

The Immediate Early Gene *Arc* Is Not Required for Hippocampal Long-Term Potentiation

Madeleine Kyrke-Smith,¹ Lenora J. Volk,^{2,3} Samuel F. Cooke,^{4,5,6} Mark F. Bear,⁴ Richard L. Huganir,² and Jason D. Shepherd^{1*}

¹Department of Neurobiology, University of Utah, Salt Lake City, Utah 84112, ²Department of Neuroscience, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205, ³Department of Neuroscience, University of Texas Southwestern Medical Center, Dallas, Texas 75390, ⁴The Picower Institute for Learning and Memory, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, ⁵Department of Basic and Clinical Neurosciences, King's College London, London, WC2R 2LS, United Kingdom, and ⁶MRC Centre for Neurodevelopmental Disorders, King's College London, London, SE1 1UL, United Kingdom

Memory consolidation is thought to occur through protein synthesis-dependent synaptic plasticity mechanisms such as long-term potentiation (LTP). Dynamic changes in gene expression and epigenetic modifications underlie the maintenance of LTP. Similar mechanisms may mediate the storage of memory. Key plasticity genes, such as the immediate early gene *Arc*, are induced by learning and by LTP induction. Mice that lack *Arc* have severe deficits in memory consolidation, and *Arc* has been implicated in numerous other forms of synaptic plasticity, including long-term depression and cell-to-cell signaling. Here, we take a comprehensive approach to determine if *Arc* is necessary for hippocampal LTP in male and female mice. Using a variety of *Arc* knock-out (KO) lines, we found that germline *Arc* KO mice show no deficits in CA1 LTP induced by high-frequency stimulation and enhanced LTP induced by theta-burst stimulation. Temporally restricting the removal of *Arc* to adult animals and spatially restricting it to the CA1 using *Arc* conditional KO mice did not have an effect on any form of LTP. Similarly, acute application of *Arc* antisense oligodeoxynucleotides had no effect on hippocampal CA1 LTP. Finally, the maintenance of *in vivo* LTP in the dentate gyrus of *Arc* KO mice was normal. We conclude that *Arc* is not necessary for hippocampal LTP and may mediate memory consolidation through alternative mechanisms.

Key words: *Arc*; hippocampus; LTP; memory; synaptic plasticity

Significance Statement

The immediate early gene *Arc* is critical for maintenance of long-term memory. How *Arc* mediates this process remains unclear, but it has been proposed to sustain Hebbian synaptic potentiation, which is a key component of memory encoding. This form of plasticity is modeled experimentally by induction of LTP, which increases *Arc* mRNA and protein expression. However, mechanistic data implicates *Arc* in the endocytosis of AMPA-type glutamate receptors and the weakening of synapses. Here, we took a comprehensive approach to determine if *Arc* is necessary for hippocampal LTP. We find that *Arc* is not required for LTP maintenance and may regulate memory storage through alternative mechanisms.

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Correspondence should be addressed to Jason D. Shepherd at Jason.Shepherd@neuro.utah.edu.

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Introduction

The maintenance of memory and Hebbian forms of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD), requires activity-dependent gene expression and *de novo* protein synthesis (Goelet et al., 1986; Bliss and Collingridge, 1993; Abraham and Williams, 2003; Takeuchi et al., 2014; Poo et al., 2016; Choi et al., 2018; Kyrke-Smith and Williams, 2018). The molecular underpinnings of LTP, in particular, are of interest because of the overlap in activity-induced genes critically involved in both LTP and memory (Alberini, 1999; 2009; Kandel et al., 2014). Thus, identifying genes that are necessary for the maintenance of LTP may be key to identifying memory molecules. The immediate early gene *Arc* is induced by neuronal activity, is necessary for memory consolidation (Guzowski et al., 2000; Plath et al., 2006; Ploski et al., 2008), and

is implicated in various forms of synaptic plasticity (Shepherd and Bear, 2011). *Arc* mRNA expression is increased in the dentate gyrus (DG) of the hippocampus after seizure or LTP induction (Link et al., 1995; Lyford et al., 1995), some of which moves out of the cell body and into dendrites where it localizes near potentiated synapses (Steward et al., 1998; Yin et al., 2002; Moga et al., 2004; Rodríguez et al., 2005). LTP maintenance has been shown to be attenuated by application of antisense RNA against *Arc* in rats (Guzowski et al., 2000; Messaoudi et al., 2007) and in *Arc* knock-out (KO) mice under urethane anesthesia (Plath et al., 2006). However, *Arc* is also critical for the maintenance of hippocampal LTD (Plath et al., 2006; Waung et al., 2008) and for homeostatic scaling of the AMPA-type glutamate receptors (i.e., AMPARs; Shepherd et al., 2006; Korb et al., 2013). How one protein can be critical for both increases and decreases in synaptic strength has remained an open question.

Arc interacts with the endocytic machinery at synapses, such as clathrin-adaptor protein 2 (AP-2; DaSilva et al., 2016), dynamin, and endophilin (Chowdhury et al., 2006). Acute overexpression of *Arc* leads to the endocytosis of AMPARs, thereby decreasing synaptic strength (Rial Verde et al., 2006; Shepherd, Rumbaugh et al., 2006). *Arc* also interacts with the calcium/calmodulin-dependent protein kinase II- β (CaMKII β) isoform to decrease synaptic strength at inactive synapses (Okuno et al., 2012). However, *Arc* can also interact with drebrin, A, which may lead to F-Actin stabilization and the enlargement of dendritic spines, indicative of increased synaptic strength (Messaoudi et al., 2007; Bramham et al., 2010; Nair et al., 2017). Further, other *Arc* protein interactions raise the possibility that *Arc* may facilitate memory through mechanisms other than direct regulation of synaptic strength. For example, *Arc* protein may be found in the nucleus where it interacts with the nuclear spectrin isoform β SpIVS5 (Bloomer et al., 2007) and the histone acetyl-transferase Tip60 (Wee et al., 2014). Additionally, we recently found that *Arc* can form virus-like capsids that can encapsidate RNA and transfer *Arc* mRNA between cells (Pastuzyn et al., 2018).

Given the extensive literature implicating LTP as a cellular correlate of memory (Bliss and Collingridge, 1993; Abraham and Williams, 2003; Whitlock et al., 2006; Rioult-Pedotti et al., 2007; Takeuchi et al., 2013; Nabavi et al., 2014; Poo et al., 2016; Choi et al., 2018), we aimed to comprehensively investigate whether *Arc* is required for the maintenance of hippocampal LTP. We used two different *Arc* KO mouse lines and an *Arc* conditional KO (*Arc^{fl/fl}*) floxed line for both *in vitro* and *in vivo* LTP analysis. We find that *Arc* is not required for the maintenance of LTP, using multiple LTP induction protocols in these mice. Additionally, acute application of *Arc* antisense oligodeoxynucleotides (ODNs) that have been previously used for *in vivo* experiments in the DG (Messaoudi et al., 2007; Kuipers, Trentani et al., 2016) had no effect on CA1 LTP. These findings suggest that *Arc*'s role in memory consolidation is likely mediated through mechanisms that do not involve the maintenance of synaptic potentiation.

Materials and Methods

Animals. Experiments were performed in three different *Arc* KO models. Two full *Arc* knock-out animal lines were used: the *Arc^{tm1Stl}* (Wang et al., 2006) line, where GFP was knocked into the endogenous *Arc* locus (*Arc^{GFP/GFP}*), and the Kuhl *Arc^{-/-}* line (Plath et al., 2006). An *Arc* conditional KO line (*Arc^{fl/fl}*), which was previously generated (provided by Richard Palmiter, University of Washington), was also used. In this line, loxP sites were inserted to enable the removal of the entire *Arc* gene on introduction of cre-recombinase (Chen et al., 2018). All lines are on the C57BL6 background, and WT littermates were used as controls. C57BL6 WT mice were used for the *Arc* antisense ODN

experiments. All procedures were approved by the Institutional Animal Care and Use Committees of the University of Utah, The Johns Hopkins University, and the Massachusetts Institute of Technology, in conjunction with National Institutes of Health guidelines.

In vitro electrophysiology. Slices were prepared from WT and KO mice at age 6–8 weeks (*Arc* cKO and *Arc^{GFP/GFP}* and WT alone for ODN) or 11–14 weeks (*Arc^{-/-}*) of either sex. Mice were anesthetized with the inhalation anesthetic isoflurane before decapitation. Four hundred μ m transverse slices were prepared using a Leica VT1200S vibratome (*Arc^{-/-}*) or a Leica VT1000S vibratome (*Arc* cKO, *Arc^{GFP/GFP}*), and ODN experiments) in ice-cold oxygenated (95% O₂/5% CO₂) dissection buffer containing the following (in mM): 2.6 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 211 sucrose, 10 glucose, 0.75 CaCl₂, 7MgCl₂. Slices were recovered in a static submersion chamber for at least 2 h in 30°C aCSF containing the following (in mM): 125 NaCl, 5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄·H₂O, 11 glucose, 2CaCl₂, 1MgCl₂. Field excitatory postsynaptic potentials (fEPSPs) were evoked at 0.033 Hz with a 125 mm platinum/iridium concentric bipolar electrode (FHC; *Arc^{-/-}*) or 75 mm Tungsten bipolar electrode (World Precision Instruments; *Arc* cKO, *Arc^{GFP/GFP}*, and ODN experiments) placed in the middle of stratum radiatum of CA1. A 1–4 M Ω glass recording electrode filled with aCSF was positioned 200–400 μ m away (orthodromic) from the stimulating electrode. Input-output (I/O) curves were obtained for each slice, and stimulus intensity for all subsequent recordings was set to elicit a fEPSP slope that was 30–40% of the maximum response. Recording aCSF was identical to recovery conditions and slices were incubated at a flow rate of 3 ml/min at 32°C. Stimulation protocols were as follows: protein-synthesis-independent early-phase LTP (E-LTP): 20 pulses at 100 Hz; moderate frequency stimulation: 900 pulses at 10 Hz; high-frequency stimulation LTP: 2 trains of 100 pulses at 100; and theta burst protein-synthesis-dependent late-phase LTP (L-LTP): four trains at 10 s intertrain intervals. Each train consisted of 10 bursts at 5 Hz, with each burst containing four stimuli at 100 Hz.

Antisense oligodeoxynucleotides. *Arc* ODNs (5'GTC CAG CTC CAT CTG CTC GC 3') and scrambled *Arc* ODNs (5'CCT GCT GAC CTC CGT ATG CC 3') previously described (Messaoudi et al., 2007; Kuipers et al., 2016) were synthesized (Integrated DNA Technologies) and used at a final concentration of 1 μ M in aCSF. After a 10 min baseline recording, slices were bathed in aCSF containing ODNs for the remainder of the experiment.

In vivo electrophysiology. Male mice were anesthetized under isoflurane (3% for induction and 1.5% for maintenance) and head-fixed in a stereotaxic frame. Rectal temperature was maintained at 37°C using a heat blanket. A dental drill was used to make craniotomies in the skull, and electrodes were placed according to established stereotaxic coordinates on the left-hand side of the brain. For LTP experiments in the dentate gyrus, a concentric bipolar stimulating electrode (Rhodes Medical Instruments) was positioned in the medial perforant path (MPP), 3 mm left of λ and at a depth of \sim 1.5 mm from brain surface. A glass micropipette was lowered into the hilus of the left dentate gyrus, 2 mm posterior to bregma, 1.6 mm left of the midline and at a depth of \sim 1.5 mm, to record positive-going evoked field responses next to the granule cell bodies. Correct placement of the stimulating electrode in the MPP was confirmed by the characteristic short latency onset of the evoked field response (2–2.5 ms) and the early onset of the population spike (\sim 4 ms). For all experiments, input–output relationships were assessed using a range of stimulus intensities from 0–220 μ A, which produced a close to maximal response. Five responses were collected at each intensity and averaged. Test responses for LTP experiments were evoked by monophasic stimuli set at an intensity to evoke a response \sim 50% of maximum fEPSP slope and a population spike of \sim 1 mV (100–220 μ A, 60 μ s). A stable 30 min baseline was acquired before delivering a theta burst tetanus to induce LTP. The tetanus comprised six series of six trains of six stimuli at 400 Hz with 200 ms between trains and 20 s between series. Pulse width was doubled during the tetanus to 120 μ s. Only the very early component of the EPSP slope was measured for analysis to ensure that there was no contamination by the population spike. The slope of the fEPSP was expressed as a percentage change relative to the averaged baseline response. At the end of each experiment, the anesthetized mouse was killed by cervical dislocation.

Viral injection. Mice were anesthetized with isoflurane (3% for induction, 1.5–2% for surgery) and placed in a stereotaxic frame following the suppression of reflexes. The antibiotic Baytril (8 mg/kg; VetOne), the analgesic Carprofen (5 mg/kg; Zoetis) and the anti-inflammatory dexamethasone (13 mg/kg; VetOne) were administered before placing the mouse in the stereotaxic frame to control pain/swelling. Lidocaine (100 mg/kg; VetOne) was then injected subcutaneously beneath the scalp before incision. The skin was incised to expose the skull and a small burr hole was made with a pneumatic dental drill above the CA1 region of the hippocampus (350 μ m lateral and 50 μ m anterior to LAMBDA). A pulled glass pipette, backfilled with mineral oil and filled with virus, was lowered (250 μ m below surface) and allowed to rest for 5 min. Bilateral injections of either AAV5/CaMKII-GFP (3.18 \times 10⁸ particles, 1 μ l volume; Ed Boyden, University of North Carolina Vector Core) or AAV5/CaMKII-GFP-cre (2.1 \times 10⁸ particles, 1 μ l volume; University of North Carolina Vector Core) were delivered using a Nanoject II Auto-Nanoliter Injector (Drummond Scientific). Following a further 5 min resting postinjection, the pipette was slowly removed. The scalp was then sutured closed and the animal was placed in a warm cage for recovery. Animals were maintained at \sim 37°C throughout the procedure and recovery, and general condition and reflex signs were monitored closely. Mice were monitored postoperatively for signs of infection or discomfort. Animals recovered for 2 weeks to allow for viral expression before slice preparation. To quantify the effectiveness of cre to knock-out Arc expression in these animals, slices used for electrophysiology were subsequently prepared for Western blotting to assess Arc protein levels. Slices were only used for experiments if GFP could be detected along the entire CA1 region of each slice before electrophysiology. GFP was visualized in slices using an LED light source at 470 nm (pE-100; CoolLED) on a SliceScope Pro-Mounted FireWire (B FWCAM X M) camera linked to the SCLight 2.2 software (Scientifica).

Immunohistochemistry. A representative image of GFP injection was made from an animal injected with AAV5:CaMKII-GFP, as described above. After 2 weeks recovery, the animal was perfused with 4% paraformaldehyde, followed by 24 h fixation in further 4% paraformaldehyde. The brain was cryoprotected in 30% sucrose before being sectioned (30 μ m) on a cryostat. Sections were stained for DAPI (catalog #R37606, Thermo Fisher Scientific; 5 min at room temperature) before mounting coverslips on slides in Fluoromount (catalog #F4680, Millipore Sigma) and dried. Images were captured on the confocal microscope (Ti2, Nikon).

Western blotting. After the CA1 region of the hippocampus was dissected from slices postelectrophysiology, tissue was mixed in 4 \times Laemlli Buffer (40% glycerol, 250 mM Tris, 4% SDS, 50 mM STT, pH 6.8) and boiled at 95°C for 10 min. SDS-PAGE gel electrophoresis was used to separate protein samples. Separated samples were transferred to a nitrocellulose membrane (GE Healthcare). Following transfer, membranes were briefly stained with 0.1% Ponceau stain, destained with 1% acetic acid to remove background, and then imaged to detect total protein. Membranes were blocked in 5% milk + 1 \times tris-buffered saline (TBS; 10 \times : 152.3 mM Tris-HCl, 46.2 mM Tris base, 1.5 M NaCl, pH 7.6, for 30 min at room temperature. Membranes were then incubated with primary antibody against Arc (1:1000, custom rabbit polyclonal, Protein Tech) in 1 \times TBS, overnight at 4°C. Membranes were washed in 1 \times TBS (3 \times 10 min) before being incubated in a secondary antibody (1:10,000 HRP-conjugated goat antirabbit, Jackson ImmunoResearch), in blocking solution described above, for 1 h at room temperature. Membranes were then washed again in 1 \times TBS (3 \times 10 min). A chemiluminescent kit was used to detect protein bands (Bio-Rad). Membranes were imaged on an Azure c300 gel dock (Azure Biosystems). Blots were analyzed and quantified using the Gel Analysis plug-in in ImageJ.

Statistics. For all LTP experiments, the magnitude of LTP over the first 5 min poststimulation and the last 5 min of recording was averaged for each slice. Unpaired two-tailed *t* tests were performed on these averages from the Arc KO and WT littermates for each experiment. For I/O curves, linear regression was fitted for each curve (\pm 95% confidence interval) and an extra-sum-of-squares *F* test was used to compare slope of the best fit line between KO and WT groups. For paired pulse, a two-way ANOVA was performed to determine whether there was a genotype

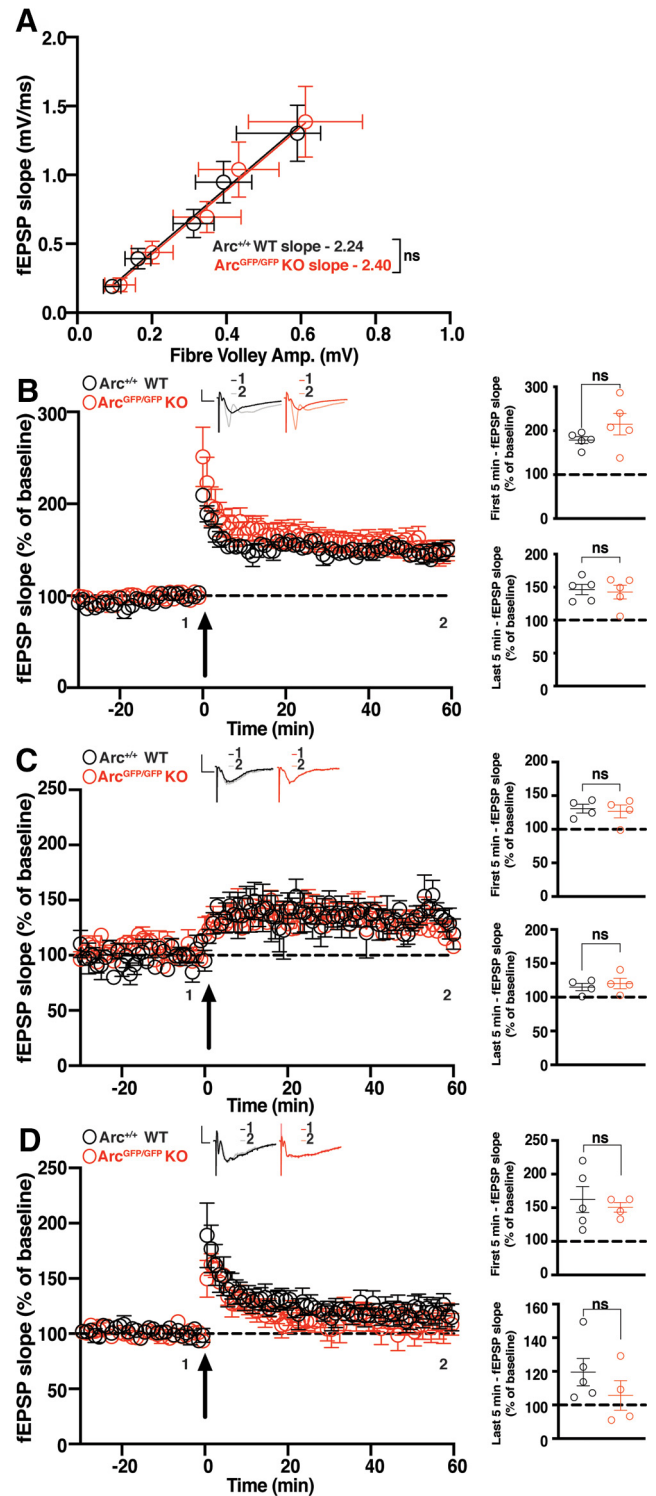


Figure 1. HFS CA1 LTP is normal in Arc KO animals. **A**, There was no significant difference in basal synaptic transmission between Arc^{GFP/GFP} KO (red circles) and WT (black circles) littermates. **B**, There was no significant difference in the average magnitude of LTP over the first 5 min poststimulation, nor over the last 5 min of recording, between Arc^{GFP/GFP} KO, and WT littermates when LTP was induced using a 2 \times HFS (100 pulses at 100 Hz) protocol. **C**, There was no significant difference in the magnitude of LTP over the first 5 min poststimulation, nor over the last 5 min of recording (**b**), between the Arc^{GFP/GFP} KO, and WT littermates when LTP was induced using a moderate frequency protocol (900 pulses at 10 Hz). **D**, There was no significant difference in the magnitude of E-LTP over the first 5 min poststimulation, nor over the last 5 min of recording, between the Arc^{GFP/GFP} KO, and WT littermates when E-LTP was induced using weak, high-frequency stimulation protocol (20 pulses at 100 Hz). Data are represented as mean \pm SEM. Scale bars: 0.5 mV (horizontal) and 5 ms (vertical).

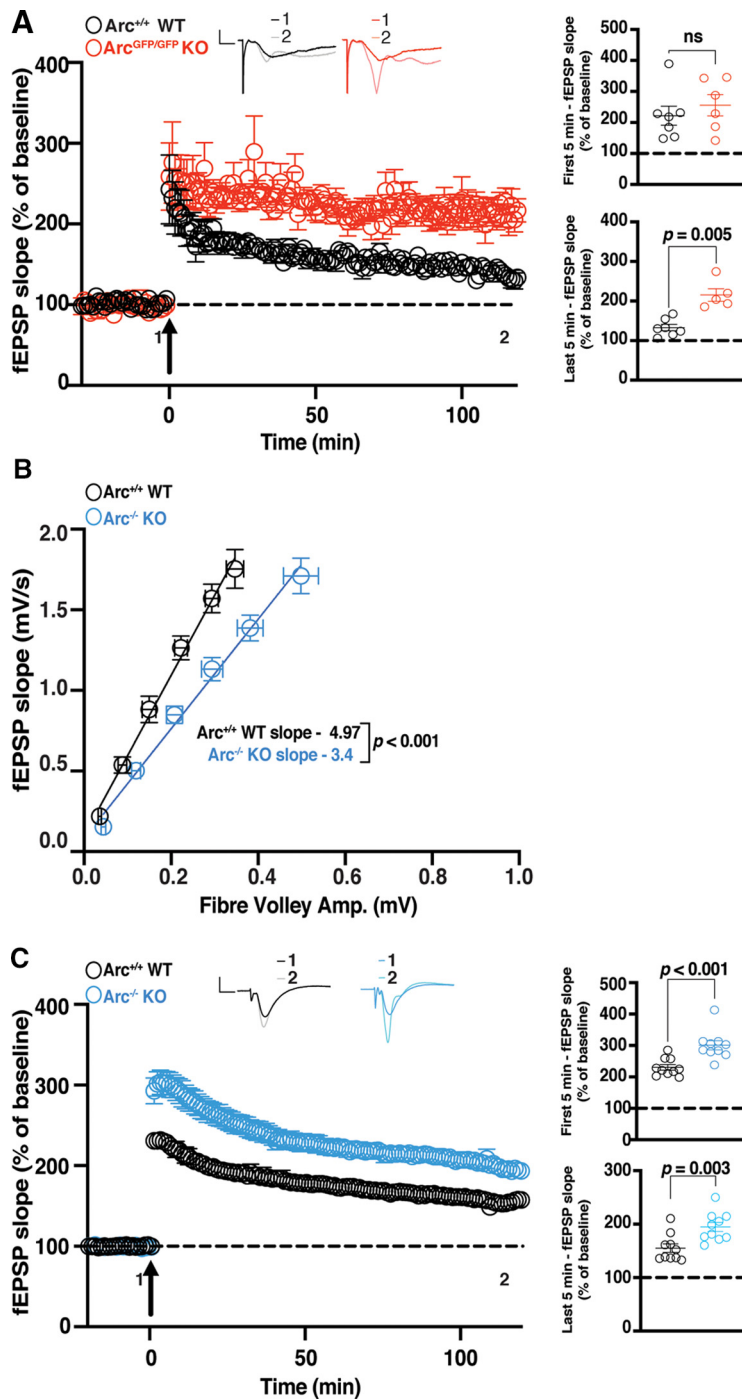


Figure 2. TBS CA1 LTP is enhanced in germline Arc KO animals. **A**, There was no significant difference in the magnitude of LTP over the first 5 min poststimulation between the Arc^{GFP/GFP} KO (red circles), and WT (black circles) littermates, but the magnitude of LTP was significantly greater in Arc^{GFP/GFP} KO mice compared with WT littermates over the last 5 min of recording, when LTP was induced using a theta-burst stimulation protocol (4 trains at 10 s intertrain intervals; each train consisted of 10 bursts at 5 Hz, with each burst containing 4 stimuli at 100 Hz). **B**, Basal synaptic transmission was significantly weaker in Arc^{-/-} KO (blue circles) mice compared with WT (black circles) littermates. **C**, The magnitude of LTP over the first 5 min poststimulation, and over the last 5 min of recording, was significantly greater in the Arc^{-/-} KO (blue circles) mice compared with WT (black circles) littermates when LTP was induced using a theta-burst stimulation protocol (4 trains at 10 s intertrain intervals; each train consisted of 10 bursts at 5 Hz, with each burst containing 4 stimuli at 100 Hz). Data are represented as mean ± SEM. Scale bars: 0.5 mV (horizontal) and 5 ms (vertical).

by ISI effect, with Sidak's *post hoc* multiple-comparison analysis of the difference between genotype at each Inter-stimulus interval (ISI). For Western blot analysis, Arc signal was normalised to total protein, and then the fold change (FC) for all samples were calculated from the average of the GFP-only group (sample/average of control group). Unpaired

two-tailed *t* tests were then performed between the two groups (GraphPad Prism; GraphPad software was used for all statistics).

Results

Arc is not required for LTP induced by high-frequency stimulation in CA1

Broadly, LTP can be divided into E-LTP or L-LTP (Nguyen et al., 1994). These types of LTP can also be induced by a variety of stimulation protocols. To test the role of Arc in different types of hippocampal LTP, we used multiple high-frequency stimulation (HFS) paradigms to induce plasticity at CA3 to CA1 synapses in hippocampal slices. As Arc expression increases within minutes of HFS, we hypothesized that Arc would be required for the maintenance of LTP induced by HFS. To test this, we used a standard HFS protocol that typically induces LTP that persists for at least 1 h. We used Arc^{GFP/GFP} mice, 6–8 weeks old, which show no differences in basal transmission (WT slope = 2.237 ± 0.64, n = 20; Arc^{GFP/GFP} KO slope = 2.397 ± 0.80, n = 21; linear regression, extra-sum-of-squares *F* test between curves: *F*_(1,190) = 0.096, *p* = 0.756; Fig. 1A). There was no significant difference in the magnitude of LTP induced by a 2 × HFS (100 Hz) protocol between Arc^{GFP/GFP} KO and WT littermates (WT 178.84% ± 7.72, n = 5; KO 214.83% ± 24.44, n = 5; *t* test: *p* = 0.20), nor was there a difference in the magnitude of LTP 1 h poststimulation (WT 146.48% ± 7.63, n = 5; Arc^{GFP/GFP} KO 141.29% ± 10.12, n = 5; *t* test: *p* = 0.69; Fig. 1B).

Surprised by this result, we hypothesised that perhaps the loss of Arc would lower the threshold for LTP induction (Shepherd and Bear, 2011) rather than mediate LTP maintenance or expression. We tested a moderate frequency stimulation protocol of 900 pulses at 10 Hz that typically induces little to no change in synaptic strength (Dudek and Bear, 1992). There was no significant difference in the magnitude of LTP induced between Arc^{GFP/GFP} KO and WT littermates (WT 124.7% ± 6.67, n = 4; Arc^{GFP/GFP} KO 125.3% ± 9.65, n = 4; *t* test: *p* = 0.96), nor a difference in the magnitude of LTP 1 h poststimulation (WT 115% ± 5.35, n = 4; Arc^{GFP/GFP} KO 120.2% ± 7.74, n = 4; *p* = 0.60; Fig. 1C). To further test the threshold hypothesis, we used a low repetition, high-frequency stimulation protocol that typically only induces E-LTP (20 pulses at 100 Hz; Frey and Morris, 1997).

There was no significant difference in the magnitude of LTP induced between Arc^{GFP/GFP} KO and WT littermates (WT 162.25% ± 19.4, n = 5, Arc^{GFP/GFP} KO 150.65% ± 7.21, n = 4; *t* test: *p* = 0.63), nor was there a difference in the

magnitude of LTP 1 h poststimulation (WT $119.54\% \pm 8.12$, $n=5$, $Arc^{GFP/GFP}$ KO $105.6\% \pm 8.81$, $n=4$; t test: $p=0.29$; Fig. 1D). These results show that Arc is not required for HFS-induced LTP, nor does loss of Arc change the threshold of LTP induction in the CA1 region of the hippocampus.

CA1 LTP induced by theta burst stimulation is increased in Arc KO mice

To further probe whether Arc mediates specific types of LTP, we tested another robust and commonly used L-LTP induction protocol, theta burst stimulation (TBS; Larson et al., 1986; Thomas et al., 1998; Volk et al., 2013). In addition, we recorded for 2 h after LTP induction to further determine whether maintenance of L-LTP was affected. The initial magnitude of LTP was not significantly different between $Arc^{GFP/GFP}$ KO and WT littermates (WT $222.0\% \pm 30.7$, $n=6$; KO $255.6\% \pm 34.3$, $n=7$; t test: $p=0.48$; Fig. 2A). However, 1 h (WT $221.96\% \pm 30.72$, $n=7$; KO $255.6\% \pm 34.3$, $n=6$; t test $p=0.004$) and 2 h poststimulation, the magnitude of LTP was significantly greater in $Arc^{GFP/GFP}$ KO mice compared with WT littermates (WT $132.9\% \pm 8.17$, $n=6$; KO $215.4\% \pm 14.46$, $n=7$; t test: $p=0.0005$; Fig. 2A). Because this Arc KO line expresses GFP from the Arc locus, we also investigated whether TBS L-LTP was altered in a different germline Arc KO line, $Arc^{-/-}$ mice (Plath et al., 2006). Interestingly, we found a small but significant difference in basal transmission in this line (WT slope = 4.97 ± 0.60 , $n=16$; $Arc^{-/-}$ KO slope = 3.4 ± 0.36 , $n=15$; linear regression, extra-sum-of-squares F test between curves: $F_{(1,175)} = 21.37$, $p < 0.0001$; Fig. 2B). The initial magnitude of L-LTP induced was significantly greater in $Arc^{-/-}$ mice compared with WT littermates (WT $230.46\% \pm 8.88$, $n=10$; KO $300.63\% \pm 14.45$, $n=10$; t test: $p=0.0006$; Fig. 2C). Similarly, the magnitude of LTP 1 h (WT $177.7\% \pm 9.91$, $n=9$; KO $226.55\% \pm 10.17$, $n=10$; t test $p=0.003$) and 2 h (WT $155.11\% \pm 8.27$, $n=10$; KO $194.72\% \pm 8.55$, $n=10$; t test: $p=0.003$) postinduction was significantly greater in $Arc^{-/-}$ mice than WT littermates (Fig. 2C). These results show that Arc is not required for the maintenance of TBS L-LTP and that loss of Arc may lead to an increase in TBS LTP magnitude when Arc is absent throughout development.

CA1 LTP induced by theta burst stimulation is normal in conditional Arc KO mice

The enhanced magnitude of L-LTP after TBS in germline Arc KO lines may be the result of compensatory mechanisms during development. To temporally restrict the loss of Arc, we used a floxed Arc KO line ($Arc^{fl/fl}$; Chen et al., 2018) injected at 4–6 weeks of

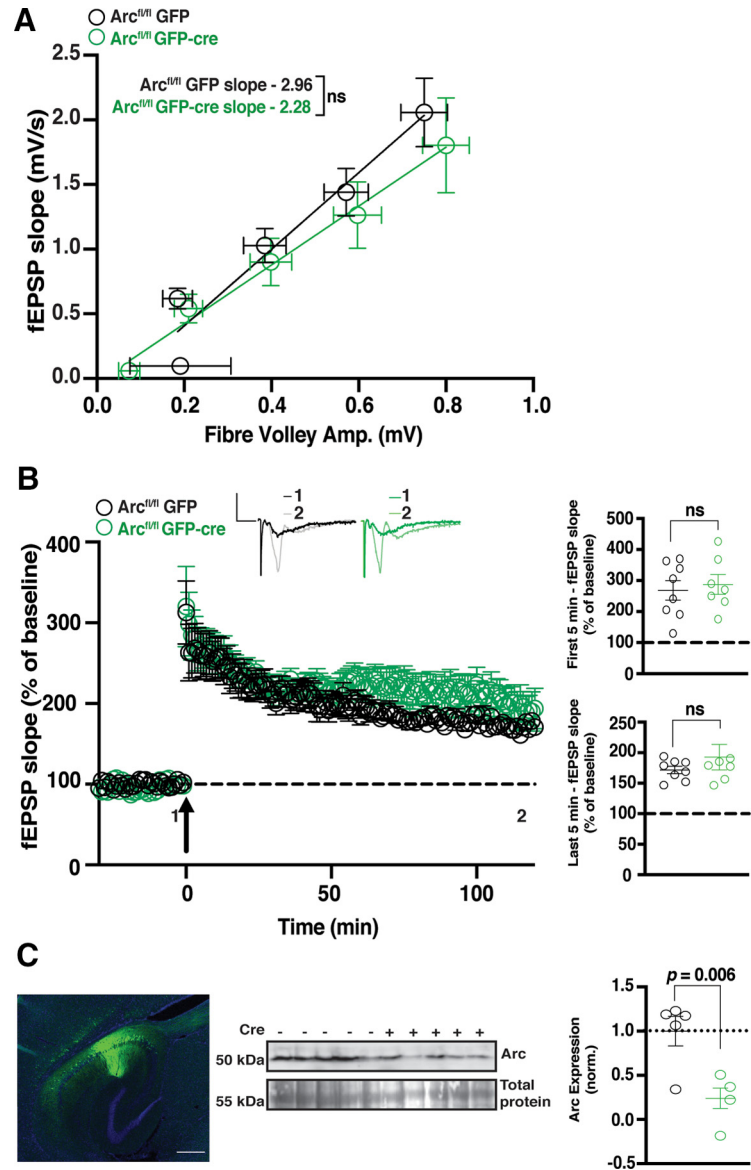


Figure 3. TBS CA1 LTP is normal in $Arc^{fl/fl}$ animals. **A**, There was no significant difference in basal synaptic transmission between $Arc^{fl/fl}$ animals injected with GFP and GFP-Cre-injected animals. **B**, There was no significant difference in the magnitude of LTP over the first 5 min poststimulation, nor over the last 5 min of recording, between $Arc^{fl/fl}$ animals injected with GFP-cre and those injected with GFP alone when LTP was induced using a theta-burst stimulation protocol (4 trains at 10 s intertrain intervals; each train consisted of 10 bursts at 5 Hz, with each burst containing 4 stimuli at 100 Hz). Data are represented as mean \pm SEM. Scale bars: 0.5 mV (horizontal) and 5 ms (vertical). **C**, Arc protein expression was significantly reduced in $Arc^{fl/fl}$ animals injected with GFP-cre (green circles) compared with GFP alone (black circles). Inset, Representative image of GFP expression (green is GFP, blue is DAPI), 2 weeks postinjection of AAV:CaMKII-GFP-cre into the CA1 region of the hippocampus. Scale bar, 500 μ m.

age with AAV5/CaMKII-GFP-cre (GFP-cre) to knock-out Arc in excitatory neurons of CA1, or AAV5/CaMKII-GFP alone (GFP) as a matched injection control. Two weeks after viral expression (6–8 weeks of age), slices were prepared for electrophysiology. Slices were used for recordings if GFP was identified along the length of the CA1 region of the hippocampus (Fig. 3, inset). Basal transmission was similar between $Arc^{fl/fl}$ animals injected with GFP and GFP-Cre injected animals (GFP slope = 2.96 ± 0.69 , $n=8$; GFP-cre slope = 2.28 ± 0.75 , $n=7$; linear regression, extra-sum-of-squares F test between curves: $F_{(1,71)} = 1.866$, $p=0.18$; Fig. 3A). We found no difference in the initial magnitude of TBS-induced LTP between $Arc^{fl/fl}$ animals injected with GFP-cre and those injected with GFP alone (GFP $271.1\% \pm 25.79$, $n=8$; GFP-

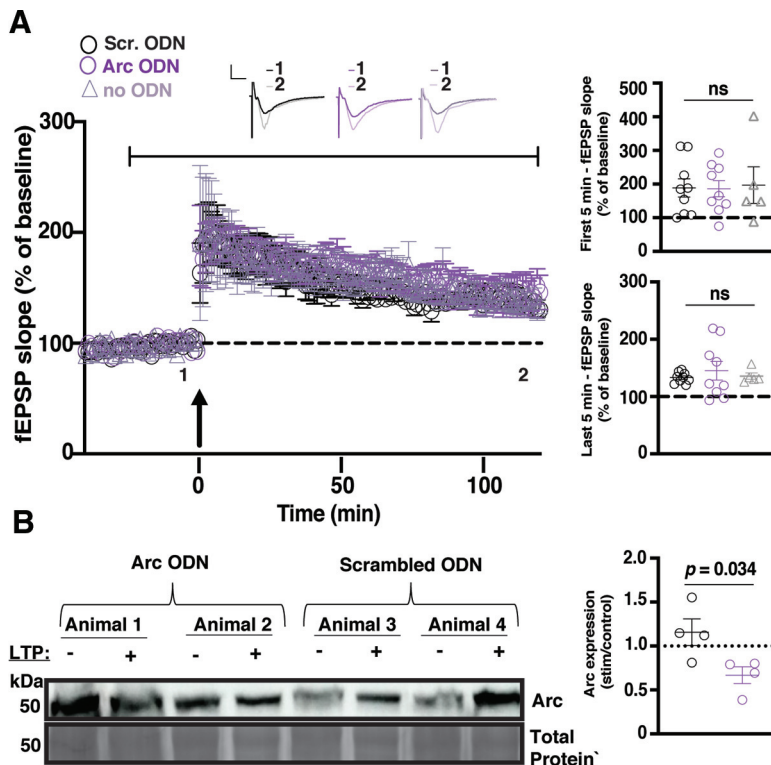


Figure 4. LTP is normal in the presence of Arc antisense ODNs. **A**, The magnitude of LTP was not significantly different between slices incubated in aCSF containing an ODN against Arc (purple circles), a scrambled ODN (black circles), or normal aCSF (gray triangles) over the first 5 min poststimulation or the last 5 min poststimulation when LTP was induced using a theta-burst stimulation protocol (4 trains at 10 s intertrain intervals; each train consisted of 10 bursts at 5 Hz, with each burst containing 4 stimuli at 100 Hz). Data are represented as mean \pm SEM. Scale bars: 5 mV (horizontal) and 5 ms (vertical). After a 10 min baseline recording, normal aCSF was switched to aCSF containing ODN for the duration of the recording, as indicated by the black bar above the graph. **B**, Arc protein expression increased after TBS in the slices that were incubated with the scrambled ODN (black circles) but not in those that were incubated with the Arc ODN (purple circles).

Cre $281.1\% \pm 37.35$, $n = 7$; t test: $p = 0.68$; Fig. 3B). There was also no significant difference in the magnitude of LTP 2 h postinduction (GFP $180.8\% \pm 20.79$, $n = 8$; GFP-Cre $193.4\% \pm 36.77$, $n = 7$; t test: $p = 0.32$; Fig. 3B). These results show that knockdown of Arc postdevelopment has no effect on the induction or maintenance of TBS L-LTP. To determine the magnitude of Arc depletion, we measured Arc protein levels by Western blot analysis from the stimulated CA1 region of a subset of slices, postrecording. Arc was significantly reduced in slices from Arc^{fl/fl} animals injected with GFP-cre compared with GFP alone (GFP 1 ± 0.17 , $n = 5$; GFP-Cre 0.24 ± 0.11 , $n = 5$; t test: $p = 0.006$; Fig. 3C).

CA1 LTP induced by theta burst stimulation is normal in slices incubated with Arc antisense ODNs

It is possible that compensatory mechanisms may still be initiated with 1–2 weeks of Arc knockdown. To determine if acute Arc knockdown could interfere with CA1 LTP, we used Arc antisense ODNs previously used in LTP studies (Messaoudi et al., 2007). After a 10 min stable baseline recording in normal aCSF, a switch was made to aCSF containing Arc ODNs or scrambled ODNs. Following a further 30 min baseline, TBS LTP was induced, and recordings were followed for 2 h. In some experiments, slices were collected for Western blot analysis. There was no change in synaptic responses after the aCSF switch during baseline recordings (Fig. 4A). We found no difference in the initial magnitude of LTP between slices treated with the Arc ODN, the scrambled ODN, and slices incubated in normal aCSF

(Scrambled ODN $188.5\% \pm 27.22$, $n = 9$; Arc ODN $186.2\% \pm 24.11$, $n = 9$; aCSF $196.9\% \pm 54.14$, $n = 5$; one-way ANOVA: $p = 0.98$; Fig. 4A). There was also no significant difference in the magnitude of LTP 2 h postinduction (Scrambled ODN $133.03\% \pm 1.04$, $n = 9$; Arc ODN $145.1\% \pm 16.10$, $n = 9$; no ODN $135.55\% \pm 5.46$, $n = 5$; one-way ANOVA: $p = 0.71$; Fig. 4A). The lack of functional effects of Arc ODNs may be because of an incomplete effect on blocking Arc synthesis during LTP induction. To test whether Arc ODNs affected Arc protein expression after LTP, we compared Arc expression in unstimulated slices with slices from the same animal where LTP was induced. We found that Arc expression was increased after LTP in slices treated with scrambled ODNs (1.16 ± 0.15 , $n = 4$; Fig. 4B). In contrast, Arc expression was decreased after LTP in slices treated with Arc ODNs and was significantly different from slices incubated with scrambled ODNs (0.69 ± 0.10 , $n = 4$ t test, $p = 0.034$; Fig. 4B), showing that Arc ODNs significantly impaired Arc protein induction after LTP. These results suggest that acutely blocking *de novo* Arc synthesis during and after LTP induction has no effect on CA1 TBS LTP.

Arc is not required for the maintenance of LTP induced by theta burst stimulation in the dentate gyrus *in vivo*

Finally, we assessed whether Arc is required for LTP *in vivo* in the DG at medial perforant path-granule cell synapses. There was no significant difference in basal transmission in the DG between Arc^{GFP/GFP} KO and WT littermates (WT slope = 0.011 ± 0.001 , $n = 6$; Arc^{tm1Sul} KO slope = 0.013 ± 0.002 , $n = 6$; linear regression, extra-sum-of-squares F test between curves: $F_{(2,272)} = 2.024$, $p = 0.13$; Fig. 5A). The initial magnitude of LTP induced by TBS was significantly enhanced in Arc^{GFP/GFP} KO animals compared with WT littermate controls (WT $133.13\% \pm 6.87$, $n = 6$; KO $158.88\% \pm 4.39$, $n = 6$; t test: $p = 0.01$; Fig. 5B). However, there was no difference in the magnitude of LTP 3 h poststimulation (WT $134.25\% \pm 13.03$, $n = 6$; KO $146.45\% \pm 12.51$, $n = 6$; $p = 0.51$; Fig. 5B). These data further suggest that Arc is not required for the expression or maintenance of L-LTP.

Discussion

The mechanisms of LTP induction and expression are well defined, but less is known about maintenance. Arc is one of the most robustly expressed genes after LTP induction, with the prevailing view that Arc protein is important for some aspect of LTP (Link et al., 1995; Lyford et al., 1995; Steward et al., 1998; Ying et al., 2002; Moga et al., 2004). Indeed, Arc protein is increased after LTP induction (Lyford et al., 1995; Steward et al., 1998; Ying et al., 2002; Moga et al., 2004) and Arc antisense ODNs have been shown to affect LTP maintenance (Guzowski et al., 2000; Messaoudi et al., 2007). However, relatively few studies have comprehensively tested the role of Arc in hippocampal LTP. Here, we show that LTP is unaffected in the hippocampus

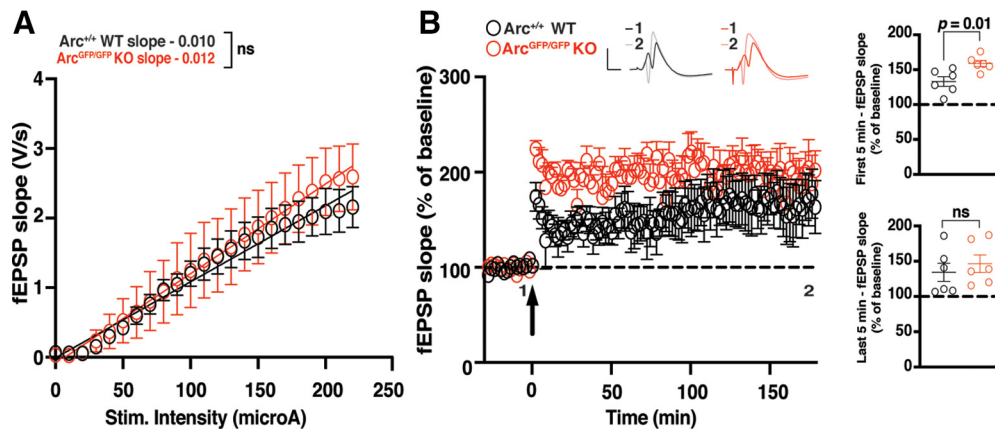


Figure 5. The maintenance of DG LTP *in vivo* is normal in Arc KO animals. **A**, There was no significant difference in basal synaptic transmission between the Arc^{GFP/GFP} KO (red circles) and WT (black circles) littermates in the dentate gyrus *in vivo*. **B**, The magnitude of LTP over the first 5 min poststimulation was significantly enhanced in Arc^{GFP/GFP} KO animals compared with WT littermates, but was not significantly different over the last 5 min of recording when LTP was induced using a theta-burst stimulation protocol (4 trains at 10 s intertrain intervals; each train consisted of 10 bursts at 5 Hz, with each burst containing 4 stimuli at 100 Hz). Data are represented as mean \pm SEM. Scale bars: 5 mV (horizontal) and 5 ms (vertical).

using multiple approaches to interfere with Arc expression and using an array of LTP induction paradigms in slices and *in vivo*. These data indicate that Arc is not essential for the expression or maintenance of LTP.

Arc is not required for the maintenance of CA1 NMDA receptor-dependent LTP in hippocampal slices

Standard NMDAR-dependent LTP in the CA1 has not previously been investigated thoroughly in Arc KO mice. A previous study, using Arc^{-/-} mice, found that LTP was disrupted in CA1 using a patch-clamp induction protocol (depolarization of the postsynaptic cell paired with 200 presynaptic pulses at 1.5 Hz; Plath et al., 2006). However, we found no significant difference in the maintenance of LTP induced using a standard extracellular field recording HFS protocol (2 \times 100 pulses at 100 Hz; Reymann et al., 1985) in Arc^{GFP/GFP} mice. An NMDAR-independent LTP that requires type 1 mGluRs receptors has also been shown to be impaired in Arc KO animals (Wang et al., 2016). The induction protocol used for this form of LTP relies on blocking of NMDAR during a 200 Hz stimulation protocol. TBS can also induce robust, persistent LTP and is arguably more physiologically relevant than HFS (Larson et al., 1986; Thomas et al., 1998). Surprisingly, we found that LTP was enhanced in Arc^{GFP/GFP} KO animals, compared with WT littermates. Recent studies have found behavioral differences between the Arc^{-/-} and the Arc^{GFP/GFP} Arc KO lines (Managò et al., 2016; Gao et al., 2019). This may be because of a neomycin cassette present in Arc^{GFP/GFP}, which may lead to off-target effects (Pham et al., 1996; Scacheri et al., 2001; Gao et al., 2019). We found that basal synaptic transmission is decreased in Arc^{-/-} mice but normal in Arc^{GFP/GFP} mice. Nevertheless, similar to the Arc^{GFP/GFP} line, we find that TBS-induced LTP is enhanced in Arc^{-/-} mice. However, TBS-induced LTP was unaffected in Arc cKO animals, suggesting that this enhanced LTP may be because of altered plasticity during development in Arc germline KO animals. Increased dendritic spine size and decreased spine density have been observed in the hippocampus of Arc KO animals (Peebles et al., 2010), and Arc has been shown to regulate developmental plasticity in the visual cortex (McCurry et al., 2010; Jenks et al., 2017; Jenks and Shepherd, 2020) and hippocampus (Gao et al., 2018). Alterations in synapse number and structure can alter the magnitude or type of subsequent plasticity (Harris et al., 1992; Spacek and Harris, 1997; Matsuzaki et al., 2004; Arendt et al.,

2013), and, therefore, disrupted plasticity during development in Arc KO animals may lead to subsequent changes in adult plasticity that are secondary to the direct functional role of Arc. Consistent with there being no direct functional role of Arc in CA1 LTP, we found the acute block of Arc protein synthesis using Arc antisense ODNs during and after LTP had no effect on LTP magnitude.

Because LTP expression or maintenance was not impaired in Arc KO mice, we wondered whether the threshold to induce LTP is altered. Metaplasticity, or the plasticity of plasticity, describes the phenomenon where alterations to the basal state of neurons changes the type of plasticity induced by a given specific stimulation paradigm (Abraham and Bear, 1996). For example, the synaptic modification rules based on stimulation frequency, as determined by the Bienenstock-Cooper-Munro model, can be altered by differences in NMDAR composition at stimulated synapses (Bienenstock et al., 1982; Dudek and Bear, 1992; Jedlicka, 2002; Cooper and Bear, 2012). Thus, we tested whether there were any differences in LTP induced using a protocol that would normally induce only E-LTP (Frey and Morris, 1997) or a moderate frequency (10 Hz) that normally induces little to no change in synaptic strength (Dudek and Bear, 1992). We found no significant difference between genotypes using these induction protocols. These data suggest that Arc is not required for CA1 LTP in hippocampal slices.

Arc does not regulate LTP in the DG *in vivo*

Previous studies found that Arc antisense ODNs affected *in vivo* LTP in the DG of rats (Guzowski et al., 2000; Messaoudi et al., 2007). We investigated the role of Arc in LTP *in vivo* in the DG of Arc^{GFP/GFP} animals. We found that the magnitude of LTP induction was significantly greater in the first 5 min poststimulation, but there was no significant difference between Arc^{GFP/GFP} and WT animals 3 h after induction. Previous studies found that the magnitude of LTP induction was initially enhanced in Arc^{-/-}, but the maintenance was significantly attenuated (Plath et al., 2006). Our experiments used the same induction protocol in the DG, but were undertaken in isoflurane-anesthetized mice, rather than urethane anesthesia (Plath et al., 2006). It is possible that maintenance of LTP in the DG is differentially affected by either the anesthetic regime or Arc knockout strategy. A different study infused Arc antisense ODNs into the DG of rats 1.5 h before LTP induction, which had no effect on the initial LTP

magnitude but significantly attenuated LTP magnitude 5 d poststimulation (Guzowski et al., 2000). Our studies do not rule out a role for Arc in the maintenance of LTP over days. Another study found that when Arc antisense ODNs were injected into the DG *in vivo* 15 min post-LTP induction, synaptic strength transiently decreased to near baseline for ~3 h before it recovered to normal levels of potentiation (Messaoudi et al., 2007) indicating that the ODNs did not alter the persistence of LTP. Further, when ODNs were injected 2 h post-LTP induction, synaptic strength also decreased to near baseline, although recordings were not made 3 h later to see whether potentiation recovered (Messaoudi et al., 2007). In germline Arc KO mice, it is possible that there is compensation by an unknown protein that performs a similar function in LTP, which still implies that Arc is not essential for LTP.

How does arc mediate long-term memory?

Although LTP has long been associated with long-term memory, and long-term memory is critically dependent on Arc expression (Guzowski et al., 2000; Plath et al., 2006; Ploski et al., 2008), the potentiation of synapses is not the only critical process (Zhang and Linden, 2003; Mozzachiodi and Byrne, 2010; Kyrke-Smith and Williams, 2018; Lisman et al., 2018; Abraham et al., 2019). Similarly, in response to stimulation paradigms that induce LTP, there are widespread changes aside from LTP, such as heterosynaptic depression, changes to intrinsic excitability and the regulation of gene expression, protein synthesis, and signaling pathways (Lynch et al., 1977; Andersen et al., 1980; Abraham and Goddard, 1983; Lopez de Armentia et al., 2007; Caroni et al., 2014; Kyrke-Smith and Williams, 2018). We posit that Arc expression driven by LTP induction may contribute to non-LTP plasticity that serves a homeostatic function to maintain overall network stability such as heterosynaptic LTD or synaptic downscaling (Shepherd and Bear, 2011). A loss of these forms of synaptic weakening in the germline Arc KO mice could account for the exaggerated LTP induction that we and others (Plath et al., 2006) have observed. This idea is also consistent with the finding that Arc interacts with CaMKII β at inactive synapses to remove AMPARs and decrease synaptic strength (Okuno et al., 2012; El-Boustani et al., 2018). Similarly, Arc may eliminate small mushroom spines after learning (Nakayama et al., 2015), although it is unclear if this occurs through heterosynaptic LTD. The net result of these processes would enhance the signal-to-noise ratio of the potentiated synapses, while still maintaining a normal dynamic range of plasticity and neuronal activity, which may be critical for long-term memory. Similarly, Arc-dependent homeostatic scaling (Shepherd et al., 2006; Korb et al., 2013) may also be important for stabilizing memory engrams. Arc-dependent plasticity mechanisms are also important for normal experience-dependent sculpting of circuits during development (Jenks et al., 2017; Jenks and Shepherd, 2020), which may be critical for normal learning and memory later in adulthood (Gao et al., 2018).

Epigenetic mechanisms are also important for long-term memory and L-LTP (Korzus et al., 2004; Levenson et al., 2004; Gräff et al., 2011; Gräff et al., 2014; Jarome and Lubin, 2014). Interestingly, Arc has been shown to localize in the nucleus and interact with histone acetyltransferase Tip60, leading to increased acH4K12 (Wee et al., 2014), which is associated with increased gene expression. Arc is also expressed in promyelocytic leukemia nuclear bodies in the nucleus, where it may regulate the expression of plasticity-related genes (Korb et al., 2013). We recently showed that Arc can form virus-like capsids that can transfer RNA cell to cell (Pastuzyn et al., 2018). Although the role of

intercellular Arc in plasticity and memory remains to be determined, it is another mechanism that may underlie long-term memory independent of LTP. Thus, many possibilities exist to explain how Arc can play a critical role in long-term memory without being necessary for L-LTP maintenance. Determining the precise molecular processes governed by Arc will likely provide great insight into the consolidation of memory.

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