HHS Public Access

Author manuscript

Behav Brain Res. Author manuscript; available in PMC 2020 December 30.

Published in final edited form as:

Behav Brain Res. 2019 December 30; 376: 112177. doi:10.1016/j.bbr.2019.112177.

Manipulation of retinoic acid signaling in the nucleus accumbens shell alters rat emotional behavior

Yafang Zhang, Ph.D.[1], **Elizabeth J. Crofton, Ph.D.**[1], **Tileena E. S. Smith, B.S.**[1], **Shyny Koshy, Ph.D.**[1], **Dingge Li, M.S.**[1], **Thomas A. Green, Ph.D.**[1,*]

[1]Center for Addiction Research, Mitchell Center for Neurodegenerativo Diseases, Dept. of Pharmacology and Toxicology, The University of Texas Medical Branch, Galveston, TX, USA

Abstract

Novel targets for depression and anxiety disorders are necessary for the development of more effective pharmacotherapeutics. Our previous study found that the retinoic acid (RA) signaling pathway is the signaling pathway most enhanced in the nucleus accumbens (NAc) shell, a region important for depression, anxiety, and addiction. Genetic manipulations of RA signaling in the NAc affecting addiction-related behavior prompted our study of the role of retinoic acid signaling in depression-related and anxiety-related behavior using *in vivo* RNA interference. Knockdown of the retinoic acid degradation enzyme cytochrome p450 family 26 subfamily b member 1 *(Cyp26b1)* in the nucleus accumbens shell increased depression-related behavior while decreasing anxiety-like behavior. Knockdown of the retinoic acid binding protein, cellular RA binding protein 2 *(Crabp2),* also increased depression-related behavior. Knockdown of another RA binding partner fatty acid binding protein 5 *(Fabp5),* did not alter these behaviors. These results further support the contention that RA signaling in the NAc shell can affect emotional behavior and that targeting some components of this pathway could be a promising avenue for developing novel treatments for depression and anxiety.

Keywords

anhedonia; behavioral phenotyping; retinol; retinal; retinoids; vitamin A; tretinoin; all-trans retinoic acid; ventral striatum; elevated plus maze; social behavior; neophobia; cold stress; sucrose preference; locomotor activity; forced swim test

1. Introduction

Mood disorders, such as major depressive disorder and generalized anxiety disorder cause severe symptoms and disrupt normal daily life. In the United States, mood disorders are very common, where the lifetime prevalence estimate for anxiety disorders is 28.8%, and the

*Thomas Green, Mental Health Research Group, Center for Addiction Research, Mitchell Center for Neurodegenerative Diseases, Department of Pharmacology and Toxicology, The University of Texas Medical Branch, 301 University Dr., T.G. Blocker Medical Research Building, 7.102C, Galveston, TX 77555-1059, tom.green@utmb.edu, Phone: (409) 747-7056, Fax: (409) 747-7050.

estimate of major depression is 16.6%[1]. Although many patients respond well to current treatments, it is estimated that 63% of patients are refractory to current pharmacotherapies[2,3]. In addition, current pharmacological treatments for depression and anxiety have side effects that render these treatments untenable[4]. Therefore, it is important to explore novel therapeutic targets for neuropsychiatric disorders. The goal of the current study is to examine retinoic acid signaling in the nucleus accumbens on depression- and anxiety-related behavior.

Retinoic acid (RA) is synthesized by the body from vitamin A (retinol) in two steps: retinol is first metabolized by retinol dehydrogenase (RDH/ADH) to retinal, and then retinal is irreversibly converted to retinoic acid via retinal dehydrogenase (RALDH/ALDH1a1–3). RA can be degraded in the cytoplasm into polar metabolites by Cytochrome P450 family proteins: CYP26A1, CYP26B1, and CYP26C1[5–7]. CYP26B1 is mainly expressed in the brain, whereas CYP26A1 dominates the liver, and CYP26C1 is mainly predominant during embryonic development[8,9]. Retinoic acid is involved in multiple intracellular signaling pathways. In the cytoplasm, retinoids are bound to different retinoic acid binding proteins including CRABP1, CRABP2, and FABP5. In the classic genomic RA signaling pathway, RA is shuttled from the cytoplasm to the nucleus with the assistance of the binding proteins and binds to its ligand- activated transcription complex, regulating transcription of downstream targets. CRABP2 delivers RA to the retinoic acid receptor (RARα, RARβ and RARΓ) and retinoid X receptor (RXRα, RXRβ and RXRΓ) complex (RAR-RXR), whereas FABP5 delivers RA to peroxisome proliferator-activated receptor β/δ (PPARβ/δ) and retinol X receptor (RXRα, RXRβ, and RXRΓ) dimer complex (PPAR-RXR)[10,11]. A diagram of the canonical intracellular pathway of RA signaling, including the targets manipulated in this study (Cyp26b1, Crabp2, and Fabp5), are shown below.

Our previous discovery-based quantitative transcriptomic study of environmental enrichment in rats and knock down of *Cyp26b1* suggested a role for retinoic acid signaling in the protective addiction phenotype of environmental enrichment[12]. Rats reared in an enriched condition, where they are exposed to novelty, exercise, and social contact, consume more sucrose in the sucrose preference test, spend more time grooming each other in the social interaction test, and display decreased immobility in the forced swim test[13]. Additionally, enriched rats self-administer stimulants less readily than isolated rats[13,14]. Thus, the environmental enrichment paradigm produces a clear protective depression and addiction phenotype, which we are utilizing to identify novel targets for the treatment of these conditions. Our previous study identified RAR signaling as the signaling pathway most enhanced (9 of 24 genes in core pathway) in the nucleus accumbens (NAc) shell (NAcSh), a region vitally important for depression- and anxiety-related behavior. We showed low RA signaling in the NAcSh of enriched rats, and increased RA signaling (by knocking down the RA degradation enzyme cytochrome P450 family 26 subfamily b member 1, *Cyp26b1)* within the NAcSh increased cocaine-taking and -seeking behaviors in rats[12]. These results suggest that the protective addiction phenotype of environmental enrichment may indeed be a function of *decreased* RA signaling. Environmentally enriched rats also show a protective depression phenotype, therefore high RA signaling may play a similar role in producing depression-related behavior. Indeed, chronic use of 13-cis-RA (isotretinoin), an isomer of

all-trans RA, has been found to produce depression-related molecular and behavioral phenotypes in both rodent models and humans[19–21].

No single behavioral paradigm is fully informative of complex conditions such as depression and anxiety. Therefore, the most robust manipulations (e.g. environmental enrichment) will produce significant results in multiple different behavioral paradigms[13]. In the current study, the role of the retinoic acid signaling pathway in depression and anxiety-related behaviors is investigated by manipulating the RA pathway in the rat NAc shell (a brain region important for addiction-, depression-, and anxiety-related behavior) in a battery of behavioral paradigms. Addiction, depression, and anxiety are all psychiatric conditions heavily reliant on nucleus accumbens signaling. To investigate the causal link between RA and anxiety-like behaviors together with depression-related behaviors in the nucleus accumbens, we manipulated three genes in the RA pathway via knocking down the expression with adeno-associated viruses (AAVs) expressing short hairpin RNAs (shRNA) targeting *Cyp26b1, Crabp2,* or *Fabp5.* We hypothesize that increasing RA signaling would increase depression-related behavior. To investigate this hypothesis, we indirectly elevated RA signaling[22–24] by knocking down the expression of *Cyp26b1* in the NAcSh. Although current technology does not allow for direct quantification of such small amounts of RA, the shRNA strategy does increase RAR signaling in RARE reporter mice[24]. Cyp26b1 was chosen specifically because it has enhanced expression in the NAcSh[25]. Next, we investigated the behavioral effects of manipulating the downstream intracellular pathways of RA signaling. We knocked down the expression of two RA binding proteins *Crabp2* and *Fabp5* in the NAc shell, hypothesizing that one or both of these manipulations would decrease depression-related behavior.

Depression, addiction, and anxiety have strong comorbidity in humans; however, manipulations of the NAcSh in rats typically produce opposite regulation of anxiety-related behavior vs. depression- and addiction-related[13,18,26]. This is most likely specific to manipulations of the NAcSh, or possibly something idiosyncratic to rats. Regardless, in keeping with previously observed increase in cocaine self-administration with *Cyp26b1* knockdown, we hypothesize increased depression-related behavior but decreased anxiety behavior from *Cyp26b1* knockdown and the opposite from knockdown of one or both of the binding proteins *(Crabp2* and *Fabp5).*

2. Experimental Procedures

2.1 Animals

Male Sprague-Dawley rats were obtained at 225–249g. Measures were taken to minimize pain or discomfort following surgical procedures. Rats were pair-housed and maintained in a controlled environment (temperature, 22°C; relative humidity, 50%; 12 h light/dark cycle, lights on 0600 h) in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved colony and procedures were approved by the UTMB Institutional Animal Care and Use Committee and conform to the NIH Guide for the Care and Use of Laboratory Animals.

2.2 Adeno-Associated viral vector knockdown of *Cyp26b1*, *Crabp2*, and *Fabp5*

In order to knock down the expression of *Cyp26b1, Crabp2,* and *Fabp5,* five 24- nucleotide sequences were identified within each of the *Cyp26b1* (Ensembl transcript ID: ENSRNOT00000020505), *Crabp2* (Ensembl transcript ID: ENSRNOT00000072198) and *Fabp5* (Ensembl transcript ID: ENSRNOT00000075493) mRNA sequences using criteria described previously[12,27–29]. The oligonucleotide sequences were synthesized and the annealed hairpin oligonucleotides were ligated into pAAV-shRNA-eGFP plasmids, where the hairpin expression is driven by the mouse U6 promoter using a pol-III mechanism. In addition to the hairpin, enhanced green fluorescent protein (eGFP) was expressed from a separate expression cassette driven by a pol-II promoter (CMV).

In order to determine the most effective hairpin, all five hairpins of each gene were screened in human embryonic kidney cells *in vitro.* To test knockdown of the rat gene sequences for *Cyp26b1, Crabp2,* and *Fabp5,* overexpression of these genes was required in the hairpin validation procedure. To create overexpression plasmids to test knockdown efficiency, the rat *Cyp26b1, Crabp2* or *Fabp5* gene sequences were amplified from rat cDNA using polymerase chain reaction *(Cyp26b1* forward primer: TAGGAATTCCTCCTGGGTTTCTTCGAGGG, reverse primer: TAGGTCGACATCCAAGAGGGTGGGAGTCA; *Crabp2* forward primer: TAGGAATTCCGGCGTCCAGTATTCTAGTTG, reverse primer: TAGCTCGAGAAAGAGTAGAGGCCAGAGAGACA; *Fabp5* forward primer: TAGGAATTCCGTCTCCTTGCTGCTTTTGT, reverse primer: TAGCTCGAGTGGGTTTCGGGTAAAGTGTC) and cloned into the pAAV-IRES-hrGFP plasmid (Agilent Technologies, CA, US). The various hairpin shRNA plasmids were co-transfected with the corresponding overexpression plasmid into HEK-293 cells using FuGENE® 6 Transfection Reagent (Promega)/Lipofectamine 2000 (Life Technologies, Grand Island, NY). The cells were harvested 24–48 hours later followed by RNA extraction and reverse transcription to cDNA. Relative knockdown was measured by Real-time PCR (SYBR Green: Applied Biosystems, Foster City, CA) on an Applied Biosystems 7500 fast thermocycler with qPCR primers *(Cyp26b1* forward primer: CCAGCAGTTTGTGGAGAATG, reverse primer: GTCCAGGGCGTCTGAGTAGT; *Crabp2* forward primer: GTGGATGGGAGACCCTGTAA, reverse primer: CGTAGACCCTGGTGCATACAA; *Fabp5* forward primer: CTTGCACCTTGGGAGAGAAG, reverse primer: CATCTTCCCGTCCTTCAGTT). The knockdown efficiency of the *Cyp26b1* shRNA has been validated in a previous publication where it was used to examine the role of RA signaling in cocaine self-administration[12]. The knockdown efficiency of *Crabp2* and *Fabp5* hairpins were validated by western blot in HEK-293 cells. HEK-293 cells transfected with the overexpressing plasmid and shRNA plasmids or the control plasmid were homogenized in a buffer containing sucrose, Hepes buffer, sodium fluoride, 10% SDS, with protease and phosphatase inhibitors (Sigma-Aldrich: P-8340, P-2850, P-5726). Protein concentration was assessed using the Pierce BCA Protein Assay Kit (Thermo Scientific, IL, USA). Protein samples were denatured at 95° for 10 min and run on a 12% gel (Criterion TGX, Bio-Rad Laboratories, CA, USA) then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). The membrane was blocked with blotting-grade blocker (nonfat dry milk), incubated with

CRABP2 primary antibody (rabbit, 1:1000, LifeSpan BioSciences, WA, USA), or FABP5 primary antibody (rabbit, 1:1000, Cell signaling, MA, USA) and GAPDH primary antibody (mouse, 1:15000, Abcam, MA, USA), washed with TBST and then incubated with fluorescent secondary antibodies (donkey anti-rabbit (780nm), donkey antimouse (680nm), 1:15000, Li-Cor Biosciences, NE, USA). Western blots were then imaged (Odyssey, Li-Cor Biosciences, NE, USA) and protein levels quantified with Odyssey software. The hairpin plasmid with the highest knockdown efficiency *(Cyp26b1* shRNA: 5'.AGTTCTTTGGTCTAGACTCCAATC.3'; *Crabp2* shRNA: 5'.TGACCAATGATGGAGAGCTAATCC.3'; *Fabp5* shRNA: 5'.GGAAGGGAAAGAAAGCACGATAAC.3') was packaged into Adeno-Associated Virus 2 (AAV2) by The University of North Carolina Vector Core and used in subsequent behavioral tests. Titer was quantified by the UNC Vector Core via qPCR (non-targeted hairpin control shRNA vector titer 1.4×10^{12} vg/mL; *Cyp26b1* shRNA vector titer 2.9×10^{12} vg/mL; *Crabp2* shRNA vector titer 4.6×10^{12}vg/mL; *Fabp5* shRNA vector titer 3.7×10^{12} vg/mL). Control vector expressed a previously validated control hairpin not targeted to any gene[27–29].

2.3 In vivo knockdown of *Cyp26b1, Crabp2,* and *Fabp5*

For behavioral tests, the AAV2-based vectors that express *Cyp26b1, Crabp2* or *Fabp5* shRNA and eGFP, or non-targeted control hairpin AAV2 vector (n=12 each) was injected bilaterally (1pl/side over 10 min) into the rat NAc shell using coordinates A/P=1.7, L=2.2, D/V=−6.7, 10 degree angle. Behavioral tests started three weeks after stereotaxic surgery to allow for maximal expression levels. Accurate placements were verified immunohistochemically after the conclusion of behavioral testing by blocking floating sections with donkey serum and incubating overnight with primary antibody chicken anti-GFP (1:500, GFP1020; Aves Labs, Tigard, OR) followed by incubation with secondary antibody donkey anti-chicken IgY (IgG) (H+L) Alexa-Fluor 488 for 2 hours (1:500, Life Technologies, Carlsbad, CA). Sections were mounted, cover slipped, and imaged on an inverted Leica True Confocal Scanner SPE with the Leica Application Suite Advanced software (Leica Microsystems, Wetzlar, Germany) (Figure1d). To validate the knockdown efficiency of AAV in vivo, *AAV-Crabp2* shRNA or *AAV-Fabp5* shRNA (n *= 4–5)* was injected in the nucleus accumbens shell of rats using coordinates A/P=1.7, L=2.4, D/V= −6.7, 10 degree angle. Two uL of AAV was injected per side of the NAc to increase the number of transduced neurons. NAc regions with eGFP fluorescence were collected and tissues were homogenized followed by RNA extraction (RNeasy, Qiagen, Venlo, Netherlands) and reverse transcription to cDNA (Superscript III, Invitrogen, Waltham, MA). Relative knockdown was detected using real-time PCR as described above.

2.4 Locomotor Activity

Locomotor activity was assessed under normal light conditions by placing the rats in clear Plexiglas chambers (40 × 40 × 40 cm) with a thin layer of bedding, surrounded by two 4 × 4 photobeam matrices, one 4 cm above the ground and one 16 cm above the ground to record horizontal ambulation and vertical (rearing) activity. Photobeam breaks were monitored for 90 minutes by a modified open field activity system for *Cyp26b1* and 60 minutes for *Crabp2* and *Fabp5* (San Diego Instruments, CA, USA).

2.5 Sucrose neophobia

To test for anxiety-like behavior, knockdown rats (n=12) and control rats (n=12) were evaluated for neophobia to a novel taste (1% sucrose). Rats were separated into individual cages and water was removed at 1600 hrs. Standard rat water bottles were filled with a 1% w/v sucrose solution in the rats' normal "tap" water and weighed before being placed in each cage at 1800 hrs. After 30 minutes, the bottles were removed to be re-weighed, and the difference in weight of the sucrose bottles before and after the test was calculated. Then, the sucrose solution was replaced on the cages for an additional 2 days to familiarize the rats to the flavor of sucrose prior to the sucrose preference test.

2.6 Elevated plus maze

Another test of anxiety-like behavior, the elevated plus maze (EPM), was tested two days after sucrose neophobia. The elevated plus maze measures vector-modified exploratory behavior in a novel and anxiety-producing environment[18]. The apparatus had two closed arms and two open arms measuring 12 × 50 cm that were 75 cm above the floor along with photobeams at the entrance of each arm (Med Associates Inc, VT, USA). Time spent on the open arms was monitored for 5 minutes by photobeam breaks using Med-PC software.

2.7 Cold stress-induced defecation

On the day after EPM, a third anxiety test was implemented: defecation in response to a mildly stressful environment (cold). Polycarbonate mouse cages (33 × 17 × 13 cm) were pre-chilled on ice for 10 min. The rats were placed in the cages on ice for 30 min and the number of fecal boli was recorded every five min.

2.8 Social contact

On the following day, depression-related behavior was measured using a social interaction test. Rats were separated for 24 hrs prior to testing. On test day, the rats were placed in a novel environment (plastic container, 45 × 40 × 45 cm) with their cage mate and their behavior was video recorded for thirty minutes. The amount of time the pair of rats spent grooming each other was measured by an investigator blinded to the rats' experimental condition.

2.9 Sucrose preference

On the day after social contact, the sucrose preference test was used as a model of anhedonia. Pair-housed rats were separated at 1600 hrs with food but not allowed access to water for 2 hours. At 1800 hrs two pre-weighed water bottles were placed in each cage, one containing water, the other a 1% sucrose solution in water. The water bottles were placed in the normal position while the sucrose was placed approximately 10 cm away. The bottles were removed to be re-weighed after 15 minutes and replaced to be weighed again 16–24 hours later.

2.10 Forced Swim Test

The last spontaneous behavioral test was the forced swim test (FST), a model sensitive to antidepressants [30,31]. Rats were placed into a Plexiglas cylinder filled with approximately 14

L of room temperature (24 ± 0.5°C) water for 15 min on Session 1, and 5 min on Session 2 the following day. The rats were dried and placed back into their home cages. Swimming activity was video recorded and the latency to the first period of immobility (1 sec) and total time immobile were determined for Session 2 by an investigator blinded to the conditions[32].

2.11 Sucrose Operant Responding

Control AAV rats and knockdown rats were regulated to 85% of free-feeding weight over 7 days. All rats were trained to bar press for banana flavored sucrose pellets (Bio-Serv, NJ, USA) on a fixed ratio 1 (FR1) schedule of reinforcement on the 8th day. On the following days, all rats were tested under FR1, FR2, and FR5 for 15-minutes per session and then on a progressive ratio (PR) schedule of reinforcement the next day. Rats were then given free access to food for 3 days and again allowed to bar-press for sucrose pellets on an FR1 and FR5 schedule for 15 minutes and under a PR schedule, this time at 100% free-feed weight.

2.12 Statistical Analysis of Behavior

Significance between only two conditions was analyzed using a Student's t-test. All t-test data passed the Shapiro-Wilk test of normality. Two-factor repeated measures analyses of variance (ANOVA) was used for FR operant sucrose responding. All data are expressed as mean ±SEM. Statistical significance was set at $p<0.05$.

3. Results

3.1 Increasing RA signaling in the NAc shell by knocking down *Cyp26b1*

To investigate the role RA signaling in anxiety- and depression-related behaviors, we knocked down expression of the RA degradation enzyme, *Cyp26b1,* specifically in the NAcSh[12] to indirectly increase RA levels[22,23]. The knockdown efficiency of *Cyp26b1* was validated previously in HEK-293 cells for mRNA as well as protein levels and validated in vivo at the protein level to then be used to assess alterations in cocaine self-administration following knockdown of *Cyp26b1* in the NAcSh[12]. To knockdown the expression of *Cyp26b1* and assess alterations in anxiety-like and depression-related behaviors, *AAV-Cyp26b1* shRNA or control AAV vector was injected into the NAcSh (n=12/treatment). Figure 1f displays the schematic diagram of the behavioral procedure. Three weeks after the virus injection surgery, control rats and knockdown rats started spontaneous behavioral tests, followed by sucrose pellet operant responding. The placements of injections were checked after all behavioral tests by immunofluorescence staining of eGFP (representative fluorescence Figure 1d)[12]. No poor placement was observed, thus no animals were removed from this study.

To examine whether knocking down *Cyp26b1* in NAc shell alters the basal locomotor activity of rats in a novel environment, the rats were first given a 90-min session of locomotor activity. The results reveal a significant increase in total locomotor activity at 30 min ($t(22) = - 2.538$, $p=0.019$) and 60 min (cumulative; $t(22) = -2.121$, $p=0.045$), but the effect washed out when looking at the full 90 min (Figure 2a-b).

Anxiety-related tests included sucrose neophobia, elevated plus maze, and cold stress-induced defecation. In the elevated plus maze, rats with *Cyp26b1* knocked down in the NAcSh exhibited more open arm entrances ($t(22) = -2.515$, $p=0.02$; Table S1) and spent significantly more time in the open arm ($t(22) = -2.240$, $p=0.036$; Figure 2c) than control rats, indicating an anxiolytic effect of the knockdown vector. However, there was no difference in sucrose consumption in the sucrose neophobia test and no difference in the number of fecal boli in the cold stress-induced defecation test (Table S1).

Depression-related behavior tests included sucrose preference, social contact, and the forced swim test. No effect of the knockdown vector was found in the sucrose preference test and the social contact behavior test (Table S1). However, in the forced swim test (FST), knocking down *Cyp26b1* in the NAc shell decreased the latency to the first period of immobility ($t(19) = 2.809$, $p=0.011$; Figure 2d) without changing total immobility time and climbing time (Table S1). The decreased latency in the FST suggests an increase in depression-related behavior in rats with knockdown of *Cyp26b1* in the NAcSh[32].

In sucrose pellet operant responding, knocking down *Cyp26b1* significantly enhanced hunger-motivated (85% bodyweight) responding for sucrose pellets under an FR1 schedule (Figure 2e; $t(22)= -2.124$, $p<0.05$). After their body weight recovered to 100%, the knockdown rats did not show increased nonhunger-motivated responses in FR1, but exhibited increased breakpoint in the progressive ratio test (Figure 2f; $t(22) = -2.224$, $p<0.05$), suggesting that knocking down *Cyp26b1* elevates motivation for sucrose pellets in rats.

3.2 Effects of knocking down *Crabp2* and *Fabp5* in rat NAc shell

To investigate the role of the two downstream intracellular signaling pathways of RA (Figure 1a) in depression and anxiety-related behaviors, either *AAV-Crabp2* shRNA, AAV- *Fabp5* shRNA or control AAV vector were injected into the NAcSh (n=12/treatment).

The knockdown efficiency of the hairpins was validated in HEK293 cells at both the mRNA and protein levels (Figure 1b-c). *pAAV-Crabp2* shRNA decreased *Crabp2* mRNA expression by 95% and protein expression by 81% (Figure 1b). *pAAV-Fabp5* shRNA reduced Fabp5 mRNA expression by 96% and protein expression by 28% (Figure 1c). Apparent discrepancies in knockdown efficiencies between mRNA and protein are due to the fact that mRNA detection is specific for overexpressed rat mRNA but antibodies detect both overexpressed rat protein and endogenous human protein from HEKs (these hairpins are specific to rat). The knockdown efficiency in vivo was validated by assessing changes in mRNA expression. *pAAV-Crabp2* shRNA decreased *Crabp2* mRNA expression by 47% ($t(3.994)=2.354$, $p<0.05$)and *pAAV-Fabp5* shRNA reduced *Fabp5* mRNA expression by 24% ($t(4.73)=4.331$, $p<0.01$, Figure 1e). The placements of injections were checked after all behavioral tests by examining endogenous eGFP fluorescence (example fluorescence shown in Figure 1d). No poor placement was observed, thus no animals were removed from this study.

3.4 Effects of knocking down *Crabp2* in rat NAc shell

The overall behavioral test procedure is the same as the previous procedure for *Cyp26b1* shRNA rats (Figure 1f). For locomotor activity, a 90-min session revealed no difference in total basal locomotor activity between either *Crabp2* or *Fabp5* knockdown rats compared to control rats (Table S1).

In an anxiety-related test, sucrose neophobia, knocking down *Crabp2* in the NAc shell revealed a trend for decreased sucrose intake in *Crabp2* shRNA rats (Figure 3a; $t(19) = 1.842$, $p=0.081$), suggesting a possible anxiogenic effect of the vector. We did not find a significant difference between *Crabp2* knockdown rats and control rats in the elevated plus maze or cold stress-induced defecation tests (Table S1).

In depression-related tests, no significant difference between *Crabp2* shRNA rats and control rats was found in the social contact behavior test or forced swim test (Table S1). However, in the sucrose preference test, knocking down *Crabp2* decreased sucrose intake at the 15 min ($t(22) = 2.418$, $p= 0.024$; Figure 3b) and 16-hour ($t(22) = 2.219$, $p= 0.037$; Figure 3c) time points, suggesting an anhedonic-like effect of the vector. However, total volume consumed was also decreased (Figure 3d).

In operant sucrose pellet responding, rats with *Crabp2* knocked down in the NAcSh exhibited less responding for sucrose pellets under FR2 and FR5 schedules at 85% of body weight (i.e. hunger motivated), with significant main effect of vector ($F(1,18) = 8.062$, $p = 0.011$; Figure 3e). Even after rats recovered to 100% body weight, *Crabp2* shRNA rats still exhibited lower responding than control rats under an FR5 schedule (Figure 3f; $t(21) = 2.091$, $p= 0.049$). There were no significant differences in progressive ratio responding at 85% or 100% free feed body weight (Table S1).

3.5 Effects of knocking down *Fabp5* in rat NAc shell

We did not find any statistically significant differences in anxiety- and depression-related behavioral tests in *Fabp5* knockdown rats in the NAcSh compared to controls (Table S1). These results suggest that *Fabp5* mediated intracellular signaling of RA might not be an important pathway in depression and anxiety.

4. Discussion

The focus of our laboratory for the past several years is to identify signaling and transcriptional mechanisms of the protective environmental enrichment phenotype for addiction and depression as they relate to the function of the NAc in order to identify and validate novel therapeutic targets[12,33–36]. Table 1 places the current results in their proper context[12–16,18,26,28,37]. The consistent overall NAc shell phenotype is that depression-related and addiction-related behaviors are regulated in concert while anxiety-related behaviors show opposite regulation.The current results, added to our previous results[12] show clearly that knockdown of Cyp26b1 produces a susceptible depression and addiction phenotype with a resilient anxiety phenotype. Knockdown of Crabp2 produced an increase in depression-related behavior (decreased sucrose preference) yet a trend ($p = 0.08$) for increased anxiety. While the results were statistically significant for sucrose preference,

these results as a whole do not satisfy our definition of a consistent accumbal phenotype due to the lack of regulation of anxiety-related behavior and lack of opposite modulation of depression- vs. anxiety-related behavior. *Fabp5* knockdown did not produce a depression- or anxiety-related phenotype. Thus, our overall conclusion is that manipulation of RA levels can produce a susceptible depression/resilient anxiety phenotype (via knockdown of Cyp26b1), whereas manipulation of the individual transcriptional pathways (RAR for *Crabp2* and PPARβ/δ for *Fabp5)* does not. It is possible, however, that these two transcriptional pathways are redundant, and that knocking down only one pathway is not enough to produce an effect, but this redundancy would be contrary to what has been shown in the literature (i.e. that the two transcriptional pathways work in opposition to each other)[38,39].

These results are consistent with the idea that the *Cyp26b1* knockdown phenotype is due to non-genomic effects of RA. Retinoic acid affects dendritic neuronal RNA granule control of mRNA translation, the AKT kinase signaling pathway, and the PKC signaling pathway independent of transcriptional control (and thus independent of *Crabp2* and Fabp5)[40–44]. Further studies are exploring some of these avenues. Alternatively, these results could be a function of differential cellular expression. One previous report suggests that Crabp2 is only expressed in what appear (by size) to be cholinergic interneurons in the striatum of adult brain[45]. Thus, it is possible that positive results with knockdown of Cyp26b1 could be restricted to medium spiny neurons that may not express Crabp2.

Chronic knockdown of Cyp26b1 in the NAc shell increased the motivation for operant sucrose taking under both hunger-motivated and free-feed states. However, there is no difference in the inactive lever responses, suggesting that increased active lever responses are not due to higher locomotion in the knockdown rats. Additionally, Cyp26b1 knockdown rats showed increased motivation under progressive ratio schedule of reinforcement. Our previous studies indicate that chronic knockdown of Cyp26b1 in the NAc shell increases cocaine self-administration and drug-primed cocaine seeking[12]. Our current results for sucrose operant responding expand these findings and suggest that the effects of increased RA signaling on motivated behaviors are not specific to drugs of abuse, but generalize to natural rewards. Operant sucrose responding has a hedonic element and a motivational element that may sometimes be discernable. In this case, knockdown of *Cyp26b1* produced no effect in sucrose preference, suggesting a lack of hedonic impact, but did increase sucrose pellet selfadministration, suggesting an increase in general motivation for an appetitive stimulus. The increase in responding was apparent under conditions of high hunger motivation (i.e. 85% free feed body weight) as well as low hunger motivation (100% free feed). In contrast, knockdown of *Crabp2* decreased responding for sucrose pellets under conditions of both low and high hunger motivation, but also decreased sucrose preference and decreased total fluid intake (sucrose + water). Thus, we cannot conclude if the decrease in operant sucrose responding was due to a decrease in motivation (as would be predicted by the *Cyp26b1* knockdown data), or due to anhedonia (as demonstrated with *Crabp2* shRNA on sucrose preference).

Retinoic acid is an active metabolite of vitamin A, playing critical roles in a variety of biological processes in animals and humans. Recently, retinoic acid has received increasing

attention for its role in psychiatric diseases. A study of human brains discovered that patients with depression have increased retinoic acid-induced gene expression in the dorsal prefrontal cortex[46]. Furthermore, chronic administration of retinoic acid affects the behavior of animals and humans. Long-term administration of 13-cis-retinoic acid (isotretinoin), a treatment for severe nodular acne, causes depression-related phenotypes in both humans[47–52] and animals[20,53]. In addition to 13-cis-RA, chronic treatment of all *trans-RA* also has impacts on the locomotor activity and emotional behaviors of animals[54]. Chronic activation of RA signaling in the CNS through intracerebroventricular RA infusion induces anhedonia in the sucrose preference test, indicating a typical depressive-like behavioral response[55]. Double knockout mice for retinoic acid receptor (RAR) and retinoid X receptor (RXR) (RARβ-RXRβ, RARB-RXRΓ, and RXRβ- RXRΓ) showed decreased expression of dopamine receptors in the ventral striatum[56] which is in support of the hypothesis that retinoic acid induces behavioral changes through dysfunction of the mesolimbic dopamine system. Therefore, the current results align with the previous literature and provide further evidence suggesting that the retinoic acid pathway contains a potential novel therapeutic target due to its role in depression and anxiety behaviors.

Depression and anxiety are widespread psychiatric conditions in the United States and environment and life experience have significant impacts on the resilience and susceptibility to these emotional conditions[57]. Understanding the mechanisms of the effects of the environment on neuropsychiatric disorders could provide potential therapeutic targets for the treatment of anxiety and depression. This study highlights the importance of the retinoic acid signaling pathway in regulating anxiety- and depression-related behaviors in the nucleus accumbens shell. In future studies, the spectrum of RAR-RXR target genes, PPAR-RXR target genes, and other retinoic acid intracellular signaling will be further explored.

Acknowledgements

This research has been funded by NIDA grants DA029091 and DA047102 (Thomas A. Green) and T32 DA007287 (Cunningham/Crofton). The funding source was not involved in study design, interpretation of data, writing of the report, or the decision to submit the article for publication. The authors have no conflicts of interest.

References

1. Kessler RC, Chiu WT, Demler O, Merikangas KR, Walters EE. Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. Archives of general psychiatry. 6 2005;62(6):617–627. Pmc2847357 [PubMed: 15939839]
2. Berlim MT, Fleck MP, Turecki G. Current trends in the assessment and somatic treatment of resistant/refractory major depression: an overview. Ann Med. 2008;40(2):149–159. [PubMed: 18293145]
3. Little A Treatment-resistant depression. Am Fam Physician. 2009;80(2):167–172. [PubMed: 19621857]
4. Gartlehner G, Thaler K, Hansen RA, Gaynes BN. The General and Comparative Efficacy and Safety of Duloxetine in Major Depressive Disorder. Drug safety. 2009;32(12):1159. [PubMed: 19916583]
5. Chen L Synaptic retinoic acid signaling and homeostatic synaptic plasticity. Neuropharmacology. 2012:1–10.
6. Maden M Retinoic acid in the development, regeneration and maintenance of the nervous system. Nature Reviews Neuroscience. 2007;8(10):755–765. [PubMed: 17882253]

7. Lane MA, Bailey SJ. Role of retinoid signalling in the adult brain. Progress in neurobiology. 2005;75(4):275–293. [PubMed: 15882777]
8. Topletz AR, Thatcher JE, Zelter A, Lutz JD, Tay S, Nelson WL, Isoherranen N. Comparison of the function and expression of CYP26A1 and CYP26B1, the two retinoic acid hydroxylases. Biochemical pharmacology. 2012;83(1):149–163. [PubMed: 22020119]
9. Catharine Ross A, Zolfaghari R. Cytochrome P450s in the regulation of cellular retinoic acid metabolism. Annual review of nutrition. 2011;31:65–87.
10. Yu S, Levi L, Siegel R, Noy N. Retinoic acid induces neurogenesis by activating both retinoic acid receptors (RARs) and peroxisome proliferator-activated receptor β/δ (PPARβ/δ). Journal of Biological Chemistry. 2012;287(50):42195–42205. [PubMed: 23105114]
11. Schug TT, Berry DC, Shaw NS, Travis SN, Noy N. Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. Cell. 2007;129(4):723–733. [PubMed: 17512406]
12. Zhang Y, Kong F, Crofton EJ, Dragosljvich SN, Sinha M, Li D, Fan X, Koshy S, Hommel JD, Spratt HM, Luxon BA, Green TA. Transcriptomics of Environmental Enrichment Reveals a Role for Retinoic Acid Signaling in Addiction. Frontiers in molecular neuroscience. 2016;9:119 PMC5110542 [PubMed: 27899881]
13. Green TA, Alibhai IN, Roybal CN, Winstanley CA, Theobald DE, Birnbaum SG, Graham AR, Unterberg S, Graham DL, Vialou V, Bass CE, Terwilliger EF, Bardo MT, Nestler EJ. Environmental enrichment produces a behavioral phenotype mediated by low cyclic adenosine monophosphate response element binding (CREB) activity in the nucleus accumbens. Biol Psychiatry. 1 1 2010;67(1):28–35. Pmc2860655 [PubMed: 19709647]
14. Green TA, Gehrke BJ, Bardo MT. Environmental enrichment decreases intravenous amphetamine self-administration in rats: dose-response functions for fixed- and progressive-ratio schedules. Psychopharmacology (Berl). 8 2002;162(4):373–378. [PubMed: 12172690]
15. Larson EB, Graham DL, Arzaga RR, Buzin N, Webb J, Green TA, Bass CE, Neve RL, Terwilliger EF, Nestler EJ, Self DW. Overexpression of CREB in the nucleus accumbens shell increases cocaine reinforcement in self-administering rats. J Neurosci. 11 9 2011; 31 (45) :16447–16457. Pmc3229171 [PubMed: 22072694]
16. Zhang Y, Crofton EJ, Li D, Lobo MK, Fan X, Nestler EJ, Green TA. Overexpression of DeltaFosB in nucleus accumbens mimics the protective addiction phenotype, but not the protective depression phenotype of environmental enrichment. Frontiers in behavioral neuroscience. 2014;8:297 PMC4148937 [PubMed: 25221490]
17. Eisch AJ, Bolanos CA, de Wit J, Simonak RD, Pudiak CM, Barrot M, Verhaagen J, Nestler EJ. Brain-derived neurotrophic factor in the ventral midbrain-nucleus accumbens pathway: a role in depression. Biol Psychiatry. 11 15 2003;54(10):994–1005. [PubMed: 14625141]
18. Green TA, Alibhai IN, Unterberg S, Neve RL, Ghose S, Tamminga CA, Nestler EJ. Induction of activating transcription factors (ATFs) ATF2, ATF3, and ATF4 in the nucleus accumbens and their regulation of emotional behavior. J Neurosci. 2 27 2008;28(9):2025–2032. [PubMed: 18305237]
19. Bremner JD, McCaffery P. The neurobiology of retinoic acid in affective disorders. Progress in Neuro-Psychopharmacology and Biological Psychiatry. 2008;32(2):315–331. [PubMed: 17707566]
20. O'Reilly KC, Shumake J, Gonzalez-Lima F, Lane MA, Bailey SJ. Chronic administration of 13-cis-retinoic acid increases depression-related behavior in mice. Neuropsychopharmacology. 9 2006;31(9):1919–1927. [PubMed: 16395305]
21. Ferguson SA, Cisneros FJ, Gough BJ, Ali SF. Four weeks of oral isotretinoin treatment causes few signs of general toxicity in male and female Sprague-Dawley rats. Food and chemical toxicology. 2005;43(8):1289–1296. [PubMed: 15950819]
22. Laue K, Jänicke M, Plaster N, Sonntag C, Hammerschmidt M. Restriction of retinoic acid activity by Cyp26b1 is required for proper timing and patterning of osteogenesis during zebrafish development. Development. 11 2008;135(22):3775–3787. PMC3608526 [PubMed: 18927157]
23. Pavez Lorie E, Li H, Vahlquist A, Torma H. The involvement of cytochrome p450 (CYP) 26 in the retinoic acid metabolism of human epidermal keratinocytes. Biochim Biophys Acta. 3 2009;1791(3):220–228. [PubMed: 19171200]

24. Kim SH, Rannals MD, Moore J, Maher BJ, Sawa A. Anterior Insula Mediates Social Behaviors that are Finely Regulated by Retinoic Acid and 5-HT2C Receptor in its Layer 5 Pyramidal Neurons Society for Neuroscience. Washington, D.C.; 2013.
25. Ng L, Bernard A, Lau C, Overly CC, Dong HW, Kuan C, Pathak S, Sunkin SM, Dang C, Bohland JW, Bokil H, Mitra PP, Puelles L, Hohmann J, Anderson DJ, Lein ES, Jones AR, Hawrylycz M. An anatomic gene expression atlas of the adult mouse brain. Nat Neurosci. 3 2009;12(3):356–362. [PubMed: 19219037]
26. Green TA, Alibhai IN, Hommel JD, DiLeone RJ, Kumar A, Theobald DE, Neve RL, Nestler EJ. Induction of inducible cAMP early repressor expression in nucleus accumbens by stress or amphetamine increases behavioral responses to emotional stimuli. J Neurosci. 8 9 2006;26(32):8235–8242. [PubMed: 16899718]
27. Benzon CR, Johnson SB, McCue DL, Li D, Green TA, Hommel JD. Neuromedin U receptor 2 knockdown in the paraventricular nucleus modifies behavioral responses to obesogenic high-fat food and leads to increased body weight. Neuroscience. 1 31 2014;258:270–279. PMC3898339 [PubMed: 24269937]
28. Crofton EJ, Nenov MN, Zhang Y, Scala F, Page SA, McCue DL, Li D, Hommel JD, Laezza F, Green TA. Glycogen synthase kinase 3 beta alters anxiety-, depression-, and addiction-related behaviors and neuronal activity in the nucleus accumbens shell. Neuropharmacology. 1 23 2017;117:49–60. [PubMed: 28126496]
29. Hommel JD, Sears RM, Georgescu D, Simmons DL, DiLeone RJ. Local gene knockdown in the brain using viral-mediated RNA interference. Nature medicine. 2003;9(12):1539–1544.
30. Mezadri T, Batista G, Portes A, Marino-Neto J, Lino-de-Oliveira C. Repeated rat-forced swim test: reducing the number of animals to evaluate gradual effects of antidepressants. Journal of neuroscience methods. 2011;195(2):200–205. [PubMed: 21167866]
31. Slattery DA, Cryan JF. Using the rat forced swim test to assess antidepressant-like activity in rodents. Nature protocols. 2012;7(6):1009. [PubMed: 22555240]
32. Castagne V, Porsolt RD, Moser P. Use of latency to immobility improves detection of antidepressant-like activity in the behavioral despair test in the mouse. Eur J Pharmacol. 8 15 2009;616(1–3):128–133. [PubMed: 19549518]
33. Fan X, Li D, Lichti CF, Green TA. Dynamic proteomics of nucleus accumbens in response to acute psychological stress in environmentally enriched and isolated rats. PloS one. 2013;8(9):e73689 Pmc3767735 [PubMed: 24040027]
34. Fan X, Li D, Zhang Y, Green TA. Differential phosphoproteome regulation of nucleus accumbens in environmentally enriched and isolated rats in response to acute stress. PloS one. 2013;8(11):e79893 Pmc3838351 [PubMed: 24278208]
35. Lichti CF, Fan X, English RD, Zhang Y, Li D, Kong F, Sinha M, Andersen CR, Spratt H, Luxon BA, Green TA. Environmental enrichment alters protein expression as well as the proteomic response to cocaine in rat nucleus accumbens. Frontiers in behavioral neuroscience. 2014;8:246 Pmc4104784 [PubMed: 25100957]
36. Zhang Y, Crofton EJ, Fan X, Li D, Kong F, Sinha M, Luxon BA, Spratt HM, Lichti CF, Green TA. Convergent transcriptomics and proteomics of environmental enrichment and cocaine identifies novel therapeutic strategies for addiction. Neuroscience. 10 4 2016;339:254–266. PMC5118094 [PubMed: 27717806]
37. Wallace DL, Vialou V, Rios L, Carle-Florence TL, Chakravarty S, Kumar A, Graham DL, Green TA, Kirk A, Iniguez SD, Perrotti LI, Barrot M, DiLeone RJ, Nestler EJ, Bolanos- Guzman CA. The influence of DeltaFosB in the nucleus accumbens on natural reward- related behavior. J Neurosci. 10 8 2008;28(41):10272–10277. PMC2653197 [PubMed: 18842886]
38. Schug TT, Berry DC, Shaw NS, Travis SN, Noy N. Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. Cell. 5 18 2007;129(4):723–733. Pmc1948722 [PubMed: 17512406]
39. Schug TT, Berry DC, Toshkov IA, Cheng L, Nikitin AY, Noy N. Overcoming retinoic acid-resistance of mammary carcinomas by diverting retinoic acid from PPARbeta/delta to RAR. Proc Natl Acad Sci U S A. 5 27 2008;105(21):7546–7551. Pmc2396692 [PubMed: 18495924]

40. Chen N, Onisko B, Napoli JL. The nuclear transcription factor RARalpha associates with neuronal RNA granules and suppresses translation. J Biol Chem. 7 25 2008;283(30):20841–20847. Pmc2475717 [PubMed: 18495661]
41. Garcia-Regalado A, Vargas M, Garcia-Carranca A, Arechaga-Ocampo E, Gonzalez-De la Rosa CH. Activation of Akt pathway by transcription-independent mechanisms of retinoic acid promotes survival and invasion in lung cancer cells. Molecular cancer. 2013;12:44 Pmc3665688 [PubMed: 23693014]
42. Hoyos B, Imam A, Chua R, Swenson C, Tong GX, Levi E, Noy N, Hammerling U. The cysteine-rich regions of the regulatory domains of Raf and protein kinase C as retinoid receptors. The Journal of experimental medicine. 9 18 2000;192(6):835–845. Pmc2193291 [PubMed: 10993914]
43. Hoyos B, Imam A, Korichneva I, Levi E, Chua R, Hammerling U. Activation of c-Raf kinase by ultraviolet light. Regulation by retinoids. J Biol Chem. 6 28 2002;277(26):23949–23957. [PubMed: 11971897]
44. Kurie JM, Younes A, Miller WH Jr.,, Burchert M, Chiu CF, Kolesnick R, Dmitrovsky E. Retinoic acid stimulates the protein kinase C pathway before activation of its beta- nuclear receptor during human teratocarcinoma differentiation. Biochim Biophys Acta. 11 7 1993;1179(2):203–207. [PubMed: 8218362]
45. Zetterstrom RH, Lindqvist E, Mata de Urquiza A, Tomac A, Eriksson U, Perlmann T, Olson L. Role of retinoids in the CNS: differential expression of retinoid binding proteins and receptors and evidence for presence of retinoic acid. Eur J Neurosci. 2 1999;11(2):407–416. [PubMed: 10051741]
46. Haybaeck J, Postruznik M, Miller CL, Dulay JR, Llenos IC, Weis S. Increased expression of retinoic acid-induced gene 1 in the dorsolateral prefrontal cortex in schizophrenia, bipolar disorder, and major depression. Neuropsychiatric disease and treatment. 2015;11:279. [PubMed: 25678793]
47. Hazen PG, Carney JF, Walker AE, Stewart JJ. Depression--a side effect of 13-cis- retinoic acid therapy. J Am Acad Dermatol. 8 1983;9(2):278–279.
48. Bigby M, Stern RS. Adverse reactions to isotretinoin: a report from the Adverse Drug Reaction Reporting System. Journal of the American Academy of Dermatology. 1988; 18(3):543–552. [PubMed: 3280622]
49. Scheinman PL, Peck GL, Rubinow DR, DiGiovanna JJ, Abangan DL, Ravin PD. Acute depression from isotretinoin. Journal of the American Academy of Dermatology. 1990;22(6):1112–1114. [PubMed: 2142496]
50. Byrne A, Hnatko G. Depression associated with isotretinoin therapy. The Canadian Journal of Psychiatry. 1995;40(9):567–567. [PubMed: 8574993]
51. Wysowski DK, Pitts M, Beitz J. Depression and suicide in patients treated with isotretinoin. N Engl J Med. 2 2001;344(6):460.
52. Barak Y, Wohl Y, Greenberg Y, Dayan YB, Friedman T, Shoval G, Knobler HY. Affective psychosis following Accutane (isotretinoin) treatment. International clinical psychopharmacology. 2005;20(1):39–41. [PubMed: 15602115]
53. Trent S, Drew CJ, Mitchell PJ, Bailey SJ. Chronic treatment with 13-cis-retinoic acid changes aggressive behaviours in the resident-intruder paradigm in rats. Eur Neuropsychopharmacol. 12 2009;19(12):876–886. [PubMed: 19651495]
54. Cai L, Yan X-B, Chen X-N, Meng Q-Y, Zhou J-N. Chronic all-trans retinoic acid administration induced hyperactivity of HPA axis and behavioral changes in young rats. European Neuropsychopharmacology. 2010;20(12):839–847. [PubMed: 20659790]
55. Hu P, Wang Y, Liu J, Meng FT, Qi XR, Chen L, van Dam AM, Joëls M, Lucassen PJ, Zhou JN. Chronic retinoic acid treatment suppresses adult hippocampal neurogenesis, in close correlation with depressive - like behavior. Hippocampus. 2016;26(7):911–923. [PubMed: 26860546]
56. Krçz W, Ghyselinck N, Samad TA, Dupé V, Kastner P, Borrelli E, Chambon P. Impaired locomotion and dopamine signaling in retinoid receptor mutant mice. Science (New York, NY). 1998;279(5352):863–867.
57. Crofton EJ, Zhang Y, Green TA. Inoculation stress hypothesis of environmental enrichment. Neurosci Biobehav Rev. 2 2015;49:19–31. PMC4305384 [PubMed: 25449533]

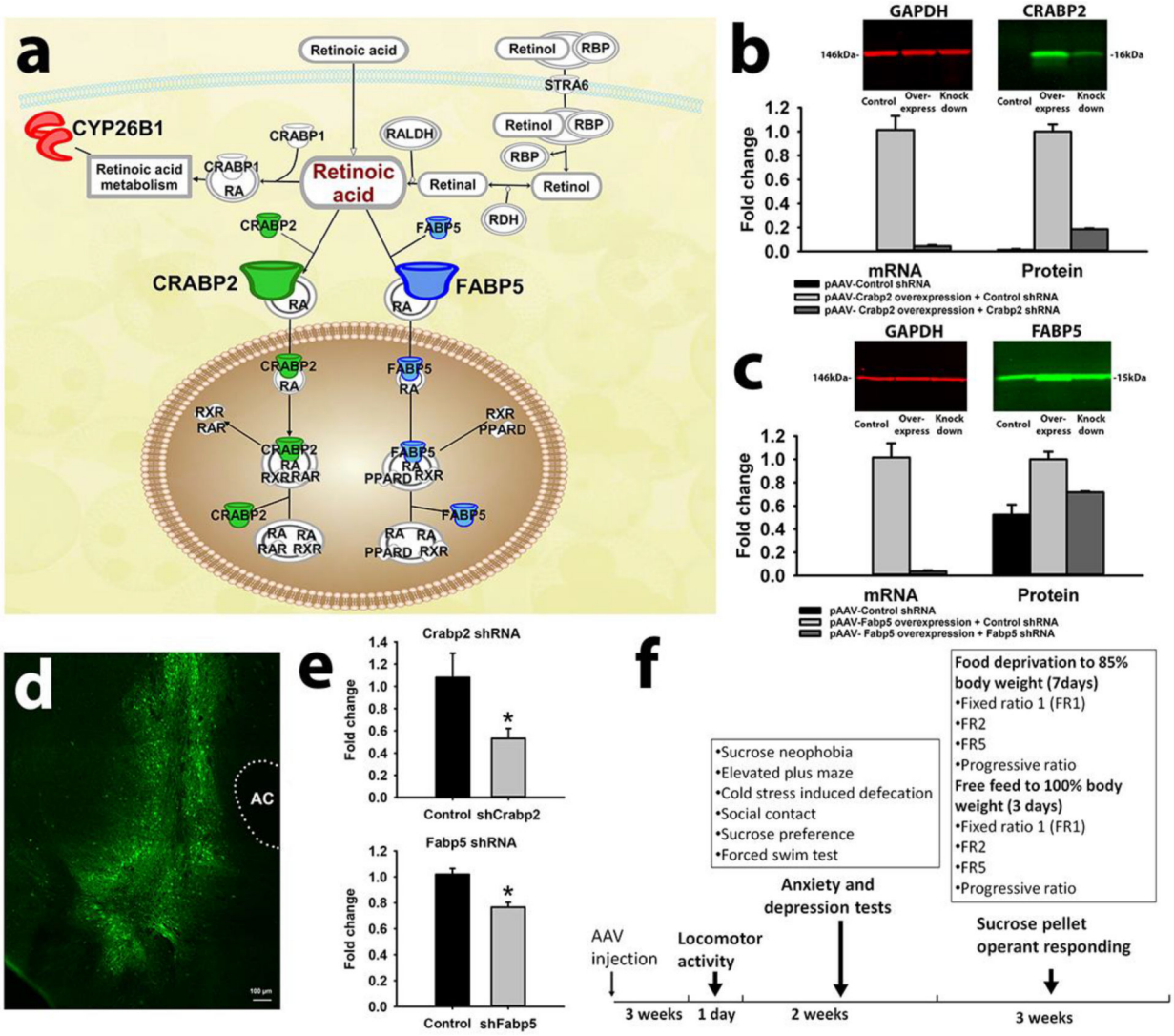

Figure 1. Manipulation of retinoic acid signaling pathway in rat nucleus accumbens shell.

a. Canonical intracellular pathway of RA signaling. Retinoic acid is synthesized by dehydrogenases and degraded by *Cyp26b1* (red) in the cytoplasm. *Crabp2* (green) and *Fabp5* (blue) shuttle RA into the cell nucleus to activate transcription through RAR-RXR or PPAR-RXR complexes, respectively. Figure created with Ingenuity Pathways Analysis. b. In vitro validation fold change of *Crabp2* mRNA (±SEM) and protein (±SEM) in HEK-293 cells transfected with pAAV-control shRNA, pAAV-control shRNA with *Crabp2* overexpression plasmid, or pAAV- Crabp2 shRNA and *Crabp2* overexpression plasmid. c. Fold change of *Fabp5* mRNA (mean ±SEM) and protein (mean ±SEM) in HEK-293 cells transfected with pAAV-control shRNA, pAAV-control shRNA with *Fabp5* overexpression plasmid, or *pAAV-Fabp5* shRNA and *Fabp5* overexpression plasmid. Full-length blot is presented in Supplementary Figure 2. d. Representative immunofluorescence staining of eGFP showing the placement and *in vivo* titer of AAV- shRNA vector. e. In vivo knockdown of *Crabp2* (top) and *Fabp5* (bottom) as measured by qPCR. f. Schematic diagram showing the order and timeline of all the behavioral tests.

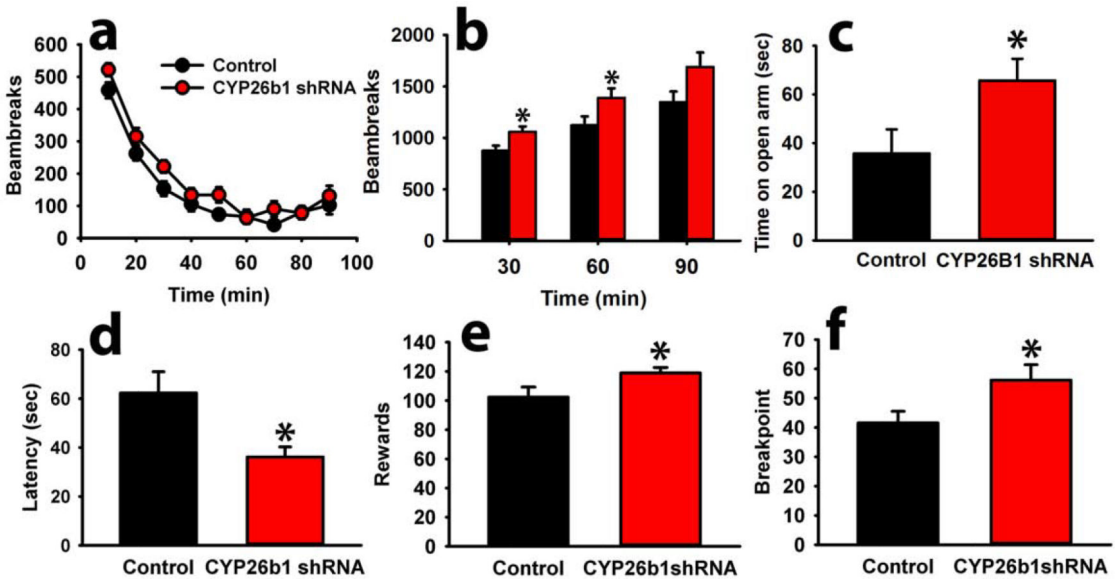

Figure 2. Effects of knocking down *Cyp26b1* in rat NAc shell on locomotor, anxiety, and depression-related behavior.

a-b. Effect of knocking down *Cyp26b1* in the NAc shell on spontaneous locomotor activity (a. timecourse and b. cumulative). c. Knocking down *Cyp26b1* in the NAc shell increases time spent in the open arm of the elevated plus maze (5 min session). d. *Cyp26b1* knockdown in the NAc shell decreases the latency to first immobility in the forced swim test. e. Knocking down *Cyp26b1* in the NAc shell increased the number of sucrose pellets earned under an FR1 schedule at 85% body weight. f. Knocking down *Cyp26b1* in the NAc shell increased breakpoint in the progressive ratio (PR) test under 100% body weight. *p<0.05, n=11 – 12/group; all plots represent mean ±SEM.

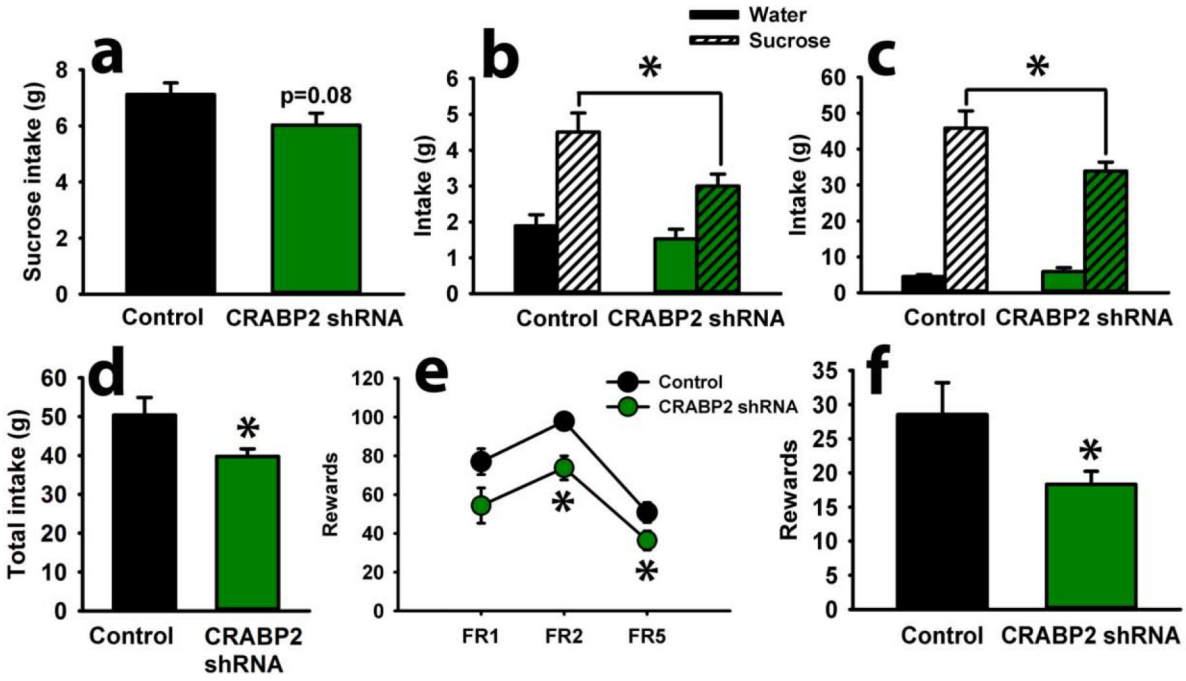

Figure 3. Effects of knocking down *Crabp2* in rat NAc shell on anxiety and depression- related behaviors.

a. Knocking down *Crabp2* in the NAc shell decreases sucrose intake in the sucrose neophobia test. b-c. Knocking down *Crabp2* decreases sucrose intake in 15min test (b) and 16 hr test (c) in the sucrose preference test. d. Total fluid intake across 16 hrs (sucrose + water). e-f. Knocking down *Crabp2* decreased sucrose pellet responding under FR2 and FR5 schedules at 85% of free-feed body weight (e), and under an FR5 schedule at 100% body weight (f) *p<0.05, n=9–11/group; all plots represent mean ±SEM.

Table 1.

Overview of current results in context of previous work

Manipulation	Cocaine Self-Administration	Depression-related behavior	Anxiety-related behavior	Spontaneous locomotor activity
Resilient Depression with Susceptible Anxiety Phenotype				
Environmental Enrichment[13]	↓↓↓↓	↓↓↓	↑↑	↓
CREB inhibition[13,15]	↓↓	↓↓↓	↑↑↑	↓
ΔFosB overexpression[16,37]	↓↓↓	↓	NC	↓
ATF2 overexpression[18]		↓↓	↑	NC
Susceptible Depression with Resilient Anxiety Phenotype				
GSK3β shRNA[28]	↑↑	↑↑	↓↓	NC
ATF3 overexpression[18]		↑	↓↓	NC
ATF4 overexpression[18]		↑↑	↓↓	↑
Cyp26b1 shRNA	↑↑↑↑[12]	↑	↑	↑
Inconsistent or No Change in Phenotype				
NR4A3 shRNA		NC	NC	NC
Scn4B shRNA		NC	NC	NC
Crabp2 shRNA		↑	NC (trend ↑)	NC
Fabp5 shRNA		NC	NC	NC

NC = no change (i.e. no significant difference from controls), blank = not assessed, shaded boxes indicate the findings of the current study.