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Regulator of calmodulin signaling (RCS) knockout mice display anxiety-like behavior and motivational deficits

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Abstract

Regulator of calmodulin (CaM) signaling (RCS), when phosphorylated by protein kinase A (PKA) on Ser55, binds to CaM and inhibits CaM-dependent signaling. RCS expression is high in the dorsal striatum, nucleus accumbens and amygdala, suggesting that the protein is involved in limbic-striatal function. To test this hypothesis, we examined RCS knockout (KO) mice in behavioral models dependent on these brain areas. Mice were tested for food-reinforced instrumental conditioning and responding under a progressive ratio (PR) schedule of reinforcement and in models of anxiety (elevated plus maze and open field). While RCS KO mice showed normal acquisition of a food-motivated instrumental response, they exhibited a lower breakpoint value when tested on responding under a PR schedule of reinforcement. RCS KO mice also displayed decreased exploration in both the open arms of an elevated plus maze and in the center region of an open field, suggesting an enhanced anxiety response. Biochemical studies revealed a reduction in the levels of dopamine and cAMP-regulated phosphoprotein (DARPP-32) in the striatum of RCS KO mice. DARPP-32 is important in reward-mediated behavior, suggestive of a possible role for DARPP-32 in mediating some of the effects of RCS. Together these results implicate a novel PKA-regulated phosphoprotein, RCS, in the etiology of motivational deficits and anxiety.

Keywords

anxiety; GluR1; motivation; mouse; regulator of calmodulin signaling; dopamine and cAMP-regulated phosphoprotein; synapsin I

Introduction

Protein kinase A (PKA) is a cAMP-regulated kinase that has been implicated in appetitive learning and performance of motivated goal-directed behavior (Baldwin *et al.*, 2002; Jentsch *et al.*, 2002; Lynch *et al.*, 2005; Paine *et al.*, 2009). PKA-dependent signaling has also been implicated in anxiety-like behavior (Pandey *et al.*, 2005). PKA has many targets in neurons, some of which have been associated with these behaviors, including the transcription factor CREB (cAMP response element-binding protein; Barrot *et al.*, 2002; Valverde *et al.*, 2004; Pandey *et al.*, 2005; Dinieri *et al.*, 2009), the glutamate receptor, GluR1 (Svenningsson *et al.*, 2002, 2005; Mead & Stephens, 2003; Bannerman *et al.*, 2004; Crombag *et al.*, 2008a, b),

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and the regulatory protein, dopamine and cAMP-regulated phosphoprotein, 32 kDa (DARPP-32; Heyser *et al.*, 2000; Risenger *et al.*, 2001; Zachariou *et al.*, 2002, 2006). Despite these studies, it is likely that other targets of PKA play a role in the etiology of goal-directed behaviors and anxiety.

Regulator of calmodulin (CaM) signaling (RCS), formerly known as ARPP-21, is a PKA-regulated phosphoprotein enriched in brain regions receiving dopaminergic innervation (Ouimet *et al.*, 1989; Brené *et al.*, 1994). Based on studies in the rodent brain, RCS has been found to be highly expressed in medium spiny neurons in striatum and nucleus accumbens (NAc), with more moderate expression in amygdala (Ouimet *et al.*, 1989). Expression of RCS in other brain regions is very low. We therefore hypothesized that behaviors dependent on limbic-striatal regions might be perturbed in RCS knockout (KO) mice. Furthermore, we hypothesized that there may also be accompanying dysregulation of CaM-dependent signaling in these two brain regions. Our results are consistent with these hypotheses, and suggest that PKA-regulated RCS is important in the etiology of limbic-striatal biochemistry and behavior.

Materials and methods

Animals

The RCS KO mice were generated as described (Rakhilin *et al.*, 2004). Mice were backcrossed more than 10 generations into a C57/Bl6 background. Male wild-type (WT) and KO mice were tested between 3 and 5 months old. All experiments were conducted during the light cycle. Mice were first tested for locomotor behavior, followed by anxiety-like behaviors, and then appetitive instrumental learning and progressive ratio (PR) tasks. Results derived from littermates and closely-related relatives were very similar. Therefore, all data from each group were combined. Mice were group housed on a 12: 12 h light: dark cycle, with lights on at 07:00 h. Food and water were supplied *ad libitum*, except during the instrumental conditioning and PR tests, when animals were kept at 85% of their body weight. All protocols and guidelines were in accordance with the National Institutes of Health ‘Guide for the Care and Use of Laboratory Animals’, and were approved by the Yale University Institutional Animal Care and Use Committee.

Open field test

The apparatus consisted of a square 50 × 50 cm base surrounded by a 35-cm-high wall. Animals were placed in the center of the field, and time spent in the central area of the field (25 × 25 cm) and total distance traveled was recorded. Testing was conducted over a 5-min session and was recorded using a Noldus video tracking system. The maze was cleaned using 70% ethanol before introduction of each animal.

Elevated plus maze

The experimental apparatus was a plus-shaped maze with two open white arms and two closed white arms surrounded by black Plexiglas walls placed on a table approximately 60 cm from the floor. Testing was conducted in a dimly lit room with 0–3 lux of white light as measured in the center of the maze. Animals were placed in the center of the maze facing an open arm, and the number of entries and time spent in each arm during the 5-min session was recorded by an experimenter blinded to genotype. An arm entry was defined as the animal placing three or more paws onto an arm. The maze was cleaned using 70% ethanol before introduction of each animal.

Food-reinforced instrumental behavior

Animals were trained for food-reinforced instrumental responding using 16 × 14 × 13 cm operant chambers controlled by MedPC software (Med Associates, Saint Albans, VT, USA) using methods previously reported (Gourley *et al.*, 2008a, 2010). Each chamber was housed within a sound-attenuating outer chamber with a white noise generator and fan to reduce the impact of external noise. A house light was mounted on the back wall to illuminate the chamber. A pellet dispenser delivered food pellets (20 mg; Bio-Serv, USA) as the reinforcer into the magazine. Three nosepoke apertures were placed on the back wall of the chambers opposite to the reinforcer magazine. Head entries were detected by photocells within the reinforcer magazine and nosepoke apertures.

Five days immediately prior to the start of training, animals were restricted to 90 min access to food per day. During the testing period, food pellets were available during the behavioral protocol as well as in unlimited amounts in the home cage for 90 min following the daily testing session. This feeding schedule reduced animal weight to 85–90% of their initial free-feeding weight and allowed for slowed weight gain throughout the course of the experiment. Animal weights were monitored daily.

Mice were habituated to the testing apparatus on the first day, during which grain-based food pellets were delivered into the reinforcer magazine on a 15-s fixed time (FT-15) schedule. On subsequent days, mice were trained using a fixed ratio 1 (FR1) schedule on which the first 10 responses resulted in reinforcer delivery. Subsequent reinforcers were delivered on a variable ratio 2 (VR2) schedule. Each session lasted for 15 min, with the same schedule of reinforcement being used in all sessions. After WT and KO mice achieved stable responding on the active aperture, they were tested on a PR schedule of reinforcement. This test employed a PR4 schedule, whereby the response requirement (r) increased linearly by four ($r = 1, 5, 9, r + 4$) for each subsequent reinforcer. The test concluded when animals stopped responding at the active aperture for a total of 5 min. Testing time was capped at 4–6 h to avoid entering the dark cycle. The highest ratio of reinforcement achieved is considered the ‘breakpoint ratio’, and was used as a measurement of goal-directed behavior.

Immunoblotting

Experimentally naïve male and female WT and KO mice were killed, and brains were harvested, frozen on dry ice and sliced into 1-mm sections using a stainless-steel brain matrix. Bilateral punches of amygdala, hippocampus and striatum (dorsal and ventral/NAc) were collected and homogenized in 1% sodium dodecyl sulfate (SDS) supplemented with 1% each of phosphatase I/II and protease inhibitors (Sigma, St Louis, MO, USA). Homogenates were boiled for 2 min at 95 °C, and debris was sedimented out by centrifugation at 15 000 ***g*** for 10 min. Protein content was measured using a BCA assay (Pierce, Rockford, IL, USA), and protein (5–10 μg) from different brain regions was analysed by SDS–polyacrylamide gel electrophoresis (PAGE). Proteins were then transferred onto 0.2 μM nitrocellulose membranes, and membranes were blocked in 1 × phosphate-buffered saline (PBS) plus 5% non-fat dry milk and incubated in primary antibody overnight. Antibodies were diluted in a 1: 1 solution of 1 × PBS:LiCor blocking buffer (LiCor) with 0.01% SDS and 0.1% Tween at the following dilutions: DARPP-32 (1: 5000, mouse; Hemmings & Greengard, 1986); striatal enriched phosphatase 46 kDa (STEP-46; 1/2000, mouse; from Novus Biologicals, Littleton, CO, USA); total synapsin, phospho-synapsin site 1 and phospho-synapsin sites 4/5 (all 1: 500, rabbit; Czernik *et al.*, 1991; Jovanovic *et al.*, 1996); total GluR1 (1: 1000, rabbit; Chemicon); pSer845 GluR1 (1: 500, rabbit; PhosphoSolutions); GluR2 (1: 1000, rabbit; AbCam); NMDA receptor (NR)1 (1: 2000, mouse; BD Pharmingen); NR2A (1: 2000, rabbit; Sigma); NR2B (1: 1000, rabbit;

Sigma); CaM-dependent kinase II (CaMKIIα; 1: 5000, mouse; Chemicon); calcineurin A (CaN A; 1: 500, mouse; BD Transduction Labs); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1: 10 000, mouse; Advanced Immunochemical, Long Beach, CA, USA). Blots were then washed in 1 × PBS + 0.1% Tween and incubated in secondary antibodies, IRDye 800 anti-mouse (Rockland) and AlexaFluor 680 anti-rabbit (Invitrogen/Molecular Probes, Eugene, OR, USA), at dilutions of 1: 10 000 each for 1 h at 4 °C. Blots were then washed twice with 1 × PBS + 0.1% Tween and twice with 1 × PBS, and scanned using the LiCor Odyssey infrared scanning system.

Statistical analyses

Experiments involving only one degree of freedom, such as elevated plus maze, open field, PR and immunoblotting, were analysed using a one-way analysis of variance (ANOVA) with Fisher's protected least significant difference test. Data from instrumental conditioning were analysed using a repeated-measures ANOVA using genotype and training day as the dependent variables. Statistical analyses were performed using SPSS Statistics 17.0.0 (SPSS, Chicago, IL, USA) and Statview 5.0.1 (SAS Institute, Cary, NC, USA) software. A value of $P < 0.05$ was considered statistically significant.

Results

RCS KO mice exhibit motivational deficits

The current series of experiments provide the first behavioral assessment of the RCS KO mice. We initially examined their general motor function and basal locomotor activity. Mice were habituated in clean cages for 30 min, and then locomotor activity was measured for an additional 30 min. No mean differences in locomotor activity were observed between WT and KO mice (5901.1 ± 318.5, 6381.5 ± 309.5, $F_{1,36} = 2.095$, $P = 0.16$), with both groups of mice similarly habituating over time.

RCS is most abundantly expressed in the striatum and NAc. Therefore, we wished to examine whether their behavior would be altered in a reward-motivated task known to involve the NAc (Balleine & Killcross, 1994; Corbit *et al.*, 2001; Parkinson *et al.*, 2002; Ito *et al.*, 2008; Meredith *et al.*, 2008). WT and KO mice (WT, $n = 21$; KO, $n = 17$) were tested for differences in acquisition in a food-reinforced instrumental learning task (Fig. 1). There was a main effect of day, indicating that the WT and KO mice acquired the task earning an increasing number of reinforcers during the sessions ($F_{1,13} = 8.261$, $P < 0.0001$; Fig. 1A). Notably, there was no significant difference in acquisition of operant responding between WT and KO mice ($F_{1,31} = 1.433$, $P = 0.24$), and there was no genotype × day interaction. There were also no differences on responding in the inactive apertures (data not shown). Moreover, there were no differences in the mean amount of pellets consumed between genotypes prior to training (WT = 34.3, KO = 32.5, $F_{1,36} = 0.591$, $P = 0.48$), indicating that there were no baseline differences in food consumption (Fig. 1B). After WT and KO mice had achieved stable responding, they were subjected to testing on a PR task using a PR4 schedule. When the task was made more demanding in this way, the RCS KO mice had a significantly lower breakpoint ratio than their WT controls ($F_{1,33} = 12.951$, $P = 0.001$; Fig. 1C), suggesting reduced motivation for food reinforcement.

RCS KO mice display behavioral deficits in open field and elevated plus maze tasks

We next analysed RCS KO mice in the open field and elevated plus maze tests that are commonly used to measure anxiety-like behavior (Pellow & File, 1986; Schmidt & Hiemke, 1998). In the open field, RCS KO mice spent less time in the center region of the open field compared with WT mice (Fig. 2A; WT = 31.0 s, KO = 18.8 s, $F_{1,42} = 13.7751$, $P = 0.0006$). There was no difference in distance traveled during this task (Fig. 2B; WT = 2713.2 cm, KO

= 2690.2 cm, $F_{1,42} = 0.8639$, $P = 0.64$), consistent with the results from the locomotor activity test. In the elevated plus maze test, RCS KO mice entered open arms significantly less often than their WT counterparts (WT = 33.47% open/total entries, KO = 22.89% open/total entries, $F_{1,42} = 11.2173$, $P = 0.001727$). RCS KO mice also entered open arms for significantly less time than WT mice (WT = 21.39% open/total time, KO = 11.73% open/total time, $F_{1,42} = 12.7998$, $P = 0.0089$). However, RCS KO and WT mice had no difference in mean number of total arm entries ($F_{1,42} = 0.4133$, $P = 0.6828$; Fig. 2D).

Analysis of signal transduction pathways in RCS KO mice

RCS has been implicated in the control of CaM-dependent enzymes, and has been found to regulate the phosphorylation state of proteins including DARPP-32, although no changes in levels of CaM expression in the striatum were found (Rahkilin *et al.*, 2004). We therefore examined whether there were any effects of RCS KO on the basal expression levels and phosphorylation state of a number of proteins implicated in signal transduction in striatal neurons. DARPP-32 levels in striatal homogenates from RCS KO mice were decreased by 38% compared with homogenates from WT mice ($F_{1,11} = 13.780$, $P = 0.004$; Fig. 3). In contrast, levels of STEP-46 were unchanged in striatal homogenates from RCS KO mice ($F_{1,11} = 0.751$, $P = 0.40$). Striatally abundant DARPP-32 and STEP-46, while present in the hippocampus and amygdala, are expressed at markedly lower levels in these regions (Ouimet *et al.*, 1984a,b; Lombroso *et al.*, 1993) and were used to confirm fidelity in regional dissections. While there appeared to be a decrease in DARPP-32 in the amygdala homogenates of KO animals, it was not statistically significant ($F_{1,10} = 3.255$, $P = 0.10$). There were no changes in Thr34 phosphorylation of DARPP-32 in the striatal samples from WT or KO mice, under the basal conditions used during which the tissue was harvested (data not shown). There were no changes in the CaM-dependent proteins, CaN A ($F_{1,11} = 2.924$, $P = 0.12$) and CaMKIIα ($F_{1,10} = 2.017$, $P = 0.19$) in the striatum, or in any of the other brain regions examined (Fig. 4).

We next examined the expression of ionotropic glutamate receptors (Fig. 5). Total GluR1 ($F_{1,10} = 5.803$, $P = 0.037$) levels were decreased in striatal homogenates from RCS KO mice, with no change in amygdala ($F_{1,11} = 0.291$, $P = 0.60$) or hippocampal ($F_{1,10} = 0.421$, $P = 0.53$) GluR1 levels. There was no change in striatal pSer845 GluR1 level when normalized to total GluR1 ($F_{1,10} = 1.869$, $P = 0.20$). In the amygdala ($F_{1,11} = 0.284$, $P = 0.20$) and hippocampus ($F_{1,10} = 0.33$, $P = 0.86$), pS845 GluR1 levels were also unchanged. Furthermore, no changes were observed in total levels of the GluR2 subunit or of the NR1, NR2A or NR2B *N*-methyl-D-aspartate (NMDA) receptor subunits (Fig. 6).

We also examined the expression and phosphorylation of the synaptic vesicle protein, synapsin I (Fig. 7). Synapsin I phosphorylation site 4/5 (Ser 62/67) levels were decreased in RCS KO striatal homogenates by 40% ($F_{1,10} = 7.475$, $P = 0.021$), but were unchanged in amygdala ($F_{1,10} = 0.023$, $P = 0.88$) and hippocampal ($F_{1,10} = 0.005$, $P = 0.95$) homogenates. Synapsin I site 1 phosphorylation was unchanged in all three homogenate samples (amygdala: $F_{1,11} = 0.094$, $P = 0.77$; striatum $F_{1,11} = 0.499$, $P = 0.49$; hippocampus: $F_{1,10} = 0.392$, $P = 0.55$). There were no changes in total levels of synapsin I (Fig. 7 and data not shown).

Discussion

In this study, we report the first behavioral characterization of RCS KO mice. RCS KO mice displayed lower breakpoints in responding for food on a PR schedule of reinforcement without evidence of altered responding of FR responses or alteration in motor behavior. These data suggest decreased motivation in mice lacking RCS. Furthermore, RCS KO mice

displayed greater thigmotaxis and less exploratory behavior into the open arms of the elevated plus maze compared with WT mice, indicative of an anxiety-like phenotype.

Inhibition of PKA signaling in the NAc can impair acquisition of a food-reinforced instrumental task (Baldwin *et al.*, 2002). Conversely, repeated exposure to drugs of abuse can cause elevations in striatal/accumbal PKA signaling (Nestler, 2001), and enhance acquisition, performance and motivation in a food-motivated instrumental task similar to the one used here (Olausson *et al.*, 2006). Dopamine depletion in the NAc reduces food-reinforced PR responding (Aberman *et al.*, 1998), and lesions of the NAc inhibit responding on operant tasks with high but not low response requirements (Aberman & Salamone, 1999; Correa *et al.*, 2002; Mingote *et al.*, 2005). We found that RCS KO and WT mice performed equally well in an operant conditioning task for a food reward, indicating that RCS is not required for the acquisition of this task. Instead, RCS KO mice had a significantly lower breakpoint ratio than their WT cohorts. RCS in the striatum may therefore be involved in the regulation of motivated behavior. Inhibition of PKA post-acquisition can affect PR responding for drug reinforcers (Lynch & Taylor, 2005). It is possible that this effect could be mediated by PKA-dependent regulation of RCS.

When RCS is phosphorylated on Ser55 by PKA, it binds to and sequesters CaM, thereby inhibiting CaM-dependent signaling (Rakhilin *et al.*, 2004). Specifically, pSer55 RCS has been shown to inhibit the activities of two CaM-dependent enzymes, the serine/threonine kinase, CaMKI, and the serine/threonine phosphatase, calcineurin (Rakhilin *et al.*, 2004). Previous studies indicated that the phosphorylation of two postsynaptic calcineurin targets in striatal medium spiny neurons, DARPP-32 (from direct analysis) and L-type calcium channels (inferred from functional analysis), are decreased in RCS KO mice (Rakhilin *et al.*, 2004). Our new biochemical data show that there are reductions in DARPP-32, GluR1 and phospho-site 4/5 in synapsin I levels selectively in striatum from RCS KO mice. The levels of other striatal cytosolic markers that are directly regulated by CaM signaling, STEP, CaN A and CaMKIIα, were unchanged in RCS KO tissue. Previous studies have found no change in CaM expression in striatum from RCS KO mice (Rakhilin *et al.*, 2004). Given the very high level of expression of RCS in the striatum compared with other areas of the brain, and the selective changes observed in a limited number of signaling proteins within the same brain region, the results suggest that the behavioral differences observed in RCS KO mice may be linked to alterations in PKA signaling in the striatum.

While the biochemical changes we observed were limited to the striatum, given the nature of the constitutive KO employed, any changes could be a fairly indirect consequence of loss of RCS, and not necessarily involved in the behavioral phenotypes observed. However, the observations that both DARPP-32 phosphorylation and DARPP-32 levels are selectively decreased in the striatum from RCS KO mice suggest a possible hierarchical relationship between these two prominent PKA targets in striatal neurons (see also Rakhilin *et al.*, 2004). KO of RCS would therefore impair PKA/DARPP-32/protein phosphatase 1 signaling through reduced levels of DARPP-32 and increased dephosphorylation of Thr34 of DARPP-32 by calcineurin. DARPP-32 is known to be involved in mediating the effects of rewarding stimuli (Risinger *et al.*, 2001; Zachariou *et al.*, 2002, 2006; Stipanovich *et al.*, 2008). DARPP-32 KO mice show reductions in instrumental responding for ethanol intake, but no differences in responding for food intake (Risinger *et al.*, 2001). These results are consistent with our findings that RCS KO mice showed no differences in acquisition of responding for food reward. Also consistent with our results, it has been shown that PR responding for food is reduced in DARPP-32 Ser97 mutant mice, which in other respects exhibit a similar phenotype to DARPP-32 KO mice (Stipanovich *et al.*, 2008). Moreover, PR responding for cocaine is associated with high levels of DARPP-32 phosphorylation by PKA in striatum/NAc (Lynch *et al.*, 2007). The overlap of the behavioral deficits in RCS

KO and DARPP-32 mutant mice further support the possibility that altered DARPP-32 signaling may be involved in some of the phenotypes of the RCS KO mice. However, it is also likely that other substrates for CaN are involved in mediating some of the actions of RCS.

Trafficking of GluR1 to the synapse is thought to be a major regulatory mechanism underlying synaptic plasticity (Malinow & Malenka, 2002; Bredt & Nicoll, 2003). The selective reduction in total GluR1 levels in the striata of RCS KO mice indicates a possible compromise in striatal plasticity. While α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors have been shown to be involved in appetitive incentive learning (Crombag *et al.*, 2008a,b) and ingestive behavior (Georgescu *et al.*, 2005; Sears *et al.*, 2010), inhibition of AMPA/kainate receptors in the NAc shell has been shown to stimulate feeding (Maldonado-Irizarry *et al.*, 1995). We find here that RCS KO mice show reductions in responding for a food reward and in striatal/accumbal GluR1. Even if very indirectly linked to KO of RCS, chronically lower levels of GluR1 in RCS KO mice vs acute pharmacological inhibition of GluR1 receptors could produce differential results on food-seeking behavior.

RCS KO mice showed an anxiety-like phenotype in the elevated plus maze and open field. We hypothesize that the absence of RCS in the striatum alone may explain the anxiety-like phenotype. Perturbation of cAMP-dependent signaling in the striatum has been shown to have an effect on anxiety-related tasks (Silvestre *et al.*, 1999; Barrot *et al.*, 2002; Green *et al.*, 2006; Favilla *et al.*, 2008; Kim *et al.*, 2008; Masood *et al.*, 2008). While anhedonia may perhaps be the best example of a dopamine-based depressive phenotype, it appears that perhaps other anxiety-like behaviors could also be dependent on dopamine signaling within the striatum as well (Millan, 2003; Nestler & Carlezon, 2006). Increased cAMP-signaling in striatum/NAc promotes anxiety-related behaviors, while inhibition of cAMP-signaling in these brain regions has been shown to produce anxiolysis (Favilla *et al.*, 2008; Kim *et al.*, 2008; Zhang *et al.*, 2008). Notably, however, some of these studies are complicated by concomitant changes in locomotion (Favilla *et al.*, 2008; Kim *et al.*, 2008), which was not the case for RCS KO mice.

Synapsin I phosphorylation at site 4/5, which is regulated by CaN activity (Jovanovic *et al.*, 2001), was decreased in RCS KO mice. In contrast, there was no difference in site 1 phosphorylation, which is regulated by phosphatase PP2A. Phosphorylation of synapsin at site 4/5 is associated with increased presynaptic glutamate release (Jovanovic *et al.*, 2000). Thus, there may be a reduction in the level of glutamate that accompanies the reduced levels of GluR1 in RCS KO mice. Because RCS is expressed apparently exclusively in medium spiny neurons in the striatum (Ouimet *et al.*, 1989), it was unexpected that altered RCS expression would influence regulation of a presynaptic protein like synapsin I. Increased CaN activity that results from reduced RCS expression may affect synapsin I phosphorylation in recurrent collateral connections between striatonigral and striatopallidal medium spiny neurons (Taverna *et al.*, 2008). Alternatively, the changes in synapsin I phosphorylation may be linked to altered postsynaptic signaling, which in turn can feed back presynaptically to reduce presynaptic signaling as well (Yin & Lovinger, 2006; Kreitzer & Malenka, 2007; Day *et al.*, 2008; Surmeier *et al.*, 2010). RCS has been implicated in Ca^{2+}-dependent inhibition of myocyte enhancer factor 2 (MEF2) activity through its actions on calcineurin (Pulipparacharuvil *et al.*, 2008). MEF2 activity may be enhanced in RCS KO mice, which in turn could influence glutamatergic synapses in the striatum (Flavell *et al.*, 2006).

In conclusion, RCS KO mice displayed decreased responding on a PR task for food reward, and increased anxiety in the open field and elevated plus maze. These results implicate RCS,

in motivation and anxiety function. Because decreased motivation and increased anxiety, though distinct phenotypes with different underlying mechanisms, are present in several psychiatric diseases, RCS could prove to be a novel target for the treatment of these disorders.

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Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CaM	calmodulin
CaMKII	calmodulin-dependant kinase II
CaN A	calcineurin A
DARPP-32	dopamine and cAMP-regulated phosphoprotein
FR	fixed ratio
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GluR	glutamate receptor
KO	knockout
MEF2	myocyte enhancer factor 2
NAc	nucleus accumbens
NMDA	*N*-methyl-D-aspartate
NR	NMDA receptor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PKA	protein kinase A
PR	progressive ratio
RCS	regulator of calmodulin signaling
SDS	sodium dodecyl sulfate
STEP-46	striatal enriched phosphatase 46 kDa
WT	wild-type

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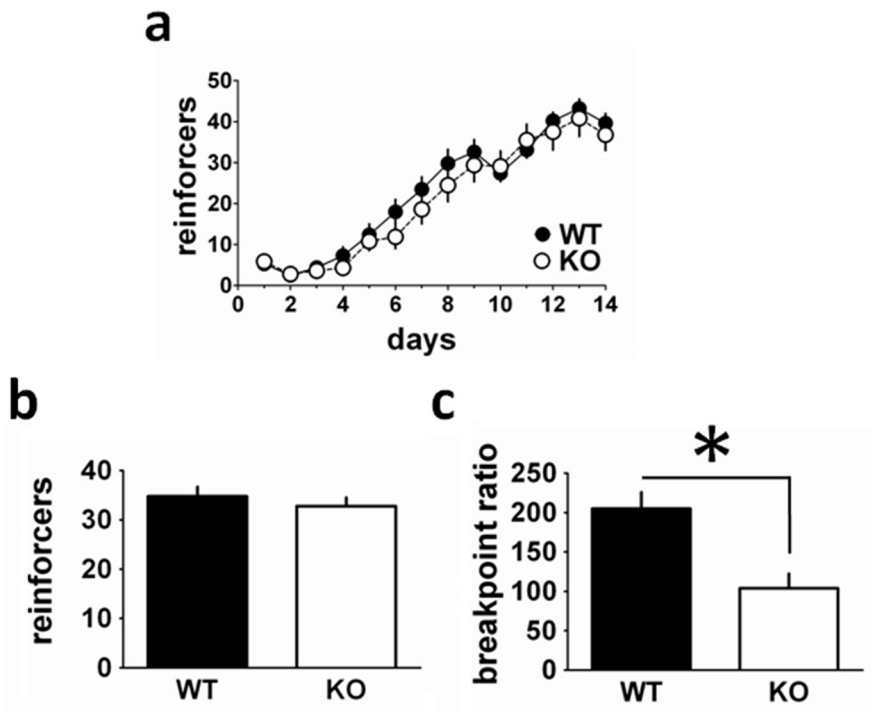

Fig. 1.

RCS knockout (KO) mice exhibit normal acquisition of food-reinforced instrumental responding, but have a lower PR breakpoint. Mice [wild-type (WT) = 21, KO = 17] were trained to nosepoke for a food pellet reward. (A) A repeated-measures ANOVA revealed a significant main effect of training day for both WT and KO mice ($F_{1,13} = 8.261$, $P < 0.0001$), but no significant differences in acquisition of operant responding between WT and KO mice ($F_{1,31} = 1.433$, $P = 0.24$). (B) On magazine training day (day 0 prior to training shown in A), there were no differences in the mean amount of pellets consumed between genotypes ($F_{1,36} = 0.591$, $P = 0.48$). (C) When subjected to a PR4 schedule of reinforcement, RCS KO mice had a significantly lower breakpoint ratio than their WT cohorts ($F_{1,33} = 12.951$, $P = 0.001$).

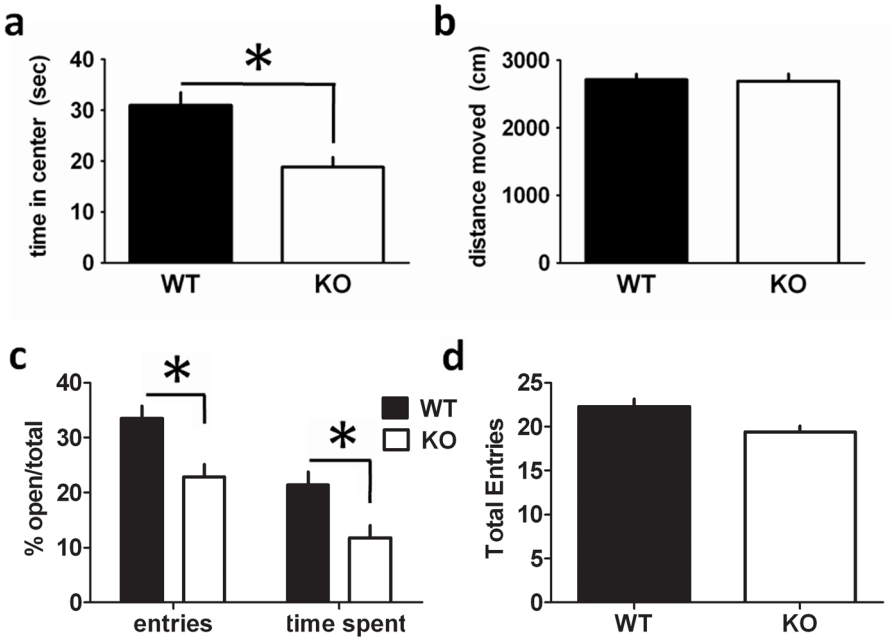

Fig. 2.

RCS knockout (KO) mice display increased anxiety-like behavior in the open field and elevated plus maze. (A) RCS KO mice spent less time in the center region of the open field compared with wild-type (WT) mice (WT = 31.0 s, KO = 18.8 s, $F_{1,42} = 13.7751$, $P = 0.0006$). (B) There was no difference between WT and KO mice in distance traveled in this test (WT = 2713.2 cm, KO = 2690.2 cm, $F_{1,42} = 0.8639$, $P = 0.64$). (C) RCS KO mice entered open arms significantly less often than their WT counterparts (WT = 33.47% open/total entries, KO = 22.89% open/total entries, $F_{1,42} = 11.2173$, $P = 0.001727$). Similarly, RCS KO mice entered open arms for significantly less time than WT mice (WT = 21.39% open/total time, KO = 11.73% open/total time, $F_{1,42} = 12.7998$, $P = 0.0089$). (D) RCS KO and WT mice had no difference in mean number of total arm entries ($F_{1,42} = 0.4133$, $P = 0.6828$). Total n for all tasks (WT = 24, KO = 20).

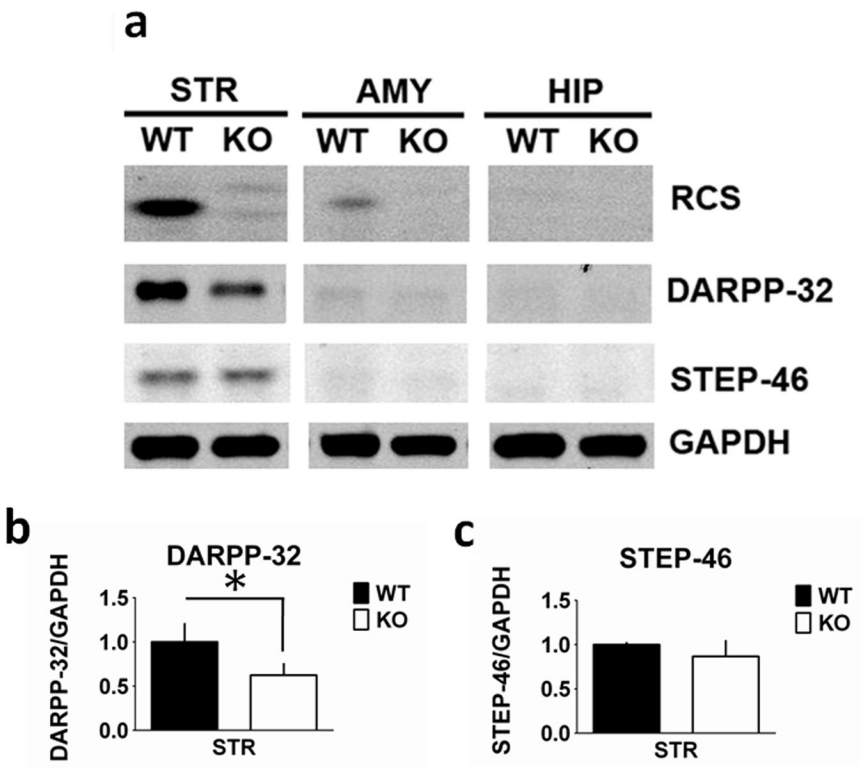

Fig. 3.

Dopamine and cAMP-regulated phosphoprotein (DARPP-32) expression is reduced in the striatum from regulator of CaM signaling (RCS) knockout (KO) mice. Proteins from striatal (STR), amygdala (AMY) and hippocampal (HIP) extracts were analysed by SDS–PAGE and immunoblotting using RCS, DARPP-32, striatal enriched phosphatase 46 kDA (STEP-46) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies. Representative immunoblots are shown in (A), and quantitation is shown in (B) and (C). Expression levels of DARPP-32 and STEP-46 were normalized to that of GAPDH. Error bars indicate SEM, $n = 5–7$ per group. DARPP-32 levels were decreased in RCS KO striatal homogenates by 38% compared with wild-type (WT) mice ($F_{1,11} = 13.780$, $P = 0.004$). Levels of STEP-46 were unchanged in striatal homogenates ($F_{1,11} = 0.751$, $P = 0.40$) from WT and RCS KO mice.

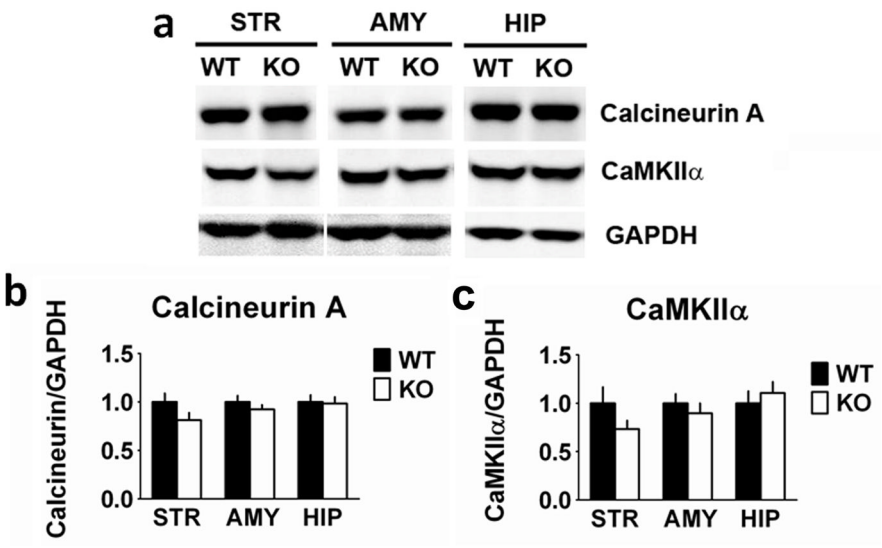

Fig. 4.

CaM-dependent proteins, calcineurin A (CaN A) subunit and CaM-dependent kinase II (CaMKIIα) levels are unchanged in RCS knockout (KO) mice. Proteins from striatal (STR), amygdale (AMY) and hippocampal (HIP) extracts were analysed by SDS–PAGE and immunoblotting using CaN A subunit, CaMKII and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies. Representative immunoblots are shown in (A), and quantitation is shown in (B) and (C). Expression levels of CaN A subunit and CaMKII were normalized to that of GAPDH. Error bars indicate SEM, $n = 5–7$ per group. There were no differences in total levels of CaN A in homogenates from wild-type (WT) and RCS KO mice from the striatum ($F_{1,11} = 2.924$, $P = 0.12$), amygdala ($F_{1,11} = 0.656$, $P = 0.44$) or hippocampus ($F_{1,10} = 2.233$, $P = 0.17$), or of CaMKII from the striatum ($F_{1,10} = 2.017$, $P = 0.19$), amygdala ($F_{1,11} = 0.466$, $P = 0.51$) or hippocampus ($F_{1,10} = 0.157$, $P = 0.70$).

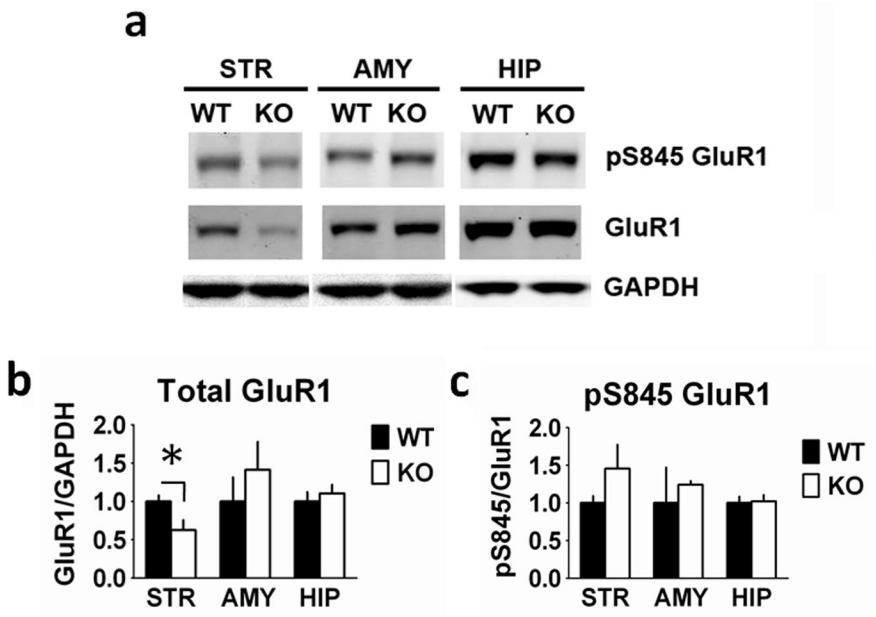

Fig. 5.

Glutamate receptor type 1 (GluR1) expression is reduced in the striatum from RCS knockout (KO) mice. Proteins from striatal (STR), amygdala (AMY) and hippocampal (HIP) extracts were analysed by SDS–PAGE and immunoblotting using GluR1, phospho-Ser845 GluR1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies. Representative immunoblots are shown in (A), and quantitation is shown in (B) and (C). Expression levels of GluR1 and phospho-Ser845 GluR1 were normalized to that of GAPDH. Error bars indicate SEM, $n = 5–7$ per group. Total GluR1 ($F_{1,10} = 5.803$, $P = 0.037$) levels were decreased in striatal homogenates from RCS KO mice compared with wild-type (WT) mice. There were no differences in amygdala ($F_{1,11} = 0.291$, $P = 0.60$) or hippocampal ($F_{1,10} = 0.421$, $P = 0.53$) GluR1 levels between WT and RCS KO mice. There were no differences in striatal pSer845 GluR1 levels when normalized to total GluR1 ($F_{1,10} = 1.869$, $P = 0.20$). In amygdala ($F_{1,11} = 0.284$, $P = 0.20$) and hippocampal ($F_{1,10} = 0.33$, $P = 0.86$) homogenates pS845 GluR1 levels were also unchanged.

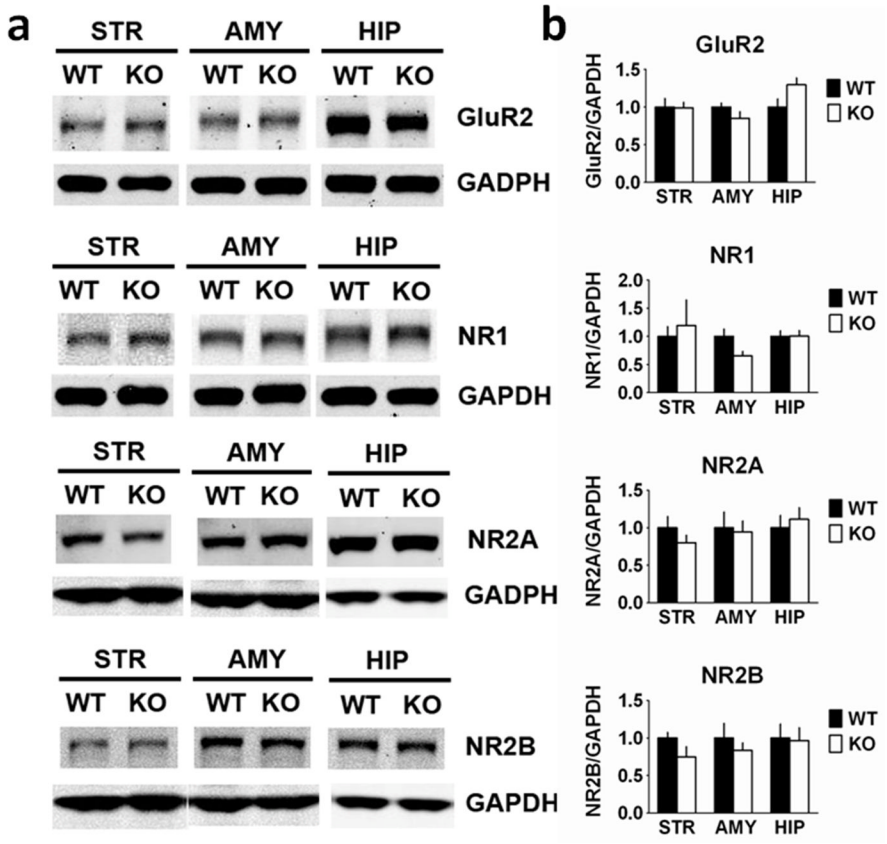

Fig. 6.

Glutamate receptor type 2 (GluR2) and NMDA receptor (NR) expression is unchanged in RCS knockout (KO) mice. Proteins from striatal (STR), amygdala (AMY) and hippocampal (HIP) extracts were analysed by SDS–PAGE and immunoblotting using GluR2, NMDA receptor type 1 (NR1), NMDA receptor type 2A (NR2A), NMDA receptor type 2B (NR2B) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies. Representative immunoblots are shown in (A), and quantitation is shown in (B) and (C). Expression levels of GluR2, NR1, NR2A and NR2B were normalized to that of GAPDH. Error bars indicate SEM, $n = 5–7$ per group. No differences in expression levels of any of these other glutamate receptor subunits were found in RCS KO mice (Table 1). WT, wild-type.

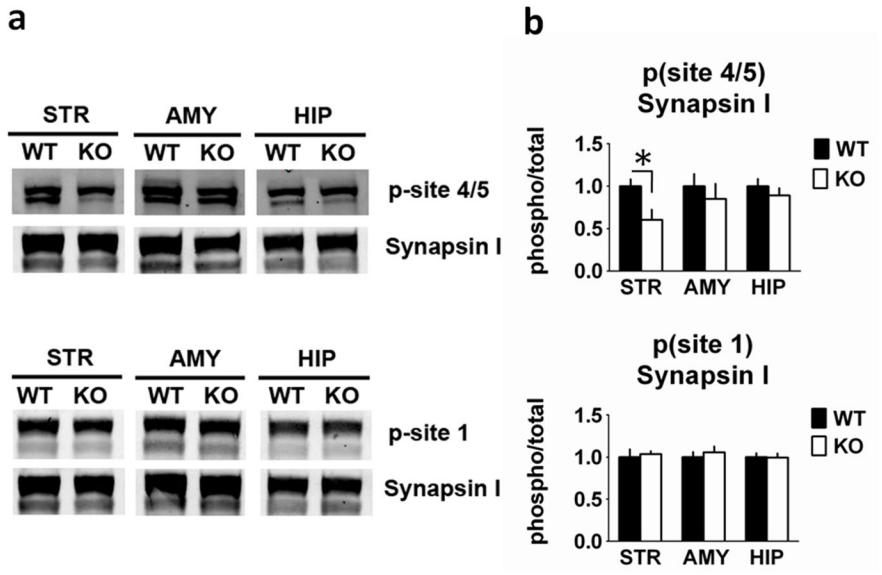

Fig. 7.

Synapsin site 4/5 phosphorylation is reduced in the striatum from RCS knockout (KO) mice. Proteins from striatal (STR), amygdala (AMY) and hippocampal (HIP) extracts were analysed by SDS–PAGE and immunoblotting using synapsin I, phospho-site 1 and phospho-site 4/5 of synapsin I antibodies. Representative immunoblots are shown in (A), and quantitation is shown in (B) and (C). The p-site 4/5 antibody detects Synapsin I as a doublet (isoforms 1a and 1b). The p-site 1 and total Synapsin I antibodies detect 1a/1b as a predominantly single band. Expression levels of phospho-site 1, phospho-site 4/5 of synapsin I were normalized to that of total synapsin I. Error bars indicate SEM, $n = 5–7$ per group. Synapsin I phosphorylation site 4/5 (Ser62/67) was decreased by 40% in striatal homogenates from RCS KO mice ($F_{1,10} = 7.475$, $P = 0.021$), but was unchanged in amygdala ($F_{1,10} = 0.023$, $P = 0.88$) and hippocampal ($F_{1,10} = 0.005$, $P = 0.95$) homogenates. Synapsin I site 1 phosphorylation was unchanged in all three homogenate samples (amygdala: $F_{1,11} = 0.094$, $P = 0.77$; striatum: $F_{1,11} = 0.499$, $P = 0.49$; hippocampus: $F_{1,10} = 0.392$, $P = 0.55$).

Table 1

GluR2 and NMDA receptor expression is unchanged in RCS KO mice

	GluR2	NR1	NR2A	NR2B
STR	$F_{1,11} = 0.018$, $P = 0.90$	$F_{1,10} = 2.355$, $P = 0.15$	$F_{1,10} = 2.700$, $P = 0.13$	$F_{1,10} = 0.20$, $P = 0.89$
AMY	$F_{1,10} = 0.024$, $P = 0.88$	$F_{1,10} = 4.109$, $P = 0.073$	$F_{1,11} = 0.049$, $P = 0.83$	$F_{1,11} = 0.610$, $P = 0.45$
HIP	$F_{1,10} = 0.152$, $P = 0.70$	$F_{1,10} = 0.309$, $P = 0.70$	$F_{1,10} = 0.309$, $P = 0.59$	$F_{1,10} = 2.700$, $P = 0.23$

These data correspond to the data in Fig. 6 where proteins from striatal, amygdala and hippocampal extracts were analysed by SDS–PAGE and immunoblotting using GluR2, NR1, NR2A, NR2B and GAPDH antibodies. No differences in expression levels of any of these other glutamate receptor subunits were found in RCS KO mice. See Fig. 6 for immunoblots.

AMY, amygdale; GluR2, glutamate receptor type 2; HIP, hippocampal; NR1, NMDA receptor type 1; NR2A, NMDA receptor type 2A; NR2B, NMDA receptor type 2B; STR, striatal.