000; Martinez-Martinez et al., 2009), also mediates emotional states and may be a potential therapeutic target for anxiety disorders (Hoeffer et al., 2013). Using *Rcan1* knockout (KO) mice, we found that RCAN1 loss resulted in anxiolytic-like behaviors. These phenotypes were associated with increased activation of cAMP response element-binding protein 1 (CREB1) throughout the brain of *Rcan1* KO mice (Hoeffer et al., 2013). CREB is a transcription factor that also has been implicated in anxiety (Wallace et al., 2009). It is regulated by reversible phosphorylation at serine-133 (S133) by kinases and phosphatases, including CaN (Schwaninger et al., 1995). Phosphorylation of CREB1 at this site enhances its transcriptional activity (Naqvi et al., 2014). In the absence of RCAN1, S133-phosphorylated CREB (pCREB1) levels were increased, in the brain of *Rcan1* KO mice (Hoeffer et al., 2013). Based on these findings, we proposed that RCAN1 mediates anxiety-related behaviors through CREB1 activity. To test this idea, the current study examined the effects of reducing CREB1 activity on anxiety-related phenotypes in *Rcan1* KO mice using conditional *Creb1* deletion from the brain.

We found that *Creb1* deletion specifically from forebrain excitatory neurons late in development leads to a compensatory increase in the pool of CREB1 protein that is phosphorylated but overall pCREB1 levels are reduced. CREB1 loss this way did not alter the display of anxiety-related behavior in mice. In the background of *Rcan1* deficiency, reducing CREB1 levels with this approach reversed the increase in CREB1 phosphorylation observed throughout different brain regions of *Rcan1* KO mice. Additionally, conditional *Creb1* deletion rescued the anxiolytic-like phenotype of *Rcan1* KO mice in the OFA test of rodent anxiety-related behavior but not in the EPM test. These findings support the idea that RCAN1 acts through CREB1 activity to mediate at least some anxiety-related behaviors but also suggest other RCAN1-regulated signaling pathways are involved in the display of anxiety-related behavior under different contexts.

Methods

Animals

Mice with null *Rcan1* alleles (Vega et al., 2003) and floxed (fl) *Creb1* alleles (Mantamadiotis et al., 2002) were generated as previously described and backcrossed on a C57BL/J6 background.

We used the T29-1 *Camk2a::Cre* (*Cre*) driver line (Tsien et al., 1996) to produce mice with conditional *Creb1* removal from forebrain excitatory neurons. Crossing *Creb1*$^{fl/+}$ males with *Cre; Creb1*$^{fl/+}$ females, we obtained experimental mice with Cre-mediated deletion of one (*Creb1* cHET) or both (*Creb1* cKO) floxed *Creb1* alleles. Littermates carrying wild-type (WT) *Creb1* alleles with and without Cre or carrying floxed *Creb1* alleles without Cre were used as controls and referred to as "WT". To obtain *Rcan1* KO/*Creb1* cKO experimental mice, which have both *Rcan1* knockout and conditional *Creb1* knockout (*Cre; Creb1*$^{fl/fl}$*; Rcan1*$^{-/-}$), we crossed male *Creb*$^{fl/fl}$; *Rcan1*$^{+/-}$ mice with *Cre; Creb*$^{fl/fl}$*; Rcan1*$^{+/-}$ females. Littermates lacking Cre and carrying WT or null *Rcan1* alleles were used for WT and *Rcan1* KO groups, respectively, in behavioral and immunoblotting studies with *Rcan1* KO/*Creb1* cKO mice. *Creb1* cKO littermates from this cross also were used in the behavioral studies. All experiments were performed using both sexes of each genotype at 3-6 months old over multiple independent cohorts. Mice were group-housed in the same facility and maintained on a 12:12 h light:dark schedule with food and water available *ad libitum*. All procedures were approved by the University of Colorado Boulder's Institutional Animal Care and Use Committee and complied with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

Open Field Arena (OFA)

Mice were acclimated to the testing room in individual holding cages for 1 h minimum prior to OFA behavior assessment. 55-dB white noise was present in the background for their entire duration in the room. After acclimation, mice were placed in the center of a white Plexiglas square arena (40 x 40 cm^2) with 195-lux overhead lighting and allowed to explore for 10 min.

Their arena activity was recorded and analyzed using the Ethovision-XT video tracking system (Noldus). The center zone was defined as the 20 x 20 cm^2 area at the center of the arena.

Elevated Plus Maze (EPM)

Mice were returned the following day to the testing room for EPM behavior assessment under similar experimental conditions to OFA. After a 1-h minimum acclimation period, mice were placed in the center of a white Plexiglas EPM arena (30-cm arm length) and allowed to explore for 5 min. Arena activity was recorded and analyzed with Ethoivison-XT (Noldus).

Immunoblotting

Tissue from different brain regions were isolated and homogenized in ice-cold lysis buffer containing 10 mM HEPES pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 10 mM $Na_4P_2O_7$, 1X protease inhibitor cocktail (Sigma), and 1X phosphatase inhibitor cocktails II and III (Sigma). Lysates were prepared in reducing Laemmli sample buffer for SDS-polyacrylamide gel electrophoresis to resolve proteins and blotted on PVDF membranes using standard techniques. Membranes were blocked and then incubated for 24 h minimum at 4 °C with primary antibodies against phospho-CREB1 S133 (1:1000; PhosphoSolutions), total CREB1 (1:750; Bethyl Laboratories), and β-tubulin (1:20000; Cell Signaling Technology) in Tris-buffered saline with 0.1% Tween-20 and 0.2% I-Block. Primaries were detected with HRP-conjugated goat anti-rabbit or anti-mouse secondary antibodies (1:5000-1:10000; Promega) diluted in the I-Block solution and visualized with ECL (GE Healthcare) on the ProteinSimple FluorChem E imaging system. Signals were obtained in the linear range for each antibody and quantified by densitometry using ImageQuant software (GE Healthcare).

Statistics

Data were statistically evaluated and presented as mean values ± SEM using GraphPad Prism software. One-way ANOVA was applied to test differences among the genotypes after outliers were excluded using the Grubbs' test. Initially, two-way ANOVA was applied with genotype and sex as between-subjects factors, but no main effects of or interactions with sex were detected, so data were collapsed by sex for all analyses. All statistical tests were two-tailed with $p<.05$ considered significant and followed by Tukey's multiple comparisons *post hoc* testing for significant effects.

Results

CREB1 activity is reduced with conditional removal of *Creb1* in different brain regions

We previously found enhanced CREB1 activation throughout different brain regions associated with reduced anxiety-related behavior in *Rcan1* KO mice (Hoeffer et al. 2013). To test if RCAN1 mediates the display of anxiety through CREB1 signaling, we took a genetic approach to reduce CREB1 activity in *Rcan1* KO mice. We used Cre-mediated deletion of *Creb1* from the brain by crossing mice with floxed *Creb1* alleles (Mantamadiotis et al., 2002) to the T29-1 *Cre* driver line (Tsien et al., 1996), which is under the control of a *Camk2a* promoter that is activated specifically in forebrain excitatory neurons late in development (Hoeffer et al., 2008). To verify that CREB1 activity is reduced with this approach, we first examined CREB1 levels and activation in the brain of conditional *Creb1* mutant mice carrying one (*Creb1* cHET) or two (*Creb1* cKO) floxed *Creb1* alleles that have been deleted by the *Camk2a* promoter-driven Cre (Fig. 1A). Protein lysates of the prefrontal cortex (PFC), nucleus accumbens (NAc), amygdala (amy), and hippocampus (HPC), brain regions involved in human and rodent anxiety (Daviu et al., 2019), were prepared from WT, *Creb1* cHET, and *Creb1* cKO littermates for western blot analysis (Fig. 1B). We confirmed significant effects of genotype on CREB1 levels in these brain regions (Fig. 1C; PFC $F(2,17)=40.31$, $p<.0001$; NAc $F(2,17)=3.796$, $p=.0434$; amy $F(2, 15)=15.88$, $p=.0002$; HPC $F(2,15)=7.252$, $p=.0063$). *Creb1* cKO mice exhibited decreased CREB1 levels in all brain regions examined (PFC $p<.0001$; NAc $p=.0345$; amy $p=.0001$; HPC $p=.0058$) while *Creb1* cHET mice only showed reduced CREB1 levels in the PFC ($p=.0072$) and amy ($p=.0479$) compared with WT littermates (Fig. 1C).

When we examined CREB1 activation levels by measuring phosphorylation of CREB1 at serine 133 (pCREB1 S133) (Fig. 1D) (Johannessen et al., 2004), we also found significant effects of genotype on the fraction of total CREB1 that is phosphorylated at this site in the PFC ($F(2,17)=34.78$, $p<.0001$) and HPC ($F(2.15)=4.873$, $p=.0234$). *Post hoc* analyses revealed that pCREB1/CREB1 levels remained the same in *Creb1* cHET mice but were upregulated in *Creb1* cKO mice in the PFC ($p<.0001$) and HPC ($p=.0477$) compared with WT controls. These results suggest that the fraction of S133-phosphorylated CREB1 is increased in the PFC and HPC to compensate for the reduced CREB1 levels in *Creb1* cKO mice.

When we examined pCREB1 levels overall, however, by comparing pCREB1 to levels of the housekeeping protein β-tubulin (Fig. 1E), there were significant genotype effects in the PFC ($F(2,17)=8.678$, $p=.0025$), NAc ($F(2,17)=3.796$, $p=.0434$), and amy ($F(2,15)=12.50$, $p=.0006$). *Creb1* cKO mice had lower absolute levels of pCREB1 in these three brain regions compared with WT controls (PFC $p=.0057$; NAc $p=.0356$; amy $p=.0005$) whereas *Creb1* cHET mice only showed a significant decrease of pCREB1 in the PFC ($p=.0088$). The HPC did not show significant differences in overall pCREB1 levels among WT, *Creb1* cHET, and *Creb1* cKO mice (Fig. 1E).

Altogether, these findings indicate that when CREB1 levels are reduced in the brain, pCREB1 increases to compensate for the CREB1 loss; however, there is still less overall pCREB1 in most of the brain regions examined in conditional *Creb1* mutants. Importantly, these results establish that *Camk2a*-Cre removal of *Creb1* can be used to reduce CREB activity in brain regions involved in anxiety.

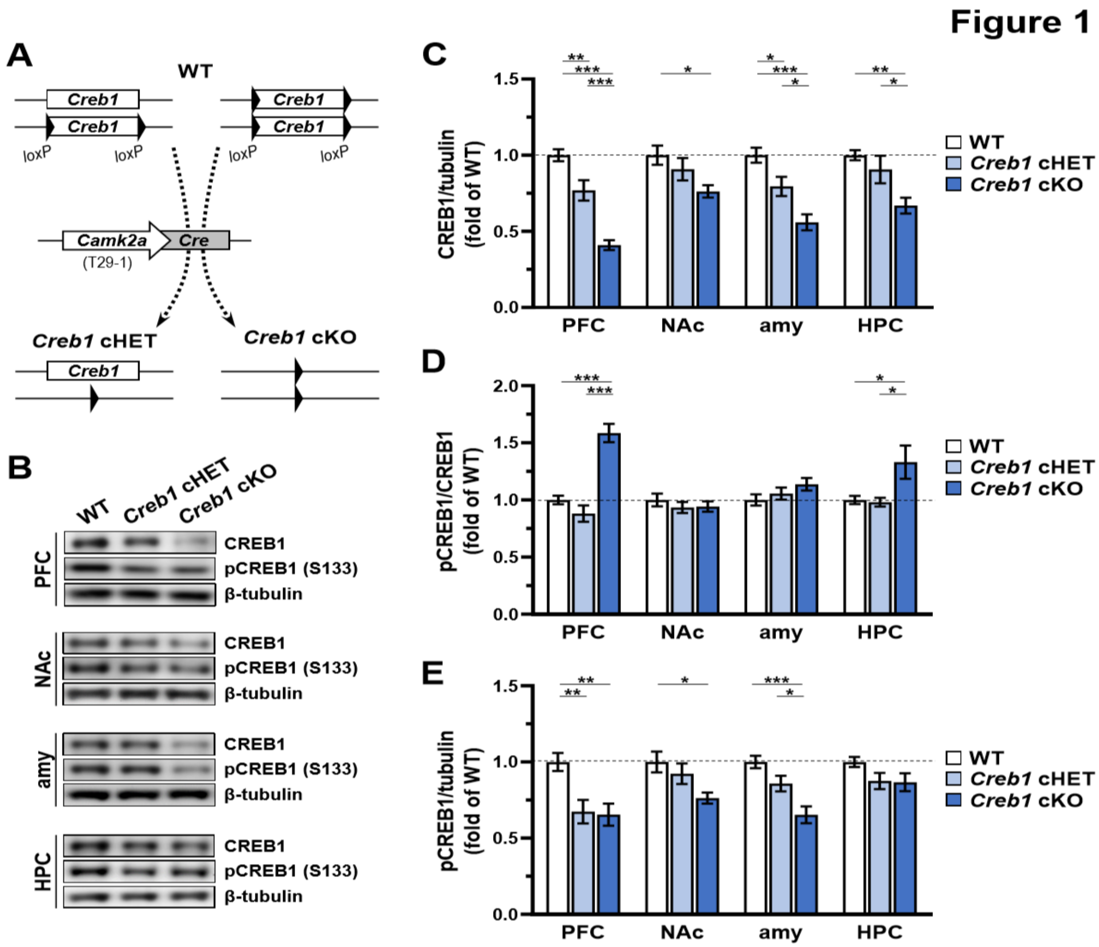

Figure 1. CREB1 activity is reduced in different brain regions with conditional removal of *Creb1*. **A.** Generation of conditional *Creb1* mutant mice carrying one (*Creb1* cHET) or two (*Creb1* cKO) floxed *Creb1* alleles that have been deleted by *Camk2α* promoter-driven Cre. **B.** Western blot analysis of CREB1 levels and activation in the PFC, NAc, amy, and HPC of WT, *Creb1* cHET, and *Creb1* cKO littermates. **C.** *Creb1* cKO mice displayed decreased CREB1 levels in all brain regions, and *Creb1* cHET mice displayed reduced CREB1 levels only in the PFC and amy compared with WT controls. β-tubulin, loading control **D.** Phosphorylation levels of CREB1 at S133 site. *Creb1* cKO mice showed a significant increase in pCREB1 levels in PFC and HPC compared to WT. pCREB1/CREB1 levels remain the same in *Creb1* cHET mice compared to WT. **E.** Examined overall pCREB1 levels by comparing to β-tubulin levels. *Creb1* cKO mice display decreased overall pCREB1 in PFC, NAc, and amy compared to WT. *Creb1* cHET showed decrease in pCREB1 in the PFC. HPC overall pCREB1 levels displayed no difference across all genotypes. *p<0.05, **p<0.01, ***p<0.001.

Conditional *Creb1* removal reverses the enhanced CREB1 phosphorylation observed in different brain regions of *Rcan1* KO mice

Because *Creb1* cKO mice showed the greatest reduction of CREB1 protein and activation levels, we used a breeding scheme that generated *Rcan1* KO mice carrying two floxed *Creb1* alleles to allow *Camk2a*-Cre deletion of *Creb1* with the most robust CREB1 reduction, producing *Rcan1* KO/*Creb1* cKO double mutants (Fig. 2A). To characterize the effect of conditional *Creb1* removal in the *Rcan1* KO background with this approach, we performed western blot analysis of CREB1 in the brains of WT, *Rcan1* KO, and *Rcan1* KO/*Creb1* cKO mice (Fig. 2B). We confirmed that genotype had a significant effect on total CREB1 levels in each brain region examined (Fig. 2C; PFC $F(2,33)=73.58$, $p<.0001$; NAc $F(2,33)=14.41$, $p<.0001$; amy $F(2,33)=19.49$, $p<.0001$; HPC $F(2,28)=36.67$, $p<.0001$), with *Rcan1* KO/*Creb1* cKO mice exhibiting lower CREB1 levels compared with WT (PFC $p<.0001$; NAc $p=.0003$; amy $p<.0001$; HPC $p<.0001$) and *Rcan1* KO mice (PFC $p<.0001$; NAc $p<.0001$; amy $p<.0001$; HPC $p<.0001$). There also was a significant effect of genotype on overall pCREB1 levels (pCREB1/β-tubulin) in each brain region examined (Fig. 2D; PFC $F(2,33)=37.74$, $p<.0001$; NAc $F(2,33)=11.43$, $p=.0002$; amy $F(2,33)=32.42$, $p<.0001$; HPC $F(2,28)=15.36$, $p<.0001$). Consistent with our previous findings (Hoeffer et al., 2013), pCREB1 levels were upregulated in *Rcan1* KO mice compared with WT controls (PFC $p=.0107$; NAc $p=.044$; amy $p=.0216$; HPC $p=.0162$). In *Rcan1* KO/*Creb1* cKO mice, pCREB1 levels were reduced relative to *Rcan1* KO mice (PFC $p<.0001$; NAc $p=.0001$; amy $p<.0001$; HPC $p<.0001$). Compared with WT controls, *Rcan1* KO/*Creb1* cKO mice also showed lower pCREB1 levels in the PFC ($p<.0001$), amy ($p<.0001$), and HPC ($p=.0302$), with a trend for reduced pCREB1 in the NAc ($p=.0748$). Taken together, these results indicate that conditional *Creb1* removal reversed the increased phosphorylation of CREB1 observed with *Rcan1* loss in brain regions involved in anxiety.

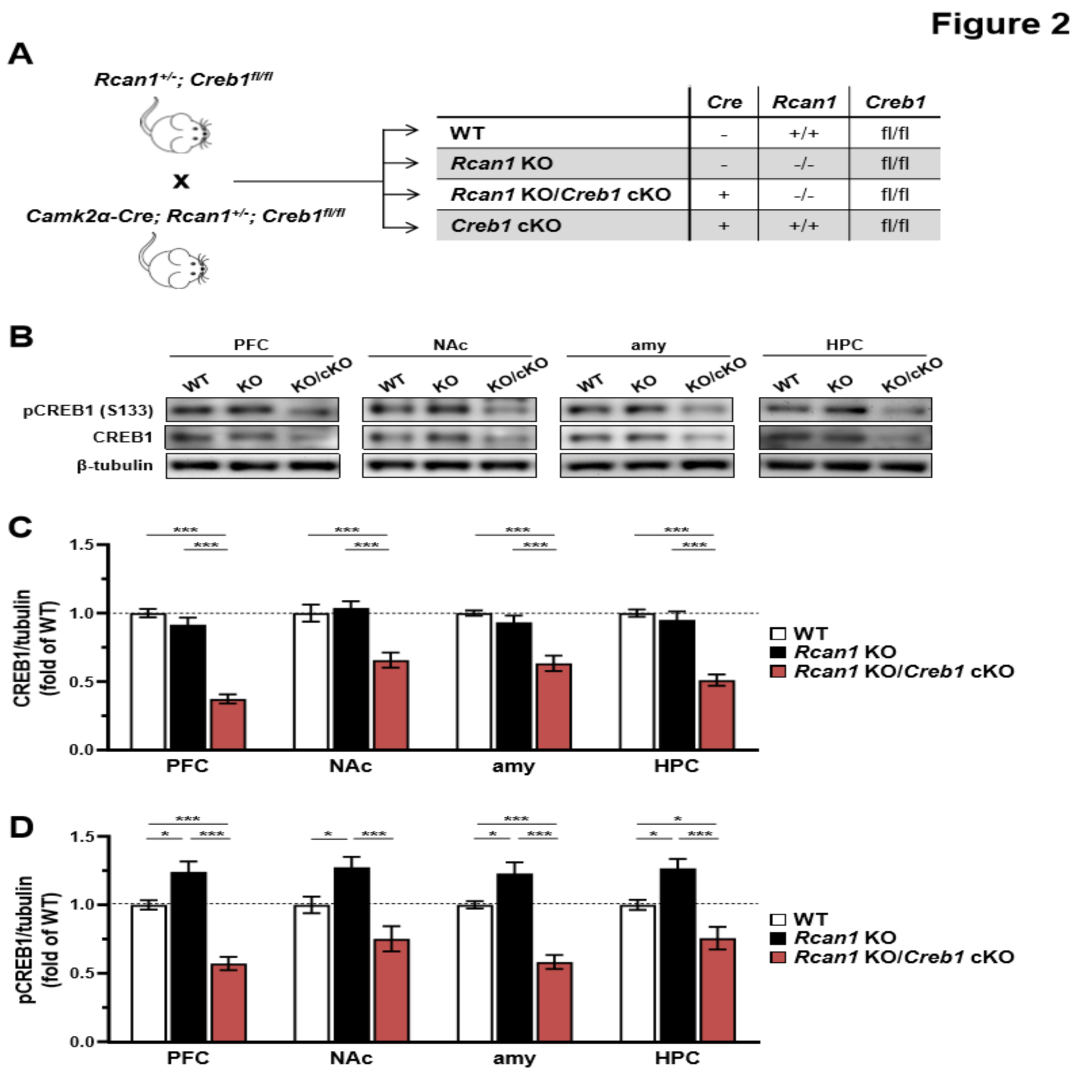

Figure 2. Conditional *Creb1* removal reverses enhanced pCREB1 in different brain regions of *Rcan1* KO mice. **A.** Generated *Rcan1* KO mice carrying two floxed *Creb1* alleles to allow *Camk2α-Cre* deletion of *Creb1*, producing *Rcan1* KO/*Creb1* cKO double mutants. **B.** Performed western blot analysis of CREB1 in the brains of WT, *Rcan1* KO, and *Rcan1* KO/*Creb1* cKO mice. **C**. *Rcan1* KO/*Creb1* cKO mice showed reduced CREB1 levels compared to WT and *Rcan1* KO. **D.** *Rcan1* KO mice exhibited increased pCREB1 levels compared with WT across all brain regions. *Rcan1* KO/*Creb1* cKO mice displayed reduced pCREB1 levels compared to Rcan1KO across all brain regions, and displayed lower levels compared to WT controls in PFC, amy, and HPC. *p<0.05, **p<0.01, ***p<0.001.

Conditional *Creb1* removal rescues the anxiolytic-like behavior of *Rcan1* KO mice in the OFA but not EPM

We next determined the effect of reducing CREB1 levels on anxiety-related phenotypes in *Rcan1* KO mice to test our hypothesis that RCAN1 acts through CREB1 signaling to mediate the display of anxiety. Similar to our previous study (Hoeffer et al., 2013), we used the open field arena (OFA) and elevated plus maze (EPM) assays, two behavioral tests that are regularly used to assess anxiety-like behavior in mice (Bailey and Crawley, 2009; Walf and Frye, 2007; Seibenhener and Wooten, 2015). When we tested WT, *Rcan1* KO, *Creb1* cKO, and *Rcan1* KO/*Creb1* cKO mice in the OFA (Fig. 3A), we found a significant effect of genotype on time spent in the center of the arena (Fig. 3B; $F(3,84)=4.419$, $p=.0062$). Consistent with our findings in the previous report, *Rcan1 KO* mice spent significantly more time in the OFA center compared with WT controls ($p=.0254$), indicating reduced anxiety-like behavior. *Rcan1* KO/*Creb1* cKO mice did not exhibit this increase in center time compared to WT controls ($p=.9163$), suggesting that CREB1 removal rescued anxiety-like behavior in *Rcan1* KO mice to the phenotype seen in WT mice. *Creb1* cKO mice also performed similarly to WT controls, indicating that CREB1 removal alone does not affect the display of anxiety-like behavior in the OFA. Although genotype had a significant effect on distance moved in the OFA (Fig. 3B; $F(3,84)=2.741$, $p=.0483$), we did not find significant differences between any genotype in *post hoc* comparisons. There was a trend for less distance moved with RCAN1 loss (*Rcan1* KO v. WT $p=.1607$; *Rcan1* KO/*Creb1* cKO v. WT $p=.1721$) but may not explain the increased time that *Rcan1* KO mice spend in the OFA center whereas *Rcan1* KO/*Creb1* cKO mice spent comparable time to WT controls.

To further examine anxiety-related behavior of these mice, the EPM test was conducted (Fig. 3C). We found a significant effect of genotype on time spent in the open arms of the EPM

(Fig. 3D; $F(3,84)=33.45$, $p<.0001$). In agreement with our OFA results (Fig. 3B) and previous study (Hoeffer et al., 2013), *Rcan1* KO mice spent more time in the open arms compared with WT controls ($p<.0001$), which indicates reduced anxiety-like behavior. Unlike the OFA results, however, conditional *Creb1* removal in *Rcan1* KO mice did not rescue the anxiolytic-like phenotype; *Rcan1* KO*/Creb1* cKO mice spent significantly more time in the open arms than both WT ($p<.0001$) and *Rcan1* KO mice (p.0006). *Creb1* cKO mice showed no difference in open arm occupancy time from WT controls, indicating that CREB1 removal alone does not affect the display of anxiety-like behavior in the EPM, similar to the OFA result. These effects were not due to differences in distance moved among the genotypes, although there was a trend for an effect of genotype ($F(3,85)=2.334$, $p=.0797$). Altogether, these findings suggest that RCAN1 regulates CREB1 signaling to affect anxiety-related behavior in the OFA but not the EPM.

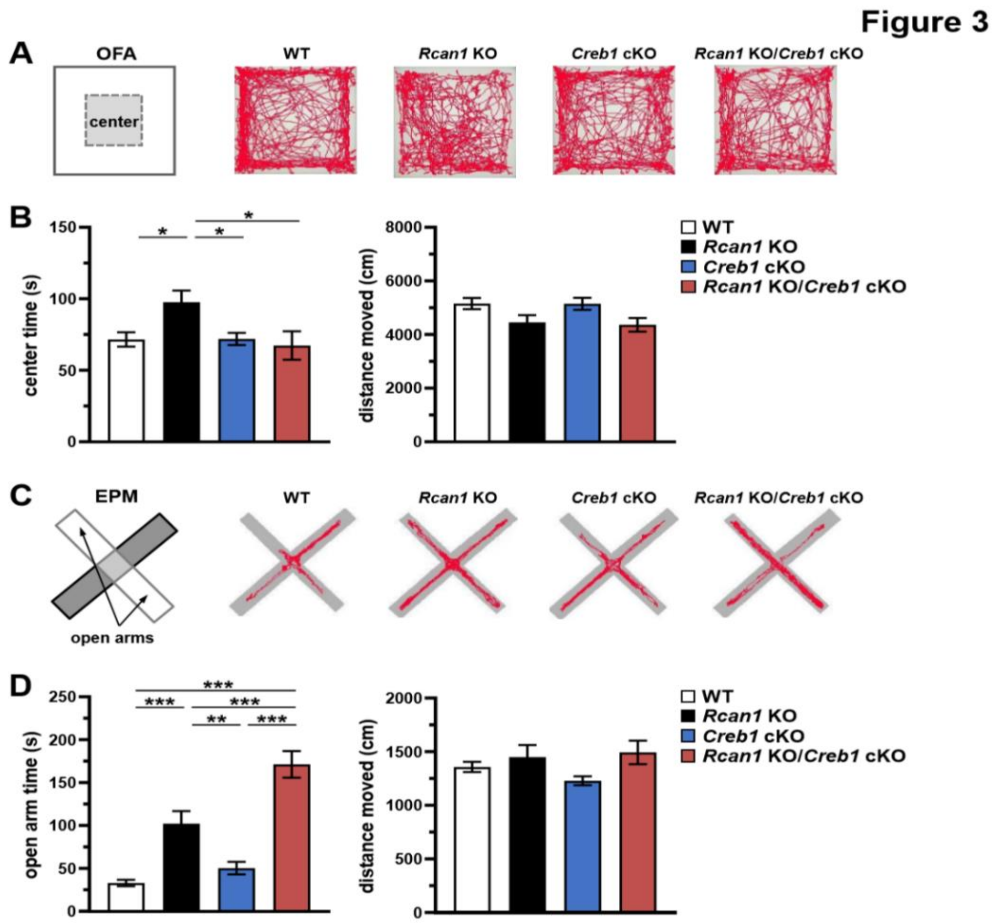

Figure 3. Conditional *Creb1* removal rescues anxiolytic-like behavior of *Rcan1* KO mice in OFA but not EPM. **A**. Representative activity tracks of WT, *Rcan1* KO, *Creb1* cKO, and *Rcan1* KO/*Creb1* cKO mice in the OFA. **B.** *Rcan1* KO mice spent more time in the OFA center compared to WT, while *Rcan1* KO/*Creb1* cKO mice displayed no difference from WT controls. *Creb1* cKO mice performed alike WT controls. There was no difference in distance moved between genotypes. **C.** Representative activity tracks of WT, *Rcan1* KO, *Creb1* cKO, and *Rcan1* KO/*Creb1* cKO mice in the EPM. **D.** *Rcan1* KO mice spent more time in the EPM open arms compared to WT controls while *Rcan1* KO/*Creb1* cKO mice spent significantly more time in the open arms compared to both *Rcan1* KO and WT. *Creb1* cKO mice showed no difference from WT controls. No difference found in distance moved between genotypes. *$p<0.05$, **$p<0.01$, ***$p<0.001$

Discussion

Using two behavioral paradigms for measuring unconditioned exploratory anxiety in rodents, we have shown that RCAN1 deficiency in mice leads to reduced anxiety-like behaviors. Our previous study found that this behavioral phenotype correlated with enhanced CREB1 activation throughout different brain regions of *Rcan1* KO mice (Hoeffer et al., 2013). In the current study, we formally tested if CREB1 activity plays a role in the anxiety-related behaviors of *Rcan1* KO mice. Using Cre-mediated removal of CREB1 specifically from forebrain excitatory neurons late in development, we observed a compensatory increase in the phosphorylated fraction of CREB1 but pCREB1 levels overall were decreased in brain regions associated with anxiety. We also found that reducing CREB1 levels with this approach rescued the anxiolytic-like phenotype of *Rcan1* KO mice in the OFA but not EPM. These findings suggest that RCAN1 regulates CREB1 activity to mediate some but not other anxiety-related behaviors.

The two behavior paradigms used for anxiety, OFA and EPM, possess differences that may attribute to different behavior results seen in the *Rcan1* KO/*Creb1* cKO mice. In OFA, *Rcan1* KO/*Creb1* cKO mice exhibited a rescued anxiety-like phenotype while in EPM they exhibited a reduced anxiety-like phenotype alike *Rcan1* KO mice. These differences in behavior may be due to the tests themselves. The stress experienced in both OFA and EPM may be to different degrees that affect which RCAN1-regulated signaling pathways are activated and whether CREB1 activity is involved. Also, the tests may stimulate certain brain regions more so (or less) than others depending on the activity being done since both OFA and EPM test anxiety to a different degree (Nosek et al. 2008).

Brain regions tend to possess many different roles in a wide variety of processes, and anxiety is one of those processes. Conditional *Creb1* deletion with the *Camk2a*-Cre driver reduced

CREB1 levels in the PFC, NAc, amy, and HPC to varying amounts. All these brain regions have been implicated in anxiety. The NAc regulates dopamine and serotonin secretion and the amy is known as the essential brain region for anxiety and emotional responses (Barrot et al, 2002; Garcia et al. 1999; Babaev et al. 2018). The PFC plays a large role in personality and emotional responses, and the HPC function not only in memory, but also in mediating emotions. With these functions, the PFC and HPC possess their own roles in anxiety (Park and Moghaddam, 2017; Engin and Treit, 2007). It is possible that some brain regions may require CREB1 more than others or do not need it to the same extent for anxiety-related behaviors. Some studies report that CREB1 may have some regional specificity, but its variety and range is not entirely understood (Tanis et al. 2008). When CREB1 is reduced by *Camk2a*-Cre removal of *Creb1*, the NAc showed the smallest reduction compared with the other brain regions examined (Fig. 1C). The NAc and amy also showed compensation for CREB1 loss with increased CREB1 phosphorylation to a lesser extent than in the HPC or PFC. These varying CREB1 responses in different brain regions may contribute to the rescue of anxiolytic-like behavior of *Rcan1* KO mice in the OFA but not EPM.

In addition, there may be an effect depending on which brain cells are being targeted for Cre-mediated *Creb1* deletion. The *Camk2a* promoter-driven Cre line used in our study is known to express Cre in forebrain excitatory neurons late in development (Hoeffer et al., 2008). It may be that CREB1 activity in other brain cell types or brain regions or during prenatal development is involved in mediating the anxiolytic-like phenotype of *Rcan1* KO mice. One possibility is CREB1 in inhibitory neurons. Future studies could use a Cre line under the control of glutamate decarboxylase (*Gad2*) promoter to remove *Creb1* specifically from inhibitory neurons in *Rcan1* KO mice. We could then determine any major differences in CREB1 activity and anxiety-related behavior when compared to *Creb1* removal from excitatory neurons (Taniguchi et al. 2011; Pan 2012).

Compensation is a common occurrence within many biological processes, with and without interference from outside sources and transgenics. In the case of *Creb1*, complete knockout of *Creb1* is not viable due to offspring being unable to survive through development. Since CREB1 cannot be removed from the body completely, conditional *Creb1* knockout (*Creb1* cKO) mice are used instead (Mantamadiotis et al. 2002). Since other studies have reported compensatory changes in response to *Creb1* deletion (Mantamadiotis et al. 2002; Vogt et al.,2014; McPherson and Lawrence, 2007), it is no surprise that *Camk2a*-Cre removal of *Creb1* in our mice led to increased activation of the remaining total CREB1 (Figures 1-2). These findings show the importance of sustaining CREB1 activity for brain function, but our behavioral results suggest it is not involved in the anxiolytic-like phenotype of *Rcan1* KO mice in the EPM.

In closing, our study provides evidence that RCAN1 mediates anxiety-related behavior through CREB1 signaling. However, RCAN1 seems to direct signaling through a different pathway in EPM than it does in OFA, which is an interesting area of potential future study. Investigating CREB1's activity by removing *Creb1* from all brain cells or using an inhibitory neuron-specific Cre-driver may be potential ways to further understand RCAN1's interaction with CREB1 and the display of anxiety-related behaviors.

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