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Activity-regulated cytoskeleton-associated protein (Arc/Arg3.1) regulates anxiety- and novelty-related behaviors

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Abstract

The activity-regulated cytoskeleton-associated protein (Arc, also known as Arg3.1) regulates glutamatergic synapse plasticity and has been linked to neuropsychiatric illness; however, its role in behaviors associated with mood and anxiety disorders remains unclear. We find that stress upregulates Arc expression in the adult mouse nucleus accumbens (NAc) – a brain region implicated in mood and anxiety behaviors. Global *Arc* knockout mice have altered AMPAR-subunit surface levels in the adult NAc, and the Arc-deficient mice show reductions in anxiety-like behavior, deficits in social novelty preference, and anti-depressive-like behavior. Viral-mediated expression of Arc in the adult NAc of male, global *Arc* KO mice restores normal levels of anxiety-like behavior in the elevated plus maze. Consistent with this finding, viral-mediated reduction of Arc in the adult NAc reduces anxiety-like behavior in male, but not female, mice in the elevated plus maze. NAc-specific reduction of Arc also produced significant deficits in both object and social novelty preference tasks. Together our findings indicate that Arc is essential for regulating normal mood-and anxiety-related behaviors and novelty discrimination, and that Arc's function within the adult NAc contributes to these behavioral effects.

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Introduction

Altered synaptic function is evident in many neuropsychiatric illnesses, including schizophrenia, major depressive disorder, and several intellectual and developmental disabilities^{1–6}. Among the genes critical for proper glutamatergic synaptic function and plasticity is the activity-regulated cytoskeleton-associated protein (*Arc*, also known as *Arg3.1*), a brain-enriched immediate early gene that is expressed in principal neuron cell types—both excitatory and inhibitory^{7,8}. *Arc* is expressed broadly at a low basal level, but following synaptic activity^{8,9}, *Arc* mRNA is rapidly and transiently induced in a process that is regulated by MEF2 (myocyte enhancer factor 2), CREB (cyclic adenosine monophosphate response element binding protein), and SRF (serum response factor) transcription factor binding at an activity-sensitive enhancer sequence¹⁰. *Arc* mRNA is trafficked to neuronal dendrites^{11,12} via RNA binding proteins (eg. FMRP), and its protein synthesis can be stimulated by neuronal activity^{13–15}, positioning it to support synaptic plasticity. *Arc* influences synapse function via multiple pathways, including interaction with F-actin⁹, and it regulates glutamatergic AMPAR trafficking^{16–18}. In addition, *Arc* protein can be trafficked to the cell nucleus where it is reported to regulate the transcription of the AMPAR subunit, *GluA1*¹⁹. *Arc* regulates AMPAR internalization, in part, by recruiting clathrin-mediated endocytic machinery to the synapse^{16,20}, thus promoting synapse weakening^{21,22}.

Arc expression is induced in a number of brain regions following emotionally-relevant experiences, including exposure to novelty^{23,24}, environmental enrichment²⁵, and stressful experiences^{26,27}. As such, *Arc* is well positioned to influence mood-related behaviors. It is also required for numerous learning and memory tasks, including memory consolidation^{28,29}, spatial learning and memory^{28,30–32}, and fear learning^{28,33,34}.

In this study, we find that global, constitutive knockout of the *Arc* gene (*Arc* KO) produces mice with reduced anxiety-like behavior, reduced depressive-like behavior, and reduced novelty discrimination. Moreover, we observe that *Arc* KO mice show an increase in surface AMPAR levels in the adult NAc – a brain region linked to mood, anxiety and reward. Stress induces *Arc* mRNA and protein expression the NAc, and reduction of *Arc* in the adult NAc is sufficient to produce some, but not all, of the behaviors observed in global *Arc* KO mice.

Materials and Methods

Experimental animals

Arc-GFP homozygous mice (Wang et. al, 2006; RRID:MGI:3694763) were backcrossed to congenicity on the C57BL/6N (Charles River Laboratories, Wilmington, MA; RRID:IM-SR_CRL:27) strain. All *Arc* KO experiments (Figures 1–3 and 4a-c) used 8–12-week old male *Arc* KO and WT littermates. *Arc* induction experiments were conducted using 8–10-week old male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) (Fig.4). The *Arc* shRNA experiments (Fig.4d–f) used 8–12-week old C57BL/6N male and female mice. Anxiety and locomotor experiments were conducted on consecutive days in each cohort. Except when noted, mice were group housed with same sex littermates (2 – 5 mice/cage). Behavioral examinations took place in the dark I phase, with mice housed in reverse dark-light cycle. Biochemistry experiments were conducted on mice housed in standard lighting

cycle. Experimenters were blind to group during experimental testing and each experiment reflects the mean of two or more independent cohorts of mice. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Southwestern Medical Center, McLean Hospital, and/or the Medical University of South Carolina.

Anxiety-related behaviors

Open field, elevated plus maze, and light/dark box testing were conducted as previously described³⁵. Briefly, mice were placed in a well-lit open field box (175 – 180 lux center, 125 – 130 lux periphery) and behavior was tracked inside and outside a predefined center zone for 5 min. For elevated plus maze (100 lux room), mice were placed in the center of a cross-maze (32" above the floor) and movement was tracked for 5 min.

Performance was tracked by video monitoring using AnyMaze 5.1 (Stoelting Co, Wood Dale, IL) or Ethovision 3.0 software (Noldus, Leesburg, VA). For light/dark box testing, mice were placed in a black Plexiglas chamber (25 cm × 26 cm) and allowed to explore (2-min habituation), after which, a small door was opened, allowing access to the illuminated side of the apparatus (25 cm × 26 cm, ~1700 lux) for 10 min. Time in each side was monitored by infrared beams. $n = 8 - 21$ mice/genotype.

Locomotor activity test

Following a 30-minute acclimation period, mice were placed into a standard housing cage filled with a thin layer of clean bedding and covered with a lid. Test cages were situated inside of black Plexiglas chambers (22.5" × 14.5" × 9.4") equipped with photobeam arrays (San Diego Instruments, San Diego, CA) under red light room illumination. Activity was collected in 5-minute bins for 2-hours via 5 evenly spaced beams across the cage length. $n = 8 - 21$ mice/genotype.

Three-chamber sociability test

Sociability tests took place in a red-light illuminated (25 – 30 lux) arena divided into 3 equal compartments, where the outer compartments each housed a small wire container and were connected to the middle compartment via a small door. Individual test animals received 3 sequential 10-min trials, always starting in the middle compartment, between which the apparatus was cleaned and cup contents were manipulated. In Trial 1, both cups were empty. In Trial 2, a novel WT mouse was placed inside one cup and the other cup either remained empty (Fig. 2) or contained a novel object (Fig. 6). In Trial 3, the familiar mouse (from prior trial) was again placed in one cup and a novel WT mouse was contained in the other cup. The sides of the apparatus for cup contents were counterbalanced between groups. The time the test animal spent in each compartment of the arena was recorded throughout the test using video monitoring software AnyMaze 5.1 (Stoelting Co, Wood Dale, IL) or Ethovision 3.0 software (Noldus, Leesburg, VA). $n = 9 - 21$ mice/group.

Acute social defeat stress

Social defeat stress (SDS) was performed essentially as described³⁶. Following 10 days of gentle handling, mice received 5 min of physical contact with a novel aggressor mouse (CD1

strain, Charles River Laboratories, Wilmington, MA; RRID:IMSR_CRL:22). Non-stressed control mice received daily transport and handling. $n = 5$ mice/group.

Porsolt (forced) swimming test (FST)

FST was performed as described previously³⁵. Mice were placed in a 4-liter beaker of water (21–25°C) and monitored for 6 minutes. Fore-and hindlimb movements were scored by an experimenter blind to genotype during the last 4 min of the 6-min test. The total time “immobile” was recorded, and “immobile” was defined as only minor movements necessary to maintain the head above water. $n = 9 - 16$ mice/group.

Sucrose preference test

Sucrose preference was performed as described previously³⁷. Briefly, mice were acclimated to 2 drinking bottles (50 ml conical tubes with sippers) alternating days of water or sucrose (1% w/v). Following 4 days of alternating solution habituation, mice receive 24 hr access to 2 tubes, one filled with water and the other with 1% sucrose. Volume consumed was measured each day and bottle position was swapped to avoid side bias. After 4 days, total sucrose preference was calculated as the volume of sucrose consumed divided by the total volume of sucrose and water consumed. $n = 9 - 11$ mice/genotype.

Novel object recognition

Novel object recognition tests were performed in an open field chamber under red-light conditions (25 – 30 lux). Mice were initially acclimated to the open field in a 5-min session. Following acclimation, mice were removed from the apparatus and two identical objects (large binder clips, metal arms removed, covered in high I or low grit sand paper) were placed in opposite corners of the field. In “same object” trials, time spent in a 16cm circular interaction zone centered around the object, was recorded for 10 min. Following the “same object” trial, mice were removed from the apparatus, objects were cleaned, and one of the now-familiar objects was replaced with a novel object (binder clip with opposite grit sandpaper). In “novel object” trials, time spent in interaction zone was recorded for ten minutes. Mice that fail to interact (movement <10m) in either trial were excluded from further analysis. The type of familiar and novel object, as well as the location of the novel object, were counterbalanced within and between groups. The total time spent interacting (sum of familiar and novel), time interacting (novel or familiar), and novelty discrimination (difference in novel vs. familiar interaction divided by total time interacting) were calculated from video recorded sessions using video monitoring (AnyMaze 5.1, Stoelting Co, Wood Dale, IL).

Tissue collection

Due to induction of immediate early genes following cage mate separation stress (M. Taniguchi, unpublished observations), single-housed mice were used for *Arc* mRNA and protein studies. One (mRNA), 2 or 4 hours (protein) after social defeat stress mice were rapidly decapitated, their brains were removed and coronal slices (4mm) were prepared in ice-cold phosphate buffered saline, as previously described³⁸. Bilateral tissue punches were taken from 1–2 slices containing the NAc (16-gauge for rostral, starting Bregma ~1.70 mm,

and either 14- or 16-gauge for caudal, starting Bregma ~1.10 mm) using the anterior commissure as a guide. A 16-gauge punch was also used to collect amygdala bilaterally from a single 1 mm slice (Bregma ~-1.82) and dorsal hippocampus and prefrontal cortex were hand-dissected bilaterally from a single slice (Bregma ~-1.82 and ~-1.94, respectively). Samples were then snap-frozen in a dry ice/ethanol bath and stored at -80°C for subsequent analysis by western blotting or qRT-PCR. For shRNA validation, freshly collected mouse brains were sliced into ~400 µm slices using a vibratome (Leica). Slices were mounted onto slides and virally transduced NAc tissue was removed using a 16-gauge punch after visualizing on a fluorescent microscope.

Immunoblotting

For western blotting, tissues were sonicated in a small amount of sucrose lysis buffer containing inhibitors (11% (w/v) sucrose, 0.005 M HEPES, 1% (w/v) SDS, 1 mM NaF, 1 mM Na₃VO₄, 0.1 µM cyclosporin A, 0.1 µM okadaic acid, 1 mM PMSF, 1 mM EDTA, 1X Roche or Pierce EDTA-free protease inhibitor tablet), heated at 98°C for 10 min, centrifuged briefly, and frozen at -80°C until protein quantification and SDS-PAGE. Protein quantification was assessed by modified-Lowry using the DC Protein Assay Kit (Biorad, Hercules, CA). SDS-PAGE gels (6–10%) were loaded with equal amounts of total protein/well (for Arc experiments, 20–30 µg/well; for GluA1 and GluA2/3, 10–20 µg/well). Proteins were transferred to Millipore Immobilon® FL PVDF membrane overnight at 40V at 4°C. Membranes were blocked in 5% (w/v) BSA in 1X TBS-T or Odyssey Blocking Buffer (diluted 1:1 in 1X PBS), rinsed in 1X TBS-T, and incubated overnight at 4°C or ~2h at room temperature in primary antibodies diluted in 3% (w/v) BSA in 1X TBS-T or Odyssey Blocking Buffer (diluted 1:1 in 1X PBS) plus 0.02% (w/v) NaN₃. Primary antibodies: GluA1 1:1000 (anti-GluR1, PA1-37776, Thermo Fisher; RRID:AB_1074654), GluA2/3 1:1000 (anti-GRIA2, AB1506, Millipore; RRID:AB_90710), Arc 1:1000 (156-002, Synaptic Systems; RRID:AB_887695) or 1:100 (sc-17839, Santa Cruz; RRID:AB_626696) and p-tubulin 1:10,000 (05-661, Millipore; RRID:AB_309885; 8226, Abcam; RRID:AB_306371). Following primary antibody exposure, membranes were rinsed in 1X TBS-T and exposed to secondary antibodies for 40–60 min at room temperature. Secondary antibodies: HRP-conjugated goat anti-rabbit and HRP-conjugated goat anti-mouse (1:10,000; Calbiochem) in 3% (w/v) BSA in 1X TBS-T or IRDye 800CW goat anti-rabbit and IRDye 680RD goat anti-mouse IgG (1:20,000; LiCor) in Odyssey Blocking Buffer (diluted 1:1 in 1X PBS) with 0.1% (w/v) Tween-20 and 0.01% (w/v) SDS. After final rinses, membranes were scanned and analyzed using Odyssey CLX and ImageStudio (LiCor) or GelDocImager and ImageLab (BioRad). *n* = 4 – 5 mice/condition (Stress Induction).

Quantitative real-time PCR

RNA isolation, reverse transcription, and quantitative real-time PCR were carried out as previously described⁴¹ on samples collected as detailed above. Briefly, total mRNA was extracted using Trizol reagent (QIAGEN, RRID:SCR_008539) and miRNA Easy kit (Qiagen). cDNA conversion was conducted using SuperScript III RT (Thermo Fisher Scientific, RRID:SCR_008452) and random hexamer primers. *Arc* expression was determined by running reactions of 6–9 ng of cDNA. Fold changes relative to *GAPDH* were determined using the AACt method, in which mean fold-change (2^{-CtAVE}) and S.E.M.

$(\text{abs}(((2^{-\text{CtAVE}} \times 2^{-\text{CtSEM}}) - (2^{-\text{CtAVE}} / 2^{-\text{CtSEM}})) / 2))$ were determined and expressed relative to control condition. The primers used to amplify *Arc* RNA were 5'-AGCAGCAGACCTGACATCCT-3' (forward) and 5'-GGCTTGTCTTCACCTTCAGC-3' (reverse). The primers used to amplify *GAPDH* were 5'-AGGTCGGTGTGAACGGATTG-3' (forward) and 5'-AGACCATGTAGTTGAGGTCA-3' (reverse). The primers used to amplify *GluA1* were 5'-CACGATCCCAGCCAGAATCA-3' (forward) and 5'-CTCCGCCATCACCTTCACAC-3' (reverse). $n = 5 - 6$ mice/genotype (*GluA1* expression); $n = 5$ mice/condition (*Arc* stress induction); $n = 2 - 3$ mice/condition (*in vivo* sh*Arc* validation).

Cell-surface protein crosslinking

Surface crosslinking was performed essentially as previously described³⁹. In brief, tissue samples were chopped into ~400 μm sections, added to pre-chilled aCSF (1 mL) containing 2 mM BS³ crosslinker (Pierce), and incubated with gentle rotation at 4°C for 30 min. Cross-linking was quenched with the addition of 100 mM glycine before sonication in tissue lysis buffer and western blot analysis. Following 100 mM glycine quenching, and samples were then pelleted by centrifugation (20,000 $\times g$) for 2 min at 4 °C, then resuspended in freshly made ice-cold tissue lysis buffer containing the following: 25 mM HEPES, pH 7.4, 500 mM NaCl, 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 20 mM NaF, 1X protease inhibitor cocktail tablet (Roche), 0.1% NP-40, 1 μM okadaic acid, 1 μM microcystin-LF, and 1 mM sodium orthovanadate. Tissue was homogenized by sonication (30% amplitude) for 5 s, or until tissue was completely dissolved in lysis buffer. After a brief centrifugation step to pellet debris, supernatant was collected and processed for western blotting, as described above. $n = 3 - 6$ mice/genotype.

Vira I-mediated Arc manipulation

pAAV2-Arc-IRES-mCherry was constructed by PCR amplification of exon 1 (encodes entire protein coding p sequence) of *Arc* from C57Bl/6 mouse genomic DNA with restriction sites (BamHI, EcoRI) added to 5' and 3' ends. The IRES-mCherry region (from pMSCV-IRES-mCherry, Addgene #52114; RRID:SCR_002037) and *Arc* exon1 were sequentially ligated into the pAAV2 vector⁴⁰. Diagnostic digests and DNA sequencing were used to confirm orientation and proper sequences. Following sequencing and endotoxin-free maxi-prep (Qiagen), plasmid DNA was packaged into high titer (10^{12} viral particles/ml) virus (Vector Biolabs, Malvern, PA). High titer (10^{12} viral particles/ml) AAV2-GFP control virus was purchased from Vector Biolabs (7005; RRID:SCR_011010). Viral-mediated expression of AAV2-Arc-IRES-mCherry or AAV2-GFP was performed as previously described⁴¹. Briefly, mice received bilateral NAc (+1.6 A/P, +1.5 M/L, -4.4 D/V from Bregma, 10° angle) infusions (500 nL each) of AAV2-Arc-IRES-mCherry or AAV-GFP at a rate of 50 nL/30 s and were allowed a 10 min post-infusion rest. Mice were given 2 weeks following injection for recovery and viral expression. Genotype, expression, and targeting was confirmed using immunohistochemistry (anti-Arc 1:1000, 156-002, Synaptic Systems; RRID:AB_887695 or 1:100, sc-17839, Santa Cruz; RRID:AB_626696 and anti-GFP, 1:100, Aves Labs, RRID:AB_10000240 or anti-NeuN, 1:250, Millipore, RRID:AB_2298772) followed by anti-chicken 488 and anti-rabbit 594 conjugated IgY (1:200, Jackson ImmunoResearch). $n = 4 - 6$ mice/condition.

pAAV2-shArc-eGFP (and shLuciferase) were constructed by the generation, in vitro oligomerization and phosphorylation of custom DNA oligos (IDT) directed against Arc or Luciferase (Arc:

AATCTGCAGGATCACATTGGGCTTCCTGTACCCCAATGTGATCCTGCAGATTTTTT
T; Luciferase:

CTTACGCTGAGTACTTCGATTCAAGAGATCGAAGTACTCAGCGTAAGTTTTT)⁴².

pAAV2-shRNA-eGFP⁴⁰ was digested with XbaI and SpaI and custom oligos were ligated into the dephosphorylated vector. Following sequencing confirmation and endotoxin-free maxi-prep (Qiagen), plasmid DNA was packaged into AAV2 and purified (University of South Carolina Viral Vector Core, Columbia, SC). Using the same coordinates described for AAV2-Arc-IRES-mCherry studies, Using the same coordinates described for AAV2-Arc-IRES-mCherry studies, 0.4 – 0.5 ul of AAV2-shArc-eGFP or AAV2-shLucif-eGFP was infused bilaterally into the NAc at a rate of 50 nl/30 s. $n = 8 - 12$ mice/condition.

Experimental Design and Statistics

Datasets were examined for normality using D'Agostino & Pearson tests and F tests to compare variances. Statistical analyses were performed using Student's t-tests (Mann Whitney U or Welch's correction as needed), Two-Way ANOVA (with or without reported measures of time). Significant interactions and planned contrasts were analyzed using Tukey's or Bonferroni's post hoc tests. All statistics were performed using GraphPad Prism (GraphPad Software), and statistical significance was set at $\alpha = 0.05$. In figures, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

Arc KO mice display reduced anxiety-like behavior.

Arc-GFP homozygous knock-in (loss-of-function) mutant mice, which have the endogenous Arc open-reading frame replaced by green fluorescent protein (GFP)⁴³, referred to here as *Arc* KO mice, were examined. We conducted a series of assessments comparing innate anxiety in *Arc* KO mice and wild-type littermates. *Arc* KO mice showed a consistent anxiolytic-like phenotype across three different behavioral tests. In the open field (OF; Fig. 1a), they spent significantly more time in the predefined centerzone (Mann-Whitney $U = 138$, $n_{WT} = 20$, $n_{KO} = 25$, $p = 0.0099$) than WT littermate controls. In the elevated plus maze (EPM; Fig. 1b), they spent more time in the open arms of the apparatus (Welch's correction, $t_{(29.84)} = 3.643$, $p = 0.0010$). Finally, when examined in the light/dark box (L/D; Fig. 1c), *Arc* KO mice had a significant increase in time spent in the illuminated compartment (Welch's correction, $t_{(28.6)} = 2.119$, $p = 0.0429$). Interestingly, the *Arc* KO mice traveled farther than their WT littermates on all of these assays (OF: Fig. S1a, $t_{(42)} = 5.335$, $p < 0.0001$; EPM: Fig. S1b, $t_{(41)} = 5.134$, $p < 0.0001$; L/D: Fig. S1c, $t_{(42)} = 2.568$, $p = 0.0139$); however, general motor hyperactivity was not detected in the locomotor test (Fig. 1d–f), suggesting that the increased distance traveled in the anxiety tests are not due to simple, general motor hyperactivity.

Arc KO mice show reduced social novelty preference and anti-depressive phenotypes

In the three-chamber sociability assay (Fig. 2a), mice were given the option of interacting with a novel conspecific mouse or an empty holding chamber located in equal-sized chambers on opposite sides of the apparatus. *Arc* KO and WT littermates performed similarly on this assay (Fig. 2a, Main Effect of Chamber, $F_{(2,66)} = 21.58$, $p < 0.001$; Main Effect of Genotype, not significant (n.s.), $p = 0.2520$). After a short (3-min) delay, mice were given the option of interacting with a novel conspecific mouse or a familiar conspecific mouse located on opposite sides of the apparatus (Fig. 2b). *Arc* KO mice showed a deficit interacting with the novel mouse (Fig. 2b; Genotype x Chamber Interaction, $F_{(2,66)} = 4.464$, $p = 0.0152$, Tukey's post hoc WT Familiar vs. Novel, $p = 0.0333$, KO Familiar vs. Novel, n.s.). Similar to the locomotor assays, *Arc* KO mice did not show motor hyperactivity in either of these tests (Fig. S2). In the forced swim test (FST), when compared to their WT littermates, *Arc* KO mice showed reduced immobility time (Fig. 2c; Welch's correction, $t_{(27.88)} = 2.157$, $p = 0.0397$) – an anti-depressive phenotype. To assess if this anti-depressive phenotype was associated with a change in hedonic state, No differences in preference for sucrose solution (1% w/v) versus regular water were observed in a naïve group of *Arc* KO and WT littermates. (Fig. 2d).

Arc KO mice show an increase in AMPAR surface localization in the adult NAc

We investigated whether *Arc* KO mice show a change in surface AMPA receptor subunits (GluA1 and GluA2/3) using a membrane-impermeable protein crosslinking reagent (BS³) that retards the electrophoretic migration of surface-accessible, crosslinked proteins, as reported previously^{39,44}. The non-crosslinked (internal) fraction of GluA1 ($t_{(6)} = 2.79$, $p = 0.0316$) and GluA2/3 ($t_{(6)} = 10.01$, $p < 0.0001$) proteins were significantly decreased in the NAc of adult *Arc* KO mice (Fig. 3a), but the BS³-crosslinked (surface) proteins failed to enter the separating portion of the SDS-PAGE, likely due to their large aggregated masses. Since we observed no difference in total AMPAR subunit levels by quantitative western blotting of untreated tissues from multiple brain regions (Fig. 3a–c), we interpret the decrease in the internal pool AMPARs in the NAc of *Arc* KO mice as an increase in surface AMPAR subunit levels. We did not detect differences in internal or total AMPAR subunit levels between adult *Arc* KO and WT mice in tissues from prefrontal cortex (PFC), amygdala, or dorsal hippocampus (Fig. 3b, c). We did not detect any changes in GluA1 protein or mRNA levels in NAc tissue from *Arc* KO mice (Fig. 3a, b, d), suggesting that loss of Arc's nuclear repressor role is unlikely account for the increase in surface AMPARs observed in the NAc of adult *Arc* KO mice. Finally, since NAc controls mood-related behaviors in both humans and animal models^{45–51}, to test the regulation of Arc expression by stress, WT mice were exposed to acute social defeat and tissue was collected from the NAc. We detected significant increases in Arc expression in the adult NAc (Fig. 4a), both at the mRNA level 1 hr after stress ($t_{(8)} = 4.469$, $p = 0.0021$; Fig. 4b), and the protein level 4 hrs after acute stress (4 hr time point; $t_{(16)} = 2.41$, $p = 0.0283$, Fig. 4c).

Arc expression in the NAc is sufficient to restore anxiety behavior in Arc KO mice

We examined whether NAc-specific expression of Arc could restore normal anxiety-like behavior. *Arc* and WT littermates received bilateral, intra-NAc infusions of AAV2-Arc-

IRES-mCherry or AAV2-GFP (Fig. 5a). Two weeks later the mice were examined for anxiety-like behavior in the EPM and OF tests. Arc overexpression in NAc did not significantly alter EPM behavior of WT mice (Fig. 5b). However, Arc expression in the NAc of *Arc* KO mice significantly reduced the amount of time they spent in the open arms (Main Effect of Virus: $F_{(17)} = 6.525$, $p = 0.0205$; Bonferroni's post-hoc *Arc* KO GFP vs. Arc, $p = 0.0094$), indicating that viral-mediated expression of Arc in the adult NAc is sufficient to restore normal anxiety-like behavior in the EPM test. Interestingly, NAc-specific expression of Arc in the *Arc* KO mice appeared to reduce center time in the OF test, but this did not quite reach statistical significance in the four-way comparisons (Fig. 5c). Finally, no effects on distance traveled were detected in either EPM or OF tasks (Fig. S5a, b).

Arc expression in the adult NAc is necessary for normal anxiety-like behaviors in male, but not female, mice.

To investigate if NAc-restricted loss of *Arc* can regulate anxiety-like behavior, we employed a viral-mediated shRNA knockdown strategy (Fig. 5d). *In vivo*, AAV2-shArc virus infused in the NAc of WT mice significantly reduced *Arc* mRNA compared to the shControl virus (Fig. 5d; $t_{(3)} = 3.653$, $p = 0.0354$). Two weeks following NAc infusion of AAV2-shArc or AAV2-shControl, male and female WT mice were examined for anxiety-like behavior in the EPM and OF tests. Male and female WT mice showed significantly different locomotor activity on both assays (Fig. S5c–d; EPM, Main Effect of Sex: $F_{(1,16)} = 3.828$, $p = 0.0681$; OF, Main Effect of Sex: $F_{(1,17)} = 21.42$, $p = 0.0002$). NAc-specific knockdown of Arc also produced a significant decrease in anxiety behavior on the EPM in male, but not mice (Fig. 5e; EPM Sex x Virus Interaction: $F_{(1,16)} = 5.92$, $p = 0.0271$, Bonferroni's posthoc, shControl v shArc Male $p = 0.0153$, Female n.s.). NAc-specific Arc knockdown appeared to increase center time in males, but not females, on the OF test (Fig. 5f), but similar to the “rescue” experiments (Fig. 5b–c), this effect did not reach statistical significance. Since we observe effects of Arc expression in the adult NAc during tests for anxiety, and since global *Arc* KO mice display a reduction in immobility time in the FST (Fig. 2c), we tested whether Arc in the adult NAc is required for this anti-depressant-like effect. However, the NAc-shArc mice showed equivalent immobility time to the NAc-shControl mice in the FST (Fig. S6e), and no differences were observed between males and females (Fig. S6f), suggesting that the decrease in FST behavior in global *Arc* KO mice is due to role for this gene in other brain regions and/or functions during development.

Arc expression in the adult NAc is necessary for normal discrimination of both social and non-social novelty.

Global *Arc* KO mice show normal sociability, but impaired social novelty preference, in the three-chamber social interaction task (Fig. 2). As the NAc plays an important role in novelty detection and preference^{52–55}, We examined whether NAc-specific Arc knockdown in WT mice would similarly influence social and novelty-related behaviors. Similar to *Arc* KO mice, shArc-NAc did not alter basic sociability (Fig. 6a) or locomotion (Fig. S6a–b) in the three-chamber sociability assay. Specifically, both shArc-NAc and shControl-NAc mice spent more time in the social zone than the center or object zone (Main Effect of Chamber; $F_{(2,57)} = 83.88$, $p < 0.0001$). Consistent with the effect of total *Arc* knockout, NAc-shArc mice failed to spend more time with the novel mouse, indicating a deficit in social novelty

preference (Fig. 6c Genotype x Chamber Interaction, $F_{(2,36)} = 5.063$, $p < 0.05$; Tukey's multiple comparison Novel vs. Familiar chamber, shControl-NAc $p < 0.05$, shArc-NAc n.s.). We observed no differences in locomotor activity between groups during the task (Fig. S6c). To assess if the deficit in social novelty preference observed in the shArc-NAc mice was specific to social novelty, we examined the performance of shArc-NAc and shControl-NAc mice in the novel object recognition task. While shControl-Arc mice spent significantly more time interacting with the novel object than the familiar object, shArc-NAc mice spend equivalent time interacting with each object (Fig. 6c; Virus x Chamber, $F_{(1,18)} = 17.35$, $p < 0.001$; Bonferroni's post-hoc, Novel vs. Familiar, shControl-NAc, $p < 0.01$; shArc-NAc, n.s.). Both groups showed comparable levels of locomotion (Fig. S6c) and total time interacting with the objects (Fig. 6d), but when a novelty discrimination index is calculated (Fig. 6h; novel – familiar interaction divided by total interaction) showed significant reductions in shArc-NAc mice compared to shControl-NAc mice ($t_{(18)} = 3.856$, $p < 0.01$). While shControl-NAc novel object discrimination was significantly greater than 0 ($t_{(7)} = 3.553$, $p < 0.01$), shArc-NAc mice spent essentially equal time interacting with both objects (discrimination of 0; n.s.). Interestingly, while shArc-NAc mice displayed novelty discrimination deficits in both sexes, there were also sex differences in the magnitude of the discrimination (Fig. 6f; Main Effect of Sex, $F_{(1,16)} = 4.826$, $p = 0.0431$, Main Effect of Virus, $F_{(1,16)} = 17.2$, $p = 0.0008$). Females had lower novelty discrimination than males, despite spending equivalent time spent interacting with the objects (Fig. S6d).

Discussion

Several synapse-associated molecules, including Arc^{56–59}, are key factors influencing the pathogenesis of multiple neuropsychiatric disorders. Here we show that *Arc* KO mice have significant decreases in anxiety-like behaviors and social novelty preference, as well as increases in anti-depressive-like behavior. Following our observations of robust Arc induction in the NAc after acute stress, as well as basal deficits in surface AMPAR subunits within the NAc of adult *arc* KO mice, we tested the effects of manipulation (shRNA and overexpression) of Arc in the adult NAc on these aviors. In particular, we demonstrate that NAc expression of Arc restores normal elevated plus behavior in *Arc* KO mice. We also find that NAc-specific Arc knockdown impairs novelty discrimination, a phenotype independent of sex. However, in male, but not female, mice, NAc-specific Arc knockdown reduces anxiety in the EPM, which phenocopies *Arc* KO behavior. Overall, our findings indicate a critical role for Arc in the NAc in anxiety and novelty preference/discrimination behaviors – a role that may partially account for sex-specific differences in some of these behaviors.

Our observations here reveal reliable anxiolytic-like effects in *Arc* KO mice across three different behavioral tests. While previous reports using the same strain of *Arc* KO mice have noted significant decreases⁶⁰ and increases⁵⁸ in locomotion, we observe no significant differences in *Arc* KO mouse activity in a locomotor assay (Fig. 1 d-f). *Arc* KO mice also did not show increased activity in the social novelty assay (Fig. 2b, d). There were several factors that differed between our studies that might contribute to these distinct observations, including background strain and behavioral testing conditions. We do, however, observe hyperactivity in *Arc* KO mice across three different tests of anxiety-like behavior. Based on our observations of increased distance traveled in short-duration tests conducted in relatively

novel arenas (Figs. 1a-c), but not in a longer duration locomotor assay conducted in a (clean) homecage environment, it appears that motor hyperactivity is not a robust phenotype in the global *Arc* KO mice and may be task-dependent.

Our observations of significant decreases in GluA1 and GluA2/3 intracellular expression levels in *Arc* KO mice (with no corresponding change in total protein) in the NAc, in contrast to several other brain regions analyzed (Fig. 4), are generally consistent with other reports for *Arc* KO mice. Normal hippocampal spine density and basal glutamatergic transmission is reported in adult KO mice²⁸, (but see also³⁰), and no change is observed in surface GluA1 or GluA2, nor in AMPAR activity, in the visual cortex of 3-week-old KO mice⁶¹. Increased AMPAR surface expression and enhanced glutamatergic strength have been previously observed in *Arc* KO mice, but only in cultured excitatory neurons^{18,62}, suggesting that intact circuits may possess compensatory mechanisms to normalize AMPAR surface balance. Our surface AMPAR results indicate that, in contrast, cells in the NAc, predominantly medium spiny neurons (MSNs), may be dependent on the presence of *Arc* to control surface AMPAR levels.

Generally consistent with a previous report on stress-dependent induction of *Arc*²⁷, we find that acute social defeat stress stimulates a robust induction of *Arc* mRNA and protein in the NAc. In support of the idea that *Arc* induction by stressful experiences contributes to behavior and is not simply a marker of increased neuronal activity, we find that altered expression of *Arc* in the NAc can influence behaviors related to anxiety. Specifically, when *Arc* is virally expressed in the NAc of *Arc* KO mice, it is sufficient to rescue anxiety behaviors in the EPM (Fig. 5b), and *Arc* expression in the adult NAc is required for normal anxiety on the EPM test (Fig. 5e), thus supporting a role for *Arc* in regulating innate anxiety in the adult NAc. Interestingly, both the rescue and knockdown effects were restricted to the EPM test and were only observed in male mice. This sex-specific effect of *Arc* has not been described elsewhere, and it implicates *Arc* in controlling sexual divergence in anxiety-related behavior. While estrous state was not tracked in this experiment, examination of the variability within the female groups does not hint at estrus-dependent effects. Nonetheless, future examination of estrous state *Arc* function and anxiety-like behavior might reveal some interesting interactions. In contrast to anxiety-like behavior, the effects of *Arc* on novelty discrimination were not sex-specific (Fig. 6), which suggests that *Arc* regulates novelty and anxiety via distinct brain circuits or cellular mechanisms.

Unlike a previous study that indicates *Arc* global KO mice have reduced sociability and social novelty preference⁵⁸, we only observed a deficit in social novelty preference (Fig. 2c). Given the short delay between familiarization and testing, these deficits are not likely to reflect previously described long-term memory/ consolidation deficits²⁸, but rather be similar to other working memory deficits⁵⁸. The NAc⁶³ and multiple upstream nuclei, including the hippocampus⁶⁴ and perirhinal cortex⁶⁵, influence novelty detection. Furthermore, exposure to novelty (*e.g.* environment, taste) can induce *Arc* expression in several brain regions^{23,24,66}, including the striatum⁶⁶, and evokes enhanced dopamine release in the NAc⁶⁷⁻⁶⁹. Interestingly, dopamine release in the NAc can induce *Arc* expression in dopamine D1-receptor expressing neurons⁷⁰. Taken together with our findings, it may be that dopamine-mediated induction of *Arc* is necessary for normal novelty

behavior. When testing the role of Arc in the adult NAc for social novelty preference, we recapitulated our global KO results; however, additional novel object recognition testing revealed an extension of the deficit to objects, indicating a broad role for NAc Arc in regulating novelty discrimination/preference.

In conclusion, our studies suggest an important role of Arc as a regulator of innate anxiety, anti-depressive-like states, and detection of novelty. Moreover, our findings implicate Arc in the NAc as a key mediator of anxiety-and novelty-related behaviors and that its role in these behaviors can be sexually dimorphic. Given Arc's complex cellular functions, future work will be important to elucidate the mechanism(s) by which it functions in the NAc to influence these important behaviors for human mental health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Anxiety and locomotion in *Arc* KO mice.

a, Time spent (seconds) by male wild-type (WT) and *Arc* KO mice in the center of the open field (OF). **b**, Time spent (seconds) spent in the open arms of the elevated plus maze (EPM) in male WT and *Arc* KO mice. **c**, Time spent (seconds) in the light side of the light/dark (L/D) box by male WT and *Arc* KO mice. **d**, Activity (beam breaks) in 5-min bins for WT and *Arc* KO mice during 1 hr locomotor test. Cumulative activity (beam breaks) during the first 5 min (**e**) or 1 hr (**f**) of the locomotor test. Bars represent Mean and SEM; n = 20 – 24 mice/group; *p < 0.05, ** p < 0.01.

Figure. 2. Social and mood behavior in *Arc* KO mice.

a, Time spent in the different compartments of the 3-chamber social interaction chamber during a sociability test for male WT and *Arc* KO mice. **b**, Time spent in the different compartments of the 3-chamber social interaction chamber during the social novelty test for male WT and *Arc* KO mice. **c**, Time spent immobile (seconds) by male WT and *Arc* KO in the forced swim test. **d**, Sucrose preference (expressed as sucrose consumption as a percentage of total sucrose and water consumption) in male WT and *Arc* KO mice. Bars represent Mean and SEM; n = 14 – 21 mice/group (3-chamber), n = 19 – 24 mice/group (forced swim), n = 9 – 11 mice/group (sucrose preference); * p < 0.05.

Fig. 3. AMPAR localization in *Arc* KO mice.

a, Approximate location of tissue collection sites. **b**, Quantification of normalized (β -tubulin) internal (top) and total (bottom) GluA1 (left) and GluA2/3 (right) protein expression in the NAc of young adult male WT and *Arc* KO mice. **c**, Quantification of normalized intracellular (top) and total (bottom) GluA1 protein expression in the prefrontal cortex (PFC), dorsal Hippocampus (dHPC), and amygdala (Amyg) of young adult male WT and *Arc* KO mice. **d**, Quantification of normalized intracellular (top) and total (bottom) GluA2/3 protein expression in the PFC, dHPC, and Amyg of young adult male WT and *Arc* KO mice. Lower panels display representative blot images, β -tubulin loading control. **e**, *Arc* mRNA expression in the NAc of male WT ($n = 5$) and *Arc* KO ($n = 6$) mice. *Arc* mRNA levels were normalized to *GAPDH* mRNA and compared using RT-qPCR. Bars represent Mean and SEM; $n = 3 - 6$ mice/genotype (protein expression), $n = 5 - 6$ mice/genotype (mRNA expression); * $p < 0.05$, *** $p < 0.001$.

Fig. 4. Stress regulation of *Arc* expression

NAc tissue (a) was collected 1, 2, and 4 hrs after an acute defeat experience. **b**, *Arc* mRNA expression (normalized to *GAPDH* mRNA) in male C57BL/6J mice exposed to gentle handling (control) or a single 10 min social defeat session (acute). **c**, *Arc* protein expression in the NAc 2 hrs (left) and 4 hrs (right) after a 10 min social defeat stress. *Arc* expression was normalized to β -tubulin and levels expressed relative to the control condition. Bars represent Mean and SEM; $n = 5$ mice/condition (mRNA expression), $n = 4 - 5$ mice/condition (protein expression); * $p < 0.05$, ** $p < 0.01$.

Fig. 5. Behavioral effects of NAc-specific *Arc* expression manipulations

a, Representative image demonstrating viral-mediated *Arc* over-expression in the NAc of *Arc* KO B mice. 2.5X and 10X images of the same field showing anti-Arc antibody staining. 20X' and 20X'' images showing independent examples of *Arc* over-expression (Green) primarily in NeuN positive (Red) cells. Scale bar = 100 μ m. **b**, Time (seconds) spent in the open arms for control (GFP-NAc) and *Arc* overexpressing (Arc-NAc) WT and *Arc* KO littermates. **c**, Time (seconds) spent in the center of the open field for GFP-NAc and Arc-NAc WT and *Arc* KO littermates. **d**, Knockdown of *Arc* in virally-infected NAc tissue from C57Bl/6N WT mice. **e**, Time (seconds) spent in the open arm for female and male shControl-NAc and shArc-NAc mice. **f**, Time (seconds) spent in the center of the open field for female and male shControl-NAc and shArc-NAc mice. Bars represent Mean and SEM; $n = 4 - 6$ mice/condition (Arc rescue), $n = 2 - 3$ mice/condition (shRNA knockdown *in vivo*), $n = 9 - 12$ e/condition (Arc shRNA behavior); * $p < 0.05$, ** $p < 0.01$.

Figure 6. Social and novelty behavior in *Arc* shRNA mice

a, Time spent in the compartments of the 3-chamber social interaction chamber for male and female shControl-NAc and shArc-NAc mice during a sociability test. **b**, Time spent in the compartments of the 3-chamber social interaction chamber for male and female shControl-NAc and shArc-NAc mice during the social novelty test. **c**, Time spent interacting with the novel or familiar object for male and female shControl-NAc and shArc-NAc mice in the novel object recognition trial. **d**, Total time spent interacting with objects for male and female shControl-NAc and shArc-NAc mice during the novel object recognition trial. Novel

object discrimination index by treatment (*e*) and by sex and treatment (*f*). Bars represent Mean and SEM; n = 9 – 12 mice/condition; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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