LTP- and LTD-inducing stimulations cause opposite changes in 

*Arc/Arg3.1* mRNA level in hippocampal area CA1 *in vivo*

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LTP- AND LTD-INDUCING STIMULATIONS CAUSE OPPOSITE CHANGES IN 
*ARC/ARG3.1* MRNA LEVEL IN HIPPOCAMPAL AREA CA1 *IN VIVO*

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Immediate early genes (IEGs) typically are the first genetic responders to a variety of cellular activations. The IEG that encodes activity-regulated cytoskeleton-associated protein (arc/arg3.1) has attracted much interest because its mRNA is transported to and translated near activated synapses. Moreover, arc has been implicated in both long-term potentiation (LTP) and long-term depression (LTD). However, little is known about the time course of altered arc expression during LTP and LTD. Here we characterized arc mRNA levels in area CA1 of the adult rat hippocampus in vivo after LTP- and LTD-inducing stimulations that were identical except for the temporal patterning of the stimulation pulses. We observed a persistent increase in arc mRNA level during LTP. In contrast, during LTD, arc mRNA level first was decreased and then transiently increased relative to control level. These findings demonstrate that arc mRNA is regulated differently during LTP and LTD, and they provide evidence for stimulation-induced down-regulation of mRNA availability during LTD. Findings of abbreviated LTD when transcription was inhibited indicate that the prolonged maintenance of the NMDA receptor-dependent LTD studied here requires de novo transcription. Furthermore, findings of no LTD-associated change in the mRNA level of the IEG zif268 show that the decrease in arc mRNA during LTD is not a general genetic response. Thus, the regulation of arc expression not only differs between LTP and LTD but also diverges from that of other IEGs implicated in activity-dependent synaptic plasticity.
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Since my early childhood I have always been fascinated by living organisms. This fascination became a passion and when it was time to choose a career path, I could not think of studying anything else than biology and biochemistry to understand life. Studying molecular foundation of life was sufficient to understand life on a cellular basis but not enough to understand human life. Specifically, I got interested in how our previous experiences shape our future life and decided to further my education by joining the CNUP to study the molecular basis of memory. I would like to thank my mentor, Dr. Edda Thiels, for letting me work in her laboratory and for everything I learned from her. I would also like to thank Drs. DeFranco, Monaghan, and Miyamae for teaching me various techniques that I needed for my projects and Drs Barrionuevo and Fanselow for serving on my dissertation committee.

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1.0 INTRODUCTION

Elucidating the biological mechanisms that underlie the information-storage and -processing capacity of the brain has been a long-standing goal of neuroscientists. In the late 19th century, Santiago Ramon y Cajal proposed that strengthening the connections between two neurons in order to increase the efficiency of their communication to be the mechanism of forming memories (Ramon y Cajal, 1894). His proposal was the first time when the synaptic strength between two neurons was considered to be important for information consolidation. About 50 years later, another neuroscientist, Donald Hebb postulated that: “When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased”. This postulate is widely known as Hebb's learning rule (Hebb, 1949) which adds to Cajal’s hypothesis the necessity of a metabolic component in order to increase the synaptic strength between two neurons.

Evidence collected within the last few decades provide insights such as the ability to change the synaptic strength between two neurons, a process called “synaptic plasticity”, is crucial for wiring of neural circuits throughout the life of an animal. Moreover, it has been shown in a variety of systems ranging from mollusk to mammals that some forms of synaptic plasticity can be long-lasting (Pittenger and Kandel, 2003).
This persistent nature of long-term synaptic plasticity makes it a suitable candidate to study memory at the circuit and cellular level. Moreover, extending Cajal's and Hebb's postulates, alterations of the synaptic strength occur bidirectionally: Strengthening of the synapses is called long-term potentiation (LTP) and the weakening of the synapses is called long-term depression (LTD). Both LTP and LTD have been observed at the excitatory synapses in many regions of the brain including the hippocampus, a region that has been shown to be essential in the acquisition of explicit memories (Malenka and Bear, 2004; Neves et al., 2008). More specifically, studies using human subjects identified hippocampus to be a main player in episodic memory, the type of memory that links experiences with time and space (Kemp and Manahan-Vaughan, 2007). In addition, interruptions of LTP and LTD in the hippocampus both interfere with spatial learning in laboratory animals, indicating that long-term synaptic plasticity is essential for hippocampal memory (Kemp and Manahan-Vaughan, 2007; Morris et al., 1986; Morris and Frey, 1997).

N-methyl-D-aspartate (NMDA) receptor-dependent forms of LTP and LTD in area CA1 of the hippocampus are the most extensively studied types of long-term synaptic plasticity (Malenka and Bear, 2004). Our laboratory studies NMDA-receptor dependent -LTP and -LTD in the adult rat hippocampus in vivo. In NMDA receptor-dependent long-term synaptic plasticity, calcium influx through the NMDA receptors is the first step of intracellular events that occur in postsynaptic neurons. Increases in calcium concentration results in the activation of calmodulin (CaM) (Malenka et al., 1989; Malenka et al., 1988). The phosphatase Calcineurin has a high affinity for CaM, thus gets activated by CaM at low levels of calcium increase, such as following LTD.
induction (Mansuy, 2003). Generally, protein kinases get activated at higher levels of calcium influx such as following LTP induction and when activated, these protein kinases lead to inactivation of protein phosphatases such as protein phosphatase 1 (PP1), as a result kinase pathways dominate LTP induction (Malenka and Bear, 2004; Mansuy, 2003). On the other hand, LTD is characterized by the dominance of phosphatase activity to dephosphorylate their target proteins (Mansuy, 2003; Winder and Sweatt, 2001). In conclusion, the amount of increase in calcium levels seem to play a decisive role in selection of the general pathway that will be turned on to direct the postsynaptic cell towards potentiation or depression.

The signaling pathways that are recruited during LTP and LTD, respectively, can affect many processes within the neuron including transcription (Malenka and Bear, 2004). It is proposed that the changes in the transcription factor phosphorylation levels during synaptic plasticity alter mRNA levels which in turn are thought to be important for the maintenance of long-term synaptic plasticity (Alberini, 2009) and changes in gene expression were shown to occur following both LTP and LTD inductions (Haberman et al., 2008).

Alteration in the expression of immediate early genes (IEGs) is the first genetic response to many kinds of cellular activations (Dragunow, 1996; Lanahan and Worley, 1998; Miyashita et al., 2008). Many IEGs regulated by neural activity encode transcription factors; however, some encode effector proteins. One effector protein-encoding gene that has received much attention in the context of activity-dependent synaptic plasticity, such as LTP and LTD, is the gene that encodes activity-regulated cytoskeleton-associated protein (arc, also known as arg3.1) (Bramham et al., 2010;
Bramham et al., 2008; Tzingounis and Nicoll, 2006). Arc mRNA levels were found to be increased and shown to be transported to activated synapses after LTP-inducing stimulation in hippocampus (Lyford et al., 1995; Steward et al., 1998; Steward and Worley, 2001a; Steward and Worley, 2001b); genetic deletion of arc was shown to be associated with impaired LTP and LTD in hippocampal slice preparations (Plath et al., 2006); and acute knock-down of arc was shown to disrupt LTP in area CA1 as well as the dentate gyrus of the hippocampus (Guzowski et al., 2000; Messaoudi et al., 2007). Furthermore, consistent with the idea of a link between activity-dependent synaptic plasticity and the establishment of long-term memories (Bliss and Collingridge, 1993; Braunewell and Manahan-Vaughan, 2001; Malenka and Bear, 2004), mice in which arc was either knocked out or acutely knocked down were found to exhibit consolidation deficits (Guzowski et al., 2000; Plath et al., 2006). However, an increase in arc expression after LTP-inducing stimulation has not been observed consistently (French et al., 2001; Miyashita et al., 2009), and overexpression of arc was found to be associated with a reduction in AMPA receptor-mediated currents and occlusion of N-methyl-D-aspartate (NMDA) receptor-dependent LTD (Rial Verde et al., 2006).

Despite much interest in arc as an early responder to plasticity-inducing synaptic input and a critical player in the establishment of long-term synaptic modification, relatively little is known about the time course of altered arc expression after LTP- or LTD-inducing stimulation. Miyashita et al. (2009) observed an increase in arc expression within minutes after LTP induction in area CA1; others reported an increase in arc mRNA levels 30 min to 4 hours after LTP induction in the dentate gyrus (Lyford et al., 1995; Steward et al., 1998). These findings suggest that arc induction in response
to LTP-inducing stimulation is very rapid and quite prolonged. Such comparisons across hippocampal subregions, however, may not be warranted, especially in light of differential rates of arc induction in areas CA3 versus CA1 in response to behavioral manipulations (Gusev et al., 2005; Miyashita et al., 2009). The profile of arc expression during LTP in a given brain region, specifically one known to undergo experience-dependent plasticity, therefore remains to be determined.

There currently is no information about changes in arc expression during LTD. Based on findings of disrupted NMDA receptor-dependent LTD in the presence of transcription inhibitors (Kauderer and Kandel, 2000), together with the aforementioned observations that arc overexpression leads to LTD occlusion (Rial Verde et al., 2006), one might predict that LTD-inducing stimulation triggers an increase in arc expression. On the other hand, results showing no effect of transcription inhibitors on NMDA receptor-dependent LTD (Manahan-Vaughan et al., 2000) suggest that arc expression is not increased during LTD or that any increase is non-consequential for the establishment of this form of LTD.

In this study we aimed to characterize arc expression during NMDA receptor-dependent LTP and LTD in area CA1 of the adult rat hippocampus in vivo. To avoid that any differences in expression profiles between LTP and LTD that could be attributed to procedural differences, such as amount or duration of stimulation, we kept all of the stimulation parameters identical but varied only the temporal pattern of the plasticity-inducing stimulation between LTP- and LTD-experiments. Our results show that arc mRNA levels are regulated very differently during the two forms of synaptic plasticity. Whereas LTP is associated with a persistent increase in arc expression, LTD is
associated a rapid decrease followed by a transient increase in arc mRNA level. These findings provide evidence for stimulation-induced down-regulation of transcriptional product during LTD.
2.0 METHODS

2.1 IN VIVO ELECTROPHYSIOLOGY

Electrophysiological methods were used as previously described (Thiels et al., 2002; Thiels et al., 1992). All procedures were in compliance with and approved by the Institutional Animal Care and Use Committee, University of Pittsburgh. Briefly, male Sprague Dawley rats (Hilltop, Scottdale, PA; 250-350 g) were anesthetized first with an intraperitoneal (i.p.) injection of 8% chloral hydrate in 150 mM NaCl (0.4 g/kg) and then maintained under constant anesthesia throughout the remainder of the recording session with intravenous (i.v.) injection of the same anesthetic (0.15 g/kg/h). Rats were placed in a stereotaxic apparatus, and an incision was made on the scalp. After the skin was retracted, one small hole was drilled into the skull on the left side (relative to bregma: AP, -1.7 mm; ML, -1.1 mm) and one on the right side (relative to bregma: AP, -3.6 mm; ML, +2.3 mm). After removal of dura mater, a pair of bipolar metal electrodes, insulated except for the 100 µm to 150 µm at the tip, was lowered into area CA3 of the left dorsal hippocampus (final DV placements relative to the surface of the brain: about -3.5 mm) and a glass electrode, filled with 2 M saline (impedance of 0.9-1.4 MΩ), was lowered to either str. pyramidale or, in separate groups of animals, str. radiatum of area CA1 of the right dorsal hippocampus (final DV placements relative to the surface of the
brain: about -1.8 mm and -2.1 mm, respectively). An input-output (I/O) function that relates the intensity of commissural stimulation (20-200 µA; 100-µs duration) to the amplitude of the evoked CA1 pyramidal cell population spike was determined at the beginning of each experiment. An additional I/O function that relates the intensity of commissural stimulation to the initial slope of the evoked CA1 population EPSP was determined for experiments involving recordings in str. radiatum. A stimulation intensity that produced a response with an amplitude that was 30% to 40% of the maximum amplitude of the evoked population spike for str. pyramidale recordings or an initial slope that was 30% to 40% of the maximum slope of the evoked population EPSP for str. radiatum recordings before delivery of high-frequency or paired-pulse stimulation (HFS and PPS, respectively) was used for test pulses (a series of 10 pulses at 0.1 Hz, delivered at 5-min intervals before and after HFS or PPS). Baseline response level was determined by delivery of successive series of test pulses for a total of 15 min to 20 min before HFS or PPS, i.e., until stable baseline responding had been established. LTP was induced by applying HFS (4 trains of 100 pulses at 100 Hz delivered at 130-sec intervals), and LTD was induced by applying PPS (200 pairs of pulses with a 25-ms interval delivered at 0.5 Hz) to the commissural fibers using a stimulation intensity that was 60% to 75% of the maximum amplitude of the population spike, as determined with the first I/O function. Thus, the total number of stimulation pulses (400 pulses), total duration of patterned stimulation (400 sec), and stimulation intensity were essentially identical between the two protocols. After termination of HFS or PPS, additional series of test pulses were delivered for a total of either 10 min, 30 min, 60 min, or 120 min using the same stimulation intensity as was used for test pulses before HFS or PPS.
delivery. Recorded data were amplified, filtered (0.1 Hz-10 kHz), digitized, and stored on computer disk for later analysis. In experiments requiring local drug infusion, a glass pipette (tip inner diameter = 35-50 µm) connected to a positive-pressure syringe pump (Harvard Apparatus, Holliston, MA) was lowered into area CA1 within 200 µm to 300 µm of the tip of the recording electrode. D-aminophosphonovaleric acid (D-APV; 0.5 mM in the drug pipette; dissolved in 150 mM NaCl; Tocris, Ellisville, MO) was infused continuously (6-8 nl/min) from 60 min before HFS or PPS until the end of recording. Actinomycin D (ActD; 0.8 mM in the drug pipette; dissolved in 0.5% methanol/95% 150 mM NaCl; Calbiochem, La Jolla, CA) was infused over the course of 15 min to 20 min (50-60 nl/min) beginning 90 min before HFS or PPS. At the end of electrophysiological recording, rats were decapitated, the brains removed rapidly, and the right hippocampus excised in the presence of ice-cold artificial cerebrospinal fluid. Within one min of brain removal, a 1 mm³-piece of tissue from dorsal area CA1 (recording site) and an equal-sized piece of tissue from ventral area CA1 (within-subject control) of the right hippocampus were dissected out and transferred to separate, color-coded 1.5-ml tubes maintained on dry ice for instantaneous freezing of the tissue samples. The samples were stored at -80°C until molecular analysis.

2.2 QUANTITATIVE PCR (QPCR)

From each tissue sample, total RNA was isolated using Trizol® reagent (Invitrogen, Carlsbad, CA) according to manufacturer instructions. Isolated RNA was treated with DNAse I (Invitrogen) prior to reverse transcription in order to remove
genomic DNA. The integrity of the RNA was confirmed by agarose gel electrophoresis, and the yield was determined by measurement of the absorbance at 260/280 nm. Using the Superscript III kit (Invitrogen) 0.5 µg total RNA was reverse-transcribed with poly-dT primers, nucleotides, and buffers in a total reaction volume of 20 µl according to manufacturer instructions. In each well, 2 µl of the appropriate cDNA was added to the qPCR reaction mixture, which consisted of 12.5 µl SYBR-green master mix (SA Biosciences, Frederick, MD), 6 µl of primer mixture containing 400 nM of each forward and reverse primers (final concentration of each ~100 nM), and 4.5 µl nuclease-free water, to bring the total volume in each well to 25 µl. A minus reverse transcriptase negative control was included in all cases. Reactions were performed in triplicates using a Biorad IQ real-time thermocycler (Hercules, CA) with an initial Taq polymerase activation at 95°C for 10 min, followed by 50 cycles each of which involved 30 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C. The efficiency (E) of each primer set was determined using a serial dilution of standard cDNA, which was generated by reverse transcription of 2 µg of total RNA extracted from whole hippocampus using polydT primers in a reaction volume of 20 µl. The threshold cycle (Ct) for each sample was chosen to lie within the early exponential rise phase, and any wells with multiple peaks during the melting-point analysis were excluded from analysis. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (gapdh) run in the same qPCR experiment. Gene expression in dorsal area CA1 (experimental tissue), relative to expression in ventral area CA1 (within-subject control tissue), was determined by the efficiency-corrected delta cycle threshold (ΔCt) method using the following formula:

\[
\text{relative quantity (RQ)}_{\text{arc,zif268}} = \frac{E_{\text{arc,zif268}}^{\text{Ct(ventral)-Ct(dorsal)}}}{E_{\text{gapdh}}^{\text{Ct(ventral)-Ct(dorsal)}}}.
\]
calibration curves used for determining primer set efficiencies and the mean Ct values used in calculations are shown in the Appendix.

All primers were selected to have the same melting temperature of 60 °C to enable running them within the same plate. In addition, all primers were designed to be on two exons to allow us to distinguish between mature versus newly synthesized mRNA by the melting curves. In all cases a single peak was observed. Arc, zif268, and gapdh PCR products were cloned using the TA cloning kit (Invitrogen) according to instructions of the manufacturer. The sequence-specific amplification was confirmed by sequencing and subsequent NCBI blast. The primers and their product sizes were as follows:

Arc (fwd): 5'-AGTCTTGGGCAGCATAGCTC-3', Arc (rev): 5'-GCCGAAGTCTGCTTTTCTTC-3' (115 bp); Zif268 (fwd): 5'-CAGCGCTTTTCAATCCTCAAA-3', Zif268 (rev): 5'-TGGGATAACTTGTCTCCACCA-3' (119 bp); Gapdh (fwd): 5'-GAAGGGCTCATGACCACAGT-3', Gapdh (rev): 5'-GGATGCAGGGATGATGTTCT-3' (117 bp).

2.3 STATISTICAL ANALYSES

To validate significant levels of synaptic potentiation or depression after HFS or PPS, respectively, two-tailed paired Student’s t-tests were carried out on data collected during the last 5 min before HFS or PPS and during the last 5 min of electrophysiological recording. Differences in level of synaptic change between groups were compared with two-tailed Student’s t-tests for independent groups. To determine
whether delivery of baseline stimulation, HFS, or PPS is associated with a significant change in arc or zif268 mRNA level, we first transformed the RQ values calculated as described above on a log$_2$ scale to achieve normal distribution of the data (Bland and Altman, 1996; Kubista et al., 2007) and then tested the resulting distributions against the null-hypothesis of equal mRNA level in dorsal and ventral samples (i.e., a population mean of 0.0) using two-tailed one-sample Student’s $t$-tests. An $\alpha$-level of $\leq 0.05$ was applied for all comparisons to determine statistical significance.
3.0 RESULTS

3.1 LTP in area CA1 *in vivo* is accompanied by a rapid and persistent increase in *arc* mRNA level.

Previous studies on changes in *arc* expression after induction of LTP in area CA1 *in vivo* used *in situ* hybridization-based techniques and have led to conflicting findings, possibly due to procedural differences (French et al., 2001; Miyashita et al., 2009). Both groups of investigators induced LTP in anesthetized rats with two trains of HFS presented 30 sec apart. French and colleagues harvested tissue 30 min after LTP induction and examined IEG induction using autoradiographic *in situ* hybridization techniques with end-labeled oligodeoxynucleotide probes, whereas Miyashita and colleagues harvested tissue 5 min after LTP induction and used fluorescent *in situ* hybridization with digoxigenin-labeled intron-enriched riboprobes. French and colleagues did not find evidence for LTP-associated *arc* induction, whereas Miyashita and colleagues observed a robust increase in *arc* expression in a large proportion of the cases. Here, we investigated *arc* expression by applying qPCR on small samples of CA1 tissue that included the recording site and were harvested at different times after LTP-inducing stimulation. To induce LTP, we delivered 4 trains of HFS 130 sec apart to the dorsal CA3 commissural projections to contralateral area CA1; to map out the time
course of *arc* expression, we collected tissue samples either 20 min, 40 min, 70 min, or 130 min after the start of HFS and conducted qPCR on those samples. Our stimulation protocol produced a persistent potentiation of the amplitude of the CA1 population spike evoked by test pulses delivered before and after HFS (Fig 1.A1). The potentiation of the evoked population spike lasted for at least 2 hr (20-min group: $t(6) = 6.36$; 40-min group: $t(6) = 4.64$; 70-min group: $t(5) = 6.34$; and 130-min group: $t(4) = 6.15$; all $p$'s < 0.01). Similarly, our HFS protocol produced a persistent potentiation of the initial slope of the CA1 population EPSP, and this potentiation lasted at least 2 hr (Fig 1.A2) (70-min group: $t(3) = 4.66$; and 130-min group: $t(4) = 5.16$; both $p$'s < 0.05). Baseline stimulation only (series of 10 test pulses delivered every 5 min), on the other hand, was not associated with a systematic change in either the amplitude of the evoked population spike (Fig. 1.A1) or the initial slope of the evoked population EPSP (Fig. 1.A2) across the 2.5-hr recording period (population spike: $t(2) = 1.15$; population EPSP: $t(2) = 1.29$, both $p$'s > 0.1). To determine whether *arc* level is altered after HFS, we compared mRNA levels in dorsal area CA1 near the recording site (experimental tissue) to that in ventral area CA1 (within-subject control). Because the CA3 commissural fibers stimulated in our experiments do not innervate ventral area CA1 (Ishizuka et al., 1990), ventral CA1 constitutes a useful within-subject control. Figure 1B depicts that before delivery of HFS, i.e., under baseline condition, *arc* mRNA level in dorsal area CA1 was comparable to that in ventral area CA1 ($t(8) < 1$). In contrast, after 4 trains of HFS, *arc* mRNA level was significantly higher in experimental relative to control tissue at each of the time points investigated (20 min: $t(6) = 3.55$, $p < 0.02$; 40 min: $t(6) = 2.52$, $p < 0.05$; 70 min: $t(9) = 3.52$, $p < 0.01$; and 130 min: $t(9) = 5.03$, $p < 0.01$). Our results confirm
Figure 1: Rapid and persistent increase of *arc* mRNA level after induction of LTP in area CA1 *in vivo*. **A1** Means ± s.e.m.s of the amplitude of the population spike evoked by commissural stimulation before and after 4 trains of high-frequency stimulation (HFS; small down-ward arrows) or only test pulse stimulation after the initial baseline stimulation period. Inserts above show representative waveforms (average of 10 recordings) recorded from an animal 5 min before (stippled line) and 127 min after HFS (solid line). Scale: 2 mV, 2 msec. **A2** Similar data as shown above for the initial slope of the population EPSP evoked by commissural stimulation before and after HFS. Scale for the insert is identical. **B** Means ± s.e.m.s of *arc* mRNA level in tissue samples harvested after either HFS or baseline stimulation (* p < 0.05, ** p < 0.01).
the findings by Miyashita et al. (2009) that arc mRNA levels increase rapidly after LTP-inducing stimulation of area CA1 in vivo. Furthermore, our results extend these earlier observations by showing that arc mRNA levels are increased for at least 2 hr after LTP induction in area CA1.

To rule out the possibility that the observed change in arc mRNA level was a non-specific consequence of patterned stimulation, we took advantage of the fact that HFS-induced LTP in area CA1 is dependent on NMDA receptor activation (Thiels et al., 1992). Thus, we infused the NMDA receptor antagonist D-APV (0.5 mM in the drug pipette) into area CA1 near the recording site throughout electrophysiological recording and assessed the effect on arc expression in CA1 tissue samples collected 20 min after HFS. Figure 2.A1 shows that in the presence of D-APV, HFS failed to produce a significant potentiation of the amplitude of the evoked CA1 population spike ($t(6) = 2.21, p > 0.07$). Figure 2.A2 shows that the increase in arc mRNA level detected 20 min after HFS in the absence of drug was abolished completely in the presence of D-APV ($t(6) < 1$). These results confirm that the increase in arc mRNA level after HFS we observed above (Figure 1B) depends on prior induction of LTP. Furthermore, the results suggest that the LTP-associated increase in arc mRNA level is mediated via an NMDA receptor-dependent mechanism.

Our observations of an HFS-associated increase in arc mRNA level do not distinguish between the possibility that LTP is associated with an increase in de novo arc transcription versus an increase in arc mRNA stability. To address this issue, we assessed the effect of the general transcription inhibitor ActinomycinD (ActD) on the increase in arc mRNA level observed immediately after HFS. Specifically, we infused
Figure 2: Increase of *arc* mRNA level after HFS fails to occur when either NMDA receptors are blocked or new RNA synthesis is inhibited.  

**A1)** Means ± s.e.m.s of the amplitude of the population spike evoked by commissural stimulation before and after 4 trains of HFS (small down-ward arrows) delivered in either the presence of D-APV (gray circles) or the absence of drug (black squares). The data from the latter group were shown in Fig. 1.A1 and are included here for purposes of comparison. Insert above shows representative waveforms (average of 10 recordings) recorded 5 min before (stippled line) and 17 min after the onset of HFS (solid line) from an animal that received HFS in the presence of D-APV. Scale: 2 mV, 2 ms.  

**A2)** Means ± s.e.m.s of *arc* mRNA level in tissue samples harvested 20 min after HFS delivered in either the presence of D-APV or the absence of drug. The data from the latter group were shown in Fig. 1.B and are included here for purposes of comparison.  

**B1)** Means ± s.e.m.s of the amplitude of the population spike evoked by commissural stimulation before and after 4 trains of HFS (small down-ward arrows) delivered in either the presence of ActD (gray circles) or the absence of drug (black squares). The data from the latter group were shown in Fig. 1.A1 and are included here for purposes of comparison. Insert above shows representative waveforms (average of 10 recordings) recorded 5 min before (stippled line) and 17 min after HFS (solid line) from an animal that received HFS in the presence of ActD. Scale: 2 mV, 2 ms.  

**B2)** Means ± s.e.m.s of *arc* mRNA in tissue samples harvested 20 min after HFS delivered in either the presence of ActD or the absence of drug. The data from the latter group were shown in Fig. 1.B and are included here for purposes of comparison.
ActD (0.8 mM in the drug pipette) into area CA1 near the recording site over the course of about 15 min beginning 90 min before HFS. Dorsal and ventral area CA1 pieces were collected 20 min after HFS. Figure 2.B1 shows that the presence of ActD did not affect the ability of 4 trains of HFS to cause a significant increase in the amplitude of the evoked population spike ($t(8) = 6.71$, $p < 0.01$). No effect of the transcriptional inhibitor on the increase in the evoked response immediately after HFS was expected because only late but not early phases of LTP depend on transcription (Abraham and Williams, 2003; Nguyen et al., 1994). Figure 2.B2 shows that the marked increase in *arc* mRNA level 20 min after HFS observed in the absence of drug failed to emerge in the presence of ActD ($t(8) = 1.15$, $p > 0.1$). These results indicate that the observed LTP-associated elevation in *arc* mRNA level stems from a stimulation-induced increase in *arc* mRNA synthesis, rather than a decrease in *arc* mRNA degradation. Taken together, our findings demonstrate that the induction of NMDA receptor-dependent LTP in area CA1 *in vivo* is associated with a robust, rapid increase in *arc* transcription and that *arc* mRNA levels remain elevated above control levels for at least 2 hr after LTP induction.

3.2 LTD in area CA1 *in vivo* is accompanied by a rapid decrease followed by a transient increase in *arc* mRNA level.

To determine the time course of any changes in *arc* mRNA level in association with LTD area CA1 *in vivo*, we followed essentially the same approach as described above. We induced LTD in area CA1 by delivering one train of PPS, which consisted of
200 pairs of pulses with a 25-ms interval presented at 0.5 Hz, to the dorsal CA3 commissural fibers, and we harvested CA1 tissue samples for qPCR analysis either 20 min, 40 min, 70 min, or 130 min after the onset of the PPS. Similar to our previous observations (Thiels et al., 1994), PPS produced a persistent depression of the amplitude of the CA1 population spike (Fig. 3.A1) as well as the initial slope of the CA1 population EPSP (Fig. 3.A2) evoked by test pulses delivered before and after PPS, and these effects lasted for at least 2 hr after PPS (population spike: 20 min: \( t(6) = 5.15, p < 0.01 \); 40 min: \( t(1) = 16.31, p < 0.05 \); 70 min: \( t(3) = 5.71, p < 0.02 \); and 130 min: \( t(4) = 7.63, p < 0.01 \); population EPSP: 40 min: \( t(4) = 10.44 \); 70 min: \( t(4) = 6.93 \); 130 min: \( t(4) = 12.60 \), all \( p's < 0.01 \)). As in the above experiments, baseline stimulation was not associated with a systematic change in either the amplitude of the evoked population spike (Fig. 3.A1) or the initial slope of the evoked population EPSP (Fig. 3.A2) across the 2.5-hr recording period (population spike: \( t(2) < 1 \); population EPSP: \( t(2) = 1.13, p > 0.1 \)). The results from the qPCR analysis are shown in Figure 3B. Before delivery of PPS, \textit{arc} mRNA level in dorsal area CA1 did not differ from that in ventral area CA1 (\( t(6) < 1 \)). However, immediately after PPS, \textit{arc} mRNA level was significantly decreased relative to the level detected in control tissue (20 min: \( t(6) = 5.22, p < 0.01 \)). This significant decrease was short-lasting, because 40 min after PPS, a systematic difference in \textit{arc} mRNA level between experimental and control tissue samples was no longer detectable (\( t(6) < 1 \)). However, as time since PPS passed, \textit{arc} mRNA levels began to increase such that 70 min after PPS, mRNA level in experimental CA1 samples was significantly higher relative to that in control samples (\( t(8) = 4.34, p < 0.01 \)). This increase in \textit{arc} mRNA level 1 hr after LTD induction, however, also did not
Figure 3: Rapid decrease followed by transient increase of arc mRNA level after induction of LTD in area CA1 in vivo.  A1) Means ± s.e.m.s of the amplitude of the population spike evoked by commissural stimulation before and after PPS or after only test pulse stimulation after the initial baseline stimulation period.  Inserts above show representative waveforms (average of 10 recordings) recorded from an animal 5 min before (stippled line) and 127 min after PPS (solid line).  Scale: 2 mV, 2 msec.  A2) Similar data as shown above for the initial slope of the population EPSP evoked by commissural stimulation before and after PPS. Scale for the insert is identical.  B) Means ± s.e.m.s of arc mRNA level in tissue samples harvested after either baseline stimulation or after PPS.
persist; mRNA level in experimental CA1 samples no longer differed significantly from control level 130 min after PPS ($t(8) < 1$). Different from what one might have expected based on previous work showing that genetic reduction of arc interferes with LTD maintenance (Plath et al., 2006) whereas overexpression of arc occludes LTD (Rial Verde et al., 2006), the present results indicate that arc mRNA level undergoes a rapid decrease after LTD-inducing stimulation. Furthermore, our results suggest a remarkable bidirectional regulation of arc mRNA level after LTD induction in area CA1 in vivo, with levels decreasing below baseline during the early phase of LTD, increasing above baseline about 1 hr after LTD induction, and returning to baseline by 2 hr after LTD induction. This profile is distinctly different from the one we observed after induction of LTP.

We previously showed that induction of LTD by PPS in area CA1 in vivo is dependent on NMDA receptor activation (Thiels et al., 1994; Thiels et al., 2000). Therefore, to rule out that the observed decrease in arc mRNA level was some non-specific effect of patterned stimulation, we infused D-APV (0.5 mM in the drug pipette) into area CA1 near the recording site throughout the electrophysiological recording session and assessed the effect on arc mRNA level in CA1 tissue samples collected 20 min after PPS. Figure 4.A1 shows that in the presence of D-APV, PPS failed to produce a depression of the amplitude of the evoked CA1 population spike ($t(5) < 1$). Figure 4.A2 shows that the decrease in arc mRNA level detected 20 min after PPS in the absence of drug failed to emerge in the presence of D-APV ($t(5) < 1$). These results confirm that the decrease in arc mRNA level after PPS observed above (Figure 3B)
Figure 4: Decrease of *arc* mRNA level after PPS fails to occur when NMDA receptors are blocked.

**A1** Means ± s.e.m.s of the amplitude of the population spike evoked by commissural stimulation before and after PPS (wide downward arrow) recorded from animals decapitated 20 min after PPS delivered in either the presence of D-APV (gray circles, n=9) or the absence of drug (black squares, n=7). The data from the latter group were shown in Fig. 3A1 and are included here for purposes of comparison. Inserts above show representative waveforms (average of 10 recordings) recorded 5 min before (stippled line) and 17 min after PPS (solid line) from an animal that received PPS in the presence of D-APV. Scale: 2 mV, 2 ms. **A2** Means ± s.e.m.s of *arc* mRNA level in tissue samples harvested 20 min after PPS delivered in either the presence of D-APV or the absence of drug. The data from the latter group were shown in Fig. 3B and are included here for purposes of comparison. **B1** Means ± s.e.m.s of the amplitude of the population spike evoked by commissural stimulation before and after PPS recorded from animals decapitated 70 min after PPS delivered in the presence of either ActD (gray circles, n=6) or vehicle solution (black squares, n=6). Insert above shows representative waveforms (average of 10 recordings) recorded 5 min before (stippled line) and 70 min after PPS (solid line) from an animal that received PPS in the presence of ActD. Scale: 2 mV, 2 ms. **B2** Means ± s.e.m.s of *arc* mRNA in tissue samples harvested 70 min after PPS delivered in the presence of either ActD or vehicle solution.
depends on prior induction of LTD and is not a non-specific consequence of prior synaptic activation.

The increase in *arc* mRNA 70 min after PPS raises the question whether the observed change stems from *de novo* transcription or a dramatic reduction in *arc* mRNA degradation. To address this issue, we assessed the effect of ActD on the increase in *arc* mRNA level observed 70 min after PPS. Specifically, we infused ActD (0.8 mM in the drug pipette) into area CA1 near the recording site over the course of about 15 min beginning 90 min before PPS. Dorsal and ventral area CA1 pieces were collected 70 min after HFS. Figure 4.B1 shows that pre-infusion of ActD did not affect the early phase of LTD, as the amplitude of the evoked population spike immediately after PPS was not distinguishable from the depressed level observed in the vehicle group (for the first 4 test-pulse series after PPS, vehicle vs. ActD, *t*(10) > 1). However, the initial depression of the evoked response did not last in the group that received ActD but had returned to baseline level by the end of the recording period (*t*(6) < 1). In contrast, the response depression lasted at least until the end of the recording period in vehicle-treated animals (*t*(6) = 8.40, *p* < 0.01) (Figure 4.B1). These findings demonstrate that the prolonged maintenance of PPS-induced LTD in area CA1 requires mRNA synthesis. Figure 4.B2 shows that the robust increase in *arc* mRNA level in dorsal hippocampus 70 min after PPS observed after infusion of vehicle solution (*t*(6) = 4.83, *p* < 0.01) was abolished completely after infusion of ActD; in fact, *arc* mRNA levels detected in dorsal CA1 tissue 70 min after PPS in the presence of ActD were slightly but significantly below control levels (*t*(6) = 2.3, *p* < 0.05). These results indicate that the LTD-associated increase in *arc* mRNA level 70 min after PPS stems from a stimulation-
induced increase in *arc* mRNA synthesis, rather than a decrease in *arc* mRNA degradation.

Taken together, these findings demonstrate that the induction of NMDA receptor-dependent LTD in area CA1 *in vivo* is associated with an immediate, pronounced decrease and a protracted, transient increase in *arc* mRNA level. The transient increase in *arc* mRNA level is the result of an increase in *arc* transcription. Importantly, this increase in *arc* transcription is necessary for the prolonged maintenance of NMDA receptor-dependent PPS-induced LTD in area CA1 *in vivo*. Relating these findings to those after LTP induction, our results show that *arc* mRNA levels are altered during both LTP and LTD in area CA1 *in vivo*. The pattern of *arc* mRNA regulation, however, differs markedly between these two forms of synaptic plasticity.

### 3.3 The patterns of change in *arc* and *zif268* mRNA level differ from one another during LTD but not during LTP in area CA1 *in vivo*.

Similar to *arc*, the IEG *zif268* was reported to be regulated in a synaptic activation-dependent manner (Jones et al., 2001; Lindcke et al., 2006). In fact, *zif268* was shown to be required for the maintenance of LTP (Davis et al., 2003; Renaudineau et al., 2009). Using the same tissue samples as we used for the analyses of *arc* mRNA level after induction of LTP, we found that *zif268* mRNA level also is increased significantly immediately after LTP induction (Fig. 5A). As was the case with *arc*, this increase in *zif268* mRNA level persisted for at least 2 hr (Base: $t(7) < 1$; 20 min: $t(6) = 2.91, p < 0.05$; 40 min: $t(6) = 3.53, p < 0.02$; 70 min: $t(9) = 3.79, p < 0.01$; and 130 min: $t(8) = 3.24, p < 0.01$).
In light of an overall similarity in the expression patterns of \textit{arc} and \textit{zif268} during LTP, it became of interest to determine whether these two IEGs also are regulated similarly during LTD. Accordingly, we probed the same LTD-tissue samples as used for the \textit{arc} analyses for \textit{zif268} mRNA. Figure 5B shows that \textit{zif268} mRNA level in dorsal area CA1 was comparable to that detected in ventral area CA1 before PPS. Although \textit{zif268} mRNA level tended to increase, relative to control level, over the course of the first hour after PPS and then decrease, relative to control level, over the course of the second hour after PPS, none of these trends was statistically significant (Base: $t(7) < 1$; 20 min: $t(6) = 1.56$; 40 min: $t(6) = 1.92$; 70 min: $t(7) = 1.74$; and 130 min: $t(7) = 1.45$, all $p$'s $> 0.1$). These results indicate that, in contrast to \textit{arc} mRNA, \textit{zif268} mRNA level does not deviate significantly from control level at any time during the first 2 hr after LTD induction. Thus, taken together, these results show that \textit{arc} and \textit{zif268} are regulated differently from one another during LTD, whereas they appear to be regulated in overall similar fashion during LTP in area CA1 \textit{in vivo}.
Figure 5: Zif268 mRNA levels also increase during LTP but do not change from control levels during LTD in area CA1 in vivo. A) Means ± s.e.m. of zif268 mRNA level in the same tissue samples used in Fig. 1.B. B) Means ± s.e.m. of zif268 mRNA level in tissue samples used in Fig. 3.B.
4.0 DISCUSSION

Previous work indicates that arc plays a critical role in LTP, LTD, and homeostatic plasticity (Bramham et al., 2008; Tzingounis and Nicoll, 2006). In this study we characterized the time course of arc expression during LTP and LTD in area CA1 of the adult rat hippocampus in vivo. Based on previous reports of changes in arc mRNA level after LTP induction (Link et al., 1995; Lyford et al., 1995; Messaoudi et al., 2007; Miyashita et al., 2009; Steward et al., 1998; Waltereit et al., 2001) and findings that arc overexpression occludes whereas arc deletion interferes with LTD induction (Plath et al., 2006; Rial Verde et al., 2006), we expected that arc mRNA expression would be increased after induction of LTP as well as LTD. To avoid that any differences in expression profiles between LTP and LTD could be attributed to procedural differences, such as amount of stimulation, we used the same number of stimulation pulses, stimulation intensity, and total duration of stimulation for the induction of LTP and LTD, but varied only the temporal pattern of the stimulation pulses between LTP and LTD experiments. Our results show that the regulation of arc expression differs markedly between LTP and LTD in area CA1 in vivo despite the similar requirement of this gene’s product for both forms of plasticity. Whereas there is a persistent up-regulation of arc during LTP, arc exhibits a biphasic regulation pattern during LTD.
The rapid induction of *arc* by a wide variety of cellular activations, and the transport to and local translation of *arc* mRNA near activated synapses (Lyford et al., 1995; Park et al., 2008; Steward et al., 1998; Steward and Worley, 2001a; Steward and Worley, 2001b; Wallace et al., 1998; Waung et al., 2008) have attracted much interest in the fields of molecular, synaptic, and behavioral neuroscience. *Arc* is a single copy gene that is highly conserved among vertebrates (Bramham et al., 2008; Miyashita et al., 2008), which suggests that *arc* serves a critical function. *Arc* is expressed in principal neurons in the brain (Vazdarjanova et al., 2006), and rapid increases in *arc* mRNA level have been observed after spatial exploration (Chawla et al., 2005; Guzowski et al., 1999; Miyashita et al., 2009; Ramirez-Amaya et al., 2005; Vazdarjanova et al., 2006), implicit and explicit memory tasks (Guzowski et al., 2000; McIntyre et al., 2005; Montag-Sallaz and Montag, 2003; Plath et al., 2006; Soule et al., 2008), LTP-inducing stimulation (Guzowski et al., 2000; Link et al., 1995; Lyford et al., 1995; Waltereit et al., 2001), seizures (Link et al., 1995; Lyford et al., 1995), and stress (Ons et al., 2004). Our findings of a reduction in *arc* mRNA level after LTD-inducing stimulation demonstrate that not all types of cellular activation lead to *arc* induction and, hence, that *arc* mRNA is not a general marker of cellular activity but regulated in differential fashion.

The differences in *arc* mRNA regulation during LTP and LTD may reflect differences in the expression of its protein product. Such differences in protein expression may include changes in the amount of Arc protein at a given time point during LTP and LTD. Future experiments using immunohistochemistry would be useful to answer whether there are differences in the amount of Arc protein during LTP and
LTD. Such a study would be valuable as a starting point to determine the specific function(s) of Arc during bidirectional synaptic plasticity. For instance, it has been proposed by several groups that Arc protein is a key player in AMPA receptor endocytosis during LTD (Park et al., 2008; Rial Verde et al., 2006). Arc protein also has been proposed for the maintenance of the actin filaments and regulation of translation factors in neurons during LTP (Bramham et al., 2010; Messaoudi et al., 2007) and LTP, but not LTD, is associated with sustained Arc translation indicating a requirement of Arc protein for a longer time period during LTP but not during LTD (Bramham et al., 2010). Based on these reports, one can hypothesize that Arc plays different roles during LTP and LTD. This possibility of Arc to play different roles during LTP and LTD would likely require the presence of synaptic plasticity-regulated binding partners of Arc that are capable of directing Arc to a context-specific function.

While it is possible that Arc serves different functions during LTP and LTD, it is also possible that Arc is involved in AMPA receptor endocytosis during both LTP and LTD. In the case of LTP, endocytosis of AMPA receptors may reduce the surface levels of defective AMPA receptors or of certain AMPA receptor subunit compositions (Rial Verde et al., 2006), and a parallel mechanism transports AMPA receptors to the synaptic surface, leading to an increase in functional AMPA receptors on the surface. In the case of LTD, endocytosis of AMPA receptors would reduce the surface expression levels of AMPA receptors. However the net effect of Arc-mediated AMPA receptor endocytosis would lead to reduction of surface AMPA receptors due to the lack of a parallel mechanism that transports AMPA receptors to the synaptic surface. In addition to the possibilities listed here, it is also possible that during LTP, Arc regulates
endocytosis of extrasynaptic AMPA receptors whereas during LTD, Arc leads to the endocytosis of AMPA receptors regardless of the location. Again, this localized activity of Arc would potentially hire binding partners to direct Arc to the correct location. The function(s) of Arc during LTP and LTD and how the mRNA expression of arc during these forms of plasticity affects Arc levels, interactions of Arc with other proteins, and Arc’s function(s) need to be determined with future studies.

The basis of the decrease in arc mRNA level after LTD induction remains to be determined. One possibility is that the transcription of arc is reduced immediately after PPS. We showed previously that PPS-induced LTD in area CA1 in vivo is associated with an increase in the activity of the serine/threonine protein phosphatases PP1 and PP2A (Thiels et al., 1998). Both PP1 and PP2A can affect the phosphorylation state of transcription factors, such as CREB (Genoux et al., 2002; Hagiwara et al., 1992), including after PPS in area CA1 (Mauna et al., submitted), and they thereby reduce the DNA binding ability of these transcriptional regulators. The promoter sequence of arc contains binding consensus sequences for several transcription factors, including CREB, MEF2, SRF, and Elk-1 (Flavell et al., 2006; Kawashima et al., 2009; Pintchovski et al., 2009; Waltereit et al., 2001). In light of findings that CREB phosphorylation is decreased below basal level during LTD (Thiels et al., 2002; Mauna et al., submitted), it is tempting to speculate that the initial decrease in arc mRNA level observed here is related to the LTD-associated decrease in CREB phosphorylation and, hence, that our results reflect a reduction in transcription. This scenario is consistent with previous work attributing to CREB a possible role in the regulation of arc expression (Kawashima et al., 2009; Waltereit et al., 2001). A prominent regulatory role by CREB does not
preclude a contribution by other transcription factors; however, we consider a significant role by either SRF/Elk-1 or MEF2 in the LTD-associated decrease of arc mRNA level less likely because (1) we previously found Elk-1 phosphorylation to be increased immediately after the induction of LTD (Thiels et al., 2002), and (2) calcineurin, a protein phosphatase activated during LTD (Mulkey et al., 1994) was shown to promote, rather than attenuate, MEF2-mediated arc expression (Flavell et al., 2006). An alternative explanation for the decrease in arc mRNA level immediately after LTD-inducing stimulation is that arc mRNA undergoes rapid degradation in response to the synaptic input pattern. Recently, arc mRNA was shown to be degraded by the nonsense-mediated mRNA decay (NMD) pathway (Giorgi et al., 2007). The NMD pathway targets newly transcribed mRNAs to degradation and thereby limits the total translation time and amount of these mRNAs (Ishigaki et al., 2001; Maquat, 2004). A key RNA-binding protein required for NMD is eIF4AIII (Palacios et al., 2004), and arc was shown to be co-localized with eIF4AIII (Giorgi et al., 2007). When NMD was inhibited in neurons by knockdown of eIF4AIII, the level of both arc mRNA and Arc protein were found to be elevated (Giorgi et al., 2007). Thus, it is possible that PPS triggers increased targeting of arc mRNA by the NMD pathway and consequently initiates degradation of arc mRNA that has rapidly been translated immediately after LTD-inducing stimulation. Future studies focused on targeting of arc mRNA by the NMD pathway and on determining pre-arc mRNA levels may help resolve the question of whether the observed decrease in arc mRNA level stems from a decrease in de novo transcription or an increase in rapid mRNA degradation.
The increase in arc mRNA level about 1 hr after LTD induction revealed to be the result of de novo transcription of arc. Interference with de novo transcription, in turn, prevented the prolonged maintenance of LTD. This effect of transcription inhibition on LTD in area CA1 in vivo is in agreement with a previous report of transcription-dependent NMDA receptor-mediated LTD in area CA1 in vitro (Kauderer and Kandel, 2000; but see Manahan-Vaughan et al., 2000). It remains to be determined whether the increase in arc transcription is critical for LTD maintenance. This intriguing possibility is suggested by findings of impaired LTD in area CA1 of arc knockout mice (Plath et al., 2006).

LTP was shown to depend on transcriptional activation, and an effect of transcriptional inhibition on LTP maintenance has been observed in a few hours after LTP-inducing stimulation (Frey et al., 1996; Nguyen et al., 1994). Even though LTP maintenance is abolished 1-2 hours after LTP-inducing stimulation, LTD maintenance was abolished within 30-60 min after LTD-inducing stimulation in the presence of ActinomycinD. The temporal difference in transcription-dependency between LTP and LTD suggests that separate gene regulatory pathways with different time scales are required for LTP and LTD. For instance, LTP maintenance may require gene products that are downstream of transcription factor IEGs induced by the HFS. On the other hand, LTD maintenance may involve translation of PPS-induced effector IEG mRNAs immediately following transcription. Our ActinomycinD infusions started 60 to 90 min before the LTD-inducing stimulation delivery, possibly reducing the levels of mRNA species with fast turnover rates. It therefore is possible that the basal level of an LTD-associated protein may have been reduced. Infusing ActinomycinD only after the start of
the LTD-inducing stimulation would be possible; this treatment would enable one to distinguish whether the reduction of fast-turnover mRNA basal levels or inhibition of LTD stimulation induced mRNA changes affect LTD maintenance.

The increase in arc mRNA level during LTD did not emerge until approximately 1 hr after PPS. It is possible that upregulation of arc transcription is triggered by the initial synaptic input, but that its manifestation at the mRNA level is masked by the mRNA degradation pathway that rapidly degrades translated arc mRNA (Giorgi et al., 2007). If Arc protein is required for early phase LTD maintenance, then this rapid transcription coupled with rapid translation and degradation could explain the disability in LTD maintenance in the presence of ActinomycinD. Alternatively, the late increase in arc mRNA level may be caused by a latent transcriptional mechanism that operates in parallel with the mechanisms that underlie the initial decrease in arc mRNA level. Future studies assessing pre-arc mRNA levels at various time points after LTD induction may inform whether a prolonged upregulation of arc transcription is masked by rapid mRNA degradation or whether de novo transcription is increased only for a short period of time approximately 1 hr after PPS. Regardless of the specific bases of the respective changes in arc mRNA level during LTD, our findings of a distinct bidirectional pattern of change highlight the dynamic nature of arc mRNA regulation during LTD. Furthermore, the findings illustrate that the time point at which mRNA levels are assessed greatly affects the direction of change one observes. Accordingly, models of arc regulation and its role in synaptic plasticity based on singular temporal measurements may be incomplete.
Similar to observations by others using a range of cellular activation paradigms (Guzowski et al., 2000; Guzowski et al., 1999; Link et al., 1995; Lyford et al., 1995; Steward and Worley, 2001a; Steward and Worley, 2001b), we found that our LTP-inducing stimulation protocol resulted in a persistent increase in \textit{arc} mRNA level that lasted at least 2 hr. Our findings that the initial increase in \textit{arc} mRNA level is abolished when transcription was blocked indicates that the change in mRNA level was due to an increase in \textit{arc} transcription. The prolonged increase in \textit{arc} mRNA level might be considered surprising given that \textit{arc} mRNA has a half-life of ~47 min (Vazdarjanova et al., 2006). One possible mechanism underlying the prolonged increase in mRNA level may be extended activation of \textit{arc} transcription. In addition to CREB, which is phosphorylated rapidly after LTP-inducing stimulation (Impey et al., 1998; Kasahara et al., 2001; Racaniello et al., 2009; Schulz et al., 1999), other transcriptional regulators, including MEF2, SRF/Elk-1, Zeste-like response element binding proteins, or Zif268, may mediate \textit{arc} transcription and may do so at later time points (Flavell et al., 2006; Kawashima et al., 2009; Li et al., 2005; Pintchovski et al., 2009). Thus, the prolonged elevation in \textit{arc} mRNA may result from enhanced transcription via a number of different transcription factors operating in sequence. Alternatively, the prolonged increase in \textit{arc} mRNA may stem from an enhancement of \textit{arc} mRNA stability after the initial increase in \textit{arc} transcription. In fact, control of mRNA stability has been implicated to play a critical role in neuronal function and synaptic plasticity (Deschenes-Furry et al., 2006; Perrone-Bizzozero and Bolognani, 2002). This type of control of mRNA stability is achieved by protein-mRNA interactions at the AU-rich elements (AREs) found on the 3'-untranslated regions (UTRs) of mRNA species (Deschenes-Furry et al., 2006). Even though \textit{arc}
mRNA contains a distinct 3’-UTR that has been linked to Arc’s interaction with the NMD pathway proteins, this 3’-UTR does not contain a definite ARE (Deschenes-Furry et al., 2006; Giorgi et al., 2007). Moreover, no changes in arc mRNA stability were observed following neural activation whereas the stability of pituitary adenylate cyclase-activating polypeptide (PACAP) mRNA is increased by calcium and cAMP following neural activation (Fukuchi et al., 2004). Other studies also failed to attribute differences in arc mRNA expression to changes in arc mRNA stability. For instance, regulation of arc by AMPA receptor activation has been linked to changes in arc transcription but not mRNA stability (Rao et al., 2006). A third possible explanation for the sustained elevation in arc mRNA we observed points to an epigenetic regulation of arc. It was shown that LTP is linked to epigenetic modifications, such as changes in DNA methylation (Levenson et al., 2006; Sweatt, 2009). The observation of differential arc expression in hippocampi of young versus old rats following similar cellular activation led to the identification of age-dependent epigenetic regulation of arc via changes in the methylation of the arc gene (Penner et al., 2010). Therefore future studies should also consider an epigenetic regulation of arc during LTP and LTD.

In conclusion, our observations reveal that LTP and LTD are associated with different profiles of arc mRNA changes that may result from different combinations of alterations in arc transcription and arc mRNA stability within as well as between LTP and LTD. Taken together with our findings of zif268 mRNA levels during these two forms of synaptic plasticity, it appears that the regulatory mechanisms underlying arc expression differ not only between and within LTP and LTD but also from those of other IEGs implicated in synaptic plasticity. We believe our study presents a useful starting
point for future dissection of the varied mechanisms that regulate arc expression during bidirectional plasticity at glutamatergic synapses in hippocampal area CA1.
APPENDIX A: EXTRACELLULAR FIELD POTENTIAL RECORDINGS FROM STR. PYRAMIDALE AND STR. RADIATUM IN AREA CA1 OF INTACT ADULT RAT BRAINS

Extracellular field potential recordings from area CA1 str. radiatum are commonly utilized to monitor synaptic transmission at Schaffer collateral commissural synapses from intact rodent brains. The most common method to assess changes in the population excitatory field potentials (pEPSPs) is to compare the initial slope of the pEPSP waveforms recorded before and after a given stimulation from str. radiatum. The initial slope of the pEPSP is less subject to contamination from other sources of current flow in the system than later stages of the pEPSP waveform, such as currents generated by feed-forward or feedback inhibition. In addition, later stages of the EPSP can be contaminated by propagation of action potentials (APs), if AP threshold has been reached. Therefore, to isolate the changes in synaptic strength to a given input stimulus, researchers prefer to measure the initial slope of the pEPSP. However, due to difficulties detecting errors with str. radiatum recordings introduced by tissue shifts in intact brains, it is easier to conduct electrophysiological recordings from str. pyramidal and measure the population spike amplitude. The population spike amplitude reflects changes in cellular output rather than changes in synaptic transmission at the dendritic
layer. Therefore, results solely based on str. pyramidale recordings shall be analyzed with caution, since some of the results may not reflect the changes at the synaptic strength. Based on the ease of recording from str. pyramidale, I initially learned to record field potentials in this layer. To assure the changes in gene expression not only reflect the changes in cellular output but synaptic transmission, I later conducted recordings from the str. radiatum. Our qPCR results did not differ between recordings from str. pyramidale and str. radiatum.
APPENDIX B: APPLICATION OF THE EFFICIENCY-CORRECTED DELTA-CT METHOD FOR QPCR ANALYSIS

It is assumed that each PCR cycle doubles the amount of the target template under ideal conditions. However, many factors may lead to amplification of the target with less efficiency. These factors include: the size of the target, buffer conditions, primer sequences and primer melting temperatures and the quality of the cDNA template. Therefore one shall assess the efficiencies of each primer set and, if needed, redesign primer sets to obtain a high efficiency (1.9 to 2.1) for each set which is within ± 0.1 of the efficiency of any other gene’s primer set that will be analyzed together (Bookout et al., 2006). The efficiencies of the primer sets used in this study was assessed using standard cDNAs generated under the same conditions with the experimental cDNAs since the efficiency of the reverse-transcription reaction thus the quality of the cDNA template can also contribute to the efficiency of the qPCR amplification of a target sequence. Figure A1 shows the calibration curves of the primer sets used for calculation of the qPCR efficiencies of arc, zif268, and gapdh. The calculated efficiencies were used in the formula:  

\[
RQ_{\text{arc}, \text{zif268}} = E_{\text{arc}, \text{zif268}} \frac{Ct(\text{ventral}) - Ct(\text{dorsal})}{E_{\text{gapdh}} Ct(\text{ventral}) - Ct(\text{dorsal})}
\]

Mean Ct values are indicated in Tables A1 (arc) and A2 (zif268) which were calculated for dorsal and ventral area CA1 tissue samples. The respective arc and zif268 mean Ct values that were normalized by gapdh Ct values for
the corresponding tissue samples. These *gapdh*-corrected Ct values, in turn, were used to determine the ratio between experimental (dorsal) and control (ventral) tissue samples, which is reflected in the mean $\Delta\Delta$Ct values (Tables A1 and A2). The mean $\Delta\Delta$Ct values are similar to the log$_2$-transformed RQ values obtained using the efficiency-corrected $\Delta$Ct method and depicted in Figures 1 through 4 (Tables A1 and A2). (* $p < 0.05$, ** $p < 0.01$).
Figure A1: Calibration curves for the primer sets. A) Calibration curve for arc. Cycle threshold (Ct) values from qPCR analysis are plotted against different levels of dilution of RNA input (undiluted, 1/5, 1/25, and 1/125) B) Calibration curve for zif368. Similar data as shown in A. C) Calibration curve for gapdh. Similar data as shown in A.
Table A1. Mean Ct values used for calculation of the relative *arc* mRNA levels.

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<td>7.77</td>
<td>7.96</td>
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<td>32.48</td>
<td>34.61</td>
<td>6.41</td>
<td>6.39</td>
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<td>3.94</td>
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<td>33.73</td>
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<td>2.39</td>
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<td>34.37</td>
<td>8.40</td>
<td>9.18</td>
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<td>9.10</td>
<td>9.04</td>
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<td>-1.00</td>
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<td>34.63</td>
<td>8.94</td>
<td>8.86</td>
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<td>ActH + PPS</td>
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<td>34.76</td>
<td>8.58</td>
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<td>-0.23</td>
<td>0.65</td>
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Table A2. Mean Ct values used for calculation of the relative *zif268* mRNA levels.

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<th>stimulation condition</th>
<th>Mean Ct</th>
<th>Mean ΔCt(d)</th>
<th>Mean ΔCt(v)</th>
<th>Mean ΔΔCt(d)/v</th>
<th>Mean 2^ΔΔCt</th>
<th>Mean log_2 efficiency corrected</th>
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BIBLIOGRAPHY


