Published in final edited form as:
Nat Neurosci. 2010 September ; 13(9): 1082–1089. doi:10.1038/nn.2611.

SRF binding to SRE 6.9 in the *Arc* promoter is essential for LTD in cultured Purkinje cells

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Abstract

It has been suggested that gene expression and protein synthesis are required for both long-term memory consolidation and late phases of long-term potentiation and long-term depression (LTD). The necessary genes and the specific transcription factor binding sites in their promoters remain unknown. We found that inhibition of the transcription factor SRF or its cofactor MAL blocked the late phase of LTD in mouse cultured cerebellar Purkinje cells, as did deletion of the immediate early gene *Arc*. Using neuronal bacterial artificial chromosome (BAC) transfection, we found that, in $Arc^{-/-}$ cells transfected with a wild-type *Arc* BAC, late-phase LTD was rescued. However, mutation of one SRF-binding site in the *Arc* promoter (SRE 6.9) blocked this rescue. Co-transfection of wild-type Arc and SRF engineered to bind mutated SRE 6.9 restored late-phase LTD in $Arc^{-/-}$, SRE 6.9 mutant BAC cells. Thus, SRF binding to SRE 6.9 in the *Arc* promoter is required for the late phase of cerebellar LTD.

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Note: Supplementary information is available on the Nature Neuroscience website.

AUTHOR CONTRIBUTIONS

C.S.-H. performed the immunohistochemistry and designed a portion of the study. B.X., R.D., Y.J. and X.Z. engineered the BACs. J.D.S. performed immunohistochemistry and wrote an early draft of the manuscript. G.P. provided mutant actin reagents and helped to design the experiments that used them. D.K. provided $Arc^{-/-}$ mice. R.L.H., D.D.G. and P.F.W. supervised the project. D.J.L. provided overall supervision of the project and conducted the electrophysiological and imaging experiments.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Consolidation and long-term storage of memory require new protein synthesis and gene expression[1]. Protein synthesis is also essential for the late phase of certain forms of synaptic plasticity that are thought to underlie memory, such as long-term potentiation (LTP) and LTD[2]. However, both the transcription factors activated by LTP and LTD induction and the identity and synaptic function of the relevant target gene products remain unclear.

Cerebellar LTD of parallel fiber–Purkinje synapses is hypothesized to form a portion of the engram for certain forms of motor learning and has proven to be a valuable model for studying the molecular mechanisms of memory[3]. This form of LTD is induced postsynaptically through a metabotropic glutamate receptor-1 (mGluR1)/protein kinase C (PKC) cascade and is expressed by clathrin-mediated endocytosis of GluR2-containing surface AMPA receptors that is dependent on the adaptor protein PICK1 (refs. [4,5]). A late phase of cerebellar LTD in cultured Purkinje cells, beginning 45–60 min after induction, is blocked by transcription or translation inhibitors[6,7] or by formation of a dendritic outside-out macropatch, thereby divorcing the synapses from the nucleus[6].

In recent years, it has been shown that inducible deletion or inhibition of the transcription factor cAMP response element binding protein (CREB) can affect LTP, LTD and memory formation in the hippocampus[8]. However, CREB is a survival factor for many neurons, and its deletion, even in adulthood, results in massive neuronal apoptosis, making analysis of synaptic plasticity and memory in CREB-deleted neurons and neuronal circuits problematic[9–11]. SRF, which is encoded by a single gene that is conserved from flies to humans, is another transcription factor that is regulated by synaptic activity, but its inducible deletion in adult brain does not cause neuronal death or gross malformation[12]. However, inducible deletion of SRF produces a near-complete blockade of induction of an important group of immediate early genes (IEGs) that respond to seizure (in the dentate gyrus) or novel environment (in somatosensory cortex). These IEGs, most or all of which contain SRF-binding sites known as serum response elements (SREs), encode transcription factors, such as c-Jun, c-Fos, Fos-B and Egr1, and synaptic proteins, such as Arc[9]. Other genes that do not contain SREs in their promoters, such as *Homer1* and *Ntrk2*, are induced normally by seizure in SRF-deleted dentate gyrus neurons. Inducible deletion of SRF results in a reduction in the amplitude of both early and late LTP at perforant path–dentate gyrus granule cell synapses[9] and, in separate studies, in alterations of the structure of the granule cell layer and dendritic spine density of hippocampal CA1 pyramidal neurons[13,14].

RESULTS

To test the hypothesis that SRF is required for the late phase of cerebellar LTD, we used a well-established cell culture model system. Perforated patch voltage-clamp recordings were made from Purkinje neurons in cultures derived from embryonic wild-type mouse cerebellum[6]. Following acquisition of baseline responses to test pulses of glutamate, we induced LTD by applying glutamate/depolarization conjunctive stimulation and then resumed test pulses of glutamate (Fig. 1a). We coated a pool of four dsRNAs specific to mouse *Srf* (together with a plasmid directing expression of enhanced green fluorescent protein, EGFP) onto gold particles for biolistic transfection. Purkinje cells treated with *Srf* dsRNA showed early-phase, but not late-phase, LTD (52 ± 7.1% of baseline at t = 40 min, 109 ± 7.7% of baseline at t = 120 min, mean ± s.e.m., n = 8). LTD did not spread to a separate control pathway in which test pulses were turned off during the induction period (101 ± 6.0% of baseline at t = 40 min). This phenotype is markedly similar to that seen with LTD recordings made with transcription or translation inhibitors or in isolated dendritic macropatches[6]. Purkinje cells treated with a control, nontargeting dsRNA showed input-specific LTD that persisted for the full duration of the monitoring period (paired pathway, 52 ± 8.3% of baseline; control pathway, 102 ± 7.0% of baseline at t = 120 min; n = 7).

As a further test of SRF involvement, LTD was measured in Purkinje cells in which the *Srf* gene was conditionally deleted (*Srf* conventional knockouts are early embryonic lethal). Previously characterized $Srf^{loxP/loxP}$ mice[9] were crossed with L7-Cre mice, in which Cre recombinase is under the control of the *L7* (also known as *Pcp2*) promoter, which drives expression in Purkinje cells[15]. LTD in the paired pathway of these Purkinje cells had an early phase, but no late phase (52 ± 7.7% of baseline at t = 40 min, 96 ± 7.8% of baseline at t = 120 min, n = 7). As a control, we assessed $Srf^{loxP/loxP}$ mice, which had normal late-phase LTD (54 ± 6.5% of baseline at t = 40 min, 50 ± 8.5% of baseline at t = 120 min, n = 7).

To assess the baseline level of postsynaptic function, we recorded miniature excitatory postsynaptic currents (mEPSCs), a measure of the level of synaptic AMPARs. This was done both before and after induction of a global, chemical form of LTD by a 10-min-long bath application of the PKC-activating phorbol ester phorbol-12,13-diactate (PDA, 200 nM)[16]. The results were consistent with those obtained using glutamate test pulses and conjunctive stimuli to induce LTD (Fig. 1b). Either knockdown of SRF with dsRNA or inducible *Srf* deletion in Purkinje cells ($Srf^{loxP/loxP}$ × *L7-cre* mice) produced Purkinje cells with normal basal mEPSC amplitude (*Srf* dsRNA, 35 ± 4.5 pA; $Srf^{loxP/loxP}$ × *L7-cre*, 30 ± 4.2 pA at t = 0 min; mean ± s.e.m., n = 10 cells per group) and chemical LTD with an early phase, but no late phase (*Srf* dsRNA: 14 ± 6.7 pA at t = 30 min, 33 ± 6.3 pA at t = 90 min; $Srf^{loxP/loxP}$ × *L7-cre*, 16 ± 5.4 pA at t = 30 min, 30 ± 5.3 pA at t = 90 min). The control for the *Srf* dsRNA was a scrambled non-targeting dsRNA, which produced normal chemical LTD (14 ± 6.9 pA at t = 30 min, 16 ± 5.6 pA at t = 90 min, n = 10), and the control for $Srf^{loxP/loxP}$ × *L7-cre* mice was Purkinje cells derived from $Srf^{loxP/loxP}$ mice, which also showed normal chemical LTD (15 ± 6.8 pA at t = 30 min, 16 ± 6.5 pA at t = 90 min, n = 10). These mEPSC and chemical LTD results are important in two respects. First, they show that basal glutamatergic synaptic strength was normal in *Srf*-deleted or SRF-knockdown Purkinje cells. Second, they argue that the effects of SRF attenuation on the late phase of LTD are unlikely to result from a side effect on the initial signals involved in LTD induction, such as mGluR1 activation, diacylglycerol production and Ca^{2+} influx, as these pathways are bypassed when PKC is activated directly by PDA.

Chemical LTD may also be induced by bath application of glutamate and the mGluR1/5 agonist DHPG if the Purkinje cells are held in current-clamp mode during the induction period with I = 0. Bath-applied glutamate also activated both AMPA and mGluR1 in Purkinje cells; in our experience, however, it is difficult to induce LTD with bath-applied glutamate alone (probably because it is a good substrate for reuptake, while DHPG is not; Supplementary Fig. 1). When the cell was voltage-clamped throughout the experiment, bath application of glutamate and DHPG failed to induce LTD (103 ± 6.4% of baseline at t = 40 min, 107 ± 8.1% of baseline at t = 120 min; n = 7). As with chemical LTD induced by PDA, induction of chemical LTD by glutamate and DHPG treatment resulted in a selectively attenuated late phase when the cells were treated with *Srf* dsRNA (54 ± 6.4% of baseline at t = 40 min, 96 ± 7.7% of baseline at t = 120 min, n = 8), but not with control nontargeting dsRNA (50 ± 8.0% of baseline at t = 40 min, 50 ± 6.7% of baseline at t = 120 min, n = 7).

If SRF is indeed activated by LTD induction, then it should be possible to measure this in individual Purkinje cells. To this end, we transfected cultured Purkinje cells with a plasmid in which tandem repeats of the SRE sequence are coupled with a minimal CMV promoter to drive expression of EGFP. We also coated the gold particles for biolistic transfection with a second marker plasmid that drives constitutive expression of dsRed. In dsRed-positive Purkinje cells, DHPG and glutamate treatment, using the same parameters as that used to induce LTD, gave rise to a delayed somatic EGFP signal (Supplementary Fig. 1). This signal reached a threshold criterion of fivefold higher than background levels in one of ten cells by 60 min and in nine of ten cells by 120 min. It should be noted that the delay between

chemical LTD induction and EGFP signal threshold detection reflects not only transcription and translation time, but also protein folding and post-translational processing time. Thus, it is not surprising that the time to detect EGFP was longer than the onset of the SRF-dependent late phase of LTD. When these two plasmids were delivered with *Srf* dsRNA, none of the ten cells showed suprathreshold EGFP signals following the chemical LTD induction protocol at 120 min. With control nontargeting dsRNA, all ten of the cells were suprathreshold for somatic EGFP signal at this time point.

Arc (also known *Arg3.1*) is an IEG that is dynamically regulated by neuronal activity in an SRF-dependent fashion[9,17,18]. Arc has been shown to be critical for hippocampal-dependent long-term memory and synaptic plasticity[19–24]. Recent studies have found that Arc regulates AMPA receptor trafficking via its interaction with the endocytic proteins endophilin and dynamin[22,23]. Overexpression of Arc in hippocampal slices and cultures depresses AMPAR-mediated synaptic currents[25,26]. We sought to determine whether Arc is required for the late-phase/protein synthesis–dependent component of cerebellar LTD.

To investigate the role of Arc in cerebellar LTD in cultured Purkinje cells, we first assessed whether Arc protein could be dynamically regulated in this preparation. Cultures were activated by bath application of glutamate (10 μM) and DHPG (100 μM) for 15 min followed by a 45-min washout recovery period, after which they were fixed and processed for Arc immunohistochemistry (the same treatment was used above; Supplementary Fig. 1). Arc expression was quite low in untreated cells, but could be upregulated ~20-fold by glutamate and DHPG treatment (Supplementary Fig. 2). Induction of Arc protein by mGluR1/5 agonist has also been observed in hippocampal neurons[23,24].

Although the early phase of LTD proceeded normally in $Arc^{-/-}$ neurons, the late phase was substantially attenuated (paired pathway, 57 ± 7.0% of baseline at t = 40 min, 90 ± 9.7% of baseline at t = 120 min, n = 8; Fig. 2a). Purkinje cells derived from wild-type littermates showed normal LTD (paired pathway, 54 ± 7.8% of baseline at t = 40 min, 58 ± 8.4% of baseline at t = 120 min, n = 9). It is possible that the LTD deficit resulted from a secondary developmental effect of *Arc* deletion. We attempted a rescue experiment using particle-mediated gene gun transfection to deliver expression constructs encoding the *Arc* open reading frame (ORF) under the expression of a constitutive CMV promoter and a second plasmid driving expression of EGFP to cultured $Arc^{-/-}$ Purkinje cells. We made recordings 22–60 h later. Transfection with the *Arc* ORF did not rescue, but instead completely occluded, both the early and late phases of LTD (paired pathway, 102 ± 7.6% of baseline at t = 40 min, 102 ± 6.9% of baseline at t = 120 min, n = 7). $Arc^{-/-}$ neurons transfected with just the EGFP plasmid showed early-phase, but not late-phase, LTD (data not shown).

As Arc has previously been implicated in AMPAR trafficking, we recorded mEPSCs as a measure of the level of synaptic AMPARs in Arc-manipulated Purkinje cells together with induction of chemical LTD. Baseline mEPSC amplitudes were normal in $Arc^{-/-}$ neurons ($Arc^{+/+}$, 30 ± 5.6 pA at t = 0 min; $Arc^{-/-}$, 35 ± 4.7 pA, n = 10 cells/group; Fig. 2b), as was the early phase of chemical LTD ($Arc^{+/+}$, 14 ± 5.6 pA; $Arc^{-/-}$, 17 ± 7.0 pA at t = 30 min), but late-phase LTD was again substantially attenuated ($Arc^{+/+}$, 16 ± 6.6 pA; $Arc^{-/-}$, 37 ± 5.9 pA at t = 90 min). Expression of the *Arc* ORF plasmid in $Arc^{-/-}$ neurons led to a substantial decrease in baseline mEPSC amplitudes (17 ± 4.3 pA at t = 0 min, n = 10 cells). Moreover, PDA treatment failed to induce a further decrease in mEPSC amplitude when the *Arc* ORF was present (16 ± 7.9 pA at t = 30 min and 15 ± 7.0 pA at t = 90 min), suggesting that overexpression of the *Arc* ORF occludes both the early and late phases of LTD by decreasing AMPAR-mediated synaptic transmission. Similar results were obtained when the *Arc* ORF was expressed in cultures derived from wild-type mice (Supplementary Fig. 3).

It has previously been shown that Arc overexpression decreases surface AMPARs and AMPAR currents in hippocampal neurons via its interaction with endophilin and dynamin[22,25]. To test whether these interactions were also required for Arc-mediated depression of mEPSCs in Purkinje neurons, we expressed Arc internal deletion mutant constructs that do not interact with dynamin (ArcΔ194–215) and endophilin (ArcΔ91–100). Expression of these Arc constructs in wild-type mouse cultures had no effect on the expression of late-phase LTD evoked by glutamate and depolarization pairing (ArcΔ194–215, 56 ± 8.0% of baseline at t = 120 min, n = 6 cells; ArcΔ91–100, 51 ± 8.0%, n = 7 cells; Supplementary Fig. 3) or on baseline mEPSCs and chemical LTD (Supplementary Fig. 3), suggesting that Arc's effects on AMPAR function are conserved in Purkinje neurons.

Because native Arc expression is low in Purkinje neurons and is induced by activity, we reasoned that we could rescue late-phase LTD in $Arc^{-/-}$ neurons by introducing an *Arc* construct that recapitulated the LTD profile of endogenous Arc. *Arc* mRNA is transcribed in response to neuronal activity and a proportion of the mRNA is then trafficked to activated dendrites, where it is translated[27]. It is uncertain whether *Arc* mRNA is trafficked to dendrites in Purkinje neurons, although it is clear that protein levels are activity regulated. To recapitulate endogenous Arc expression, we used an *Arc* construct that contained the *Arc* ORF, as well as the 3′ and 5′ untranslated regions, the *Arc* promoter and ~200 kb of surrounding genomic sequence in a BAC expression vector. We transfected $Arc^{-/-}$ neurons with the BAC-Arc construct and a conventional EGFP plasmid (as a marker) using the gene gun technique and assessed the ability of the BAC to confer expression of Arc protein by glutamate and DHPG treatment; BAC-transfected $Arc^{-/-}$ Purkinje cells recovered a ~16-fold induction of Arc expression (Supplementary Fig. 4). We then measured LTD. BAC-Arc–transfected $Arc^{-/-}$ neurons had normal, input- specific early- and late-phase LTD (56 ± 9.0% of baseline at t = 40 min, 52 ± 9.7% of baseline at t = 120 min, n = 8 cells; Fig. 3a). In addition, BAC-Arc–transfected cells had normal baseline mEPSCs (32 ± 6.0 pA at t = 0 min, n = 10 cells) and normal chemical LTD (16 ± 7.8 pA at t = 30 min, 15 ± 6.9 pA at t = 90 min; Fig. 3b). When the BAC-Arc construct was delivered to wild-type Purkinje cells, they showed normal early- and late-phase LTD (50 ± 9.0% of baseline at t = 40 min, 52 ± 9.2% of baseline at t = 120 min, n = 7 cells), normal baseline mEPSCs (32 ± 6.0 pA at t = 0 min, n = 10 cells), and normal chemical LTD (13 ± 8.6 pA at t = 30 min, 15 ± 6.9 pA at t = 90 min). Thus, BAC-Arc expression was able to fully rescue the LTD deficits in $Arc^{-/-}$ neurons.

There are several transcription factor binding sites in the *Arc* promoter, including two functional SRF-bindings sites (SREs) at locations approximately 1.1 and 6.9 kb upstream from the translation start site[14,15]. We sought to test the hypothesis that SRF binding at SRE 1.1, SRE 6.9 or both is necessary for the late phase of cerebellar LTD in cultured Purkinje cells. As an initial test of this hypothesis, we constructed mutant BACs in which either SRE 1.1 or SRE 6.9 were deleted and replaced with three repeats of a sequence constituting the binding domain of the yeast transcription factor Gal4 (Fig. 3c). These mutant BACs were then coated onto gold particles, together with an EGFP marker plasmid, for delivery to cerebellar cultures derived from $Arc^{-/-}$ mice. Purkinje cells transfected with the SRE 1.1 mutant BAC showed normal early- and late-phase LTD evoked by glutamate and depolarization pairing (55 ± 7.8% of baseline at t = 40 min, 54 ± 6.7% of baseline at t = 120 min, n = 7). mEPSC analysis revealed normal basal synaptic strength (31 ± 5.6 pA at t = 0 min, n = 10) and normal chemical LTD (14 ± 6.3 pA at t = 30 min, 16 ± 6.4 pA at t = 90 min). The SRE 1.1 mutant BAC was indistinguishable from wild-type BAC-Arc. However, when the SRE 6.9 mutant BAC was used, early-phase LTD was intact and late-phase LTD was abolished (51 ± 7.4% of baseline at t = 40 min, 94 ± 7.0% of baseline at t = 120 min, n = 8). Chemical LTD was similarly impaired (32 ± 4.2 pA at t = 0 min; 14 ± 7.4 pA at t = 30

min, 34 ± 8.2 pA at *t* = 90 min). These findings indicate that the integrity of SRE 6.9 is necessary for the late phase of LTD in cultured Purkinje cells.

The most straightforward explanation for these data is that SRF binding to SRE 6.9 is required for the late phase of LTD. However, SRE 6.9 is adjacent to functional binding sites for CREB and MEF2 in the *Arc* promoter[17] and it is possible that disruption of SRE 6.9 could affect late-phase LTD by altering MEF2 or CREB binding[28]. It is also possible that a transcription factor other than SRF binds to SRE 6.9. To address these issues, we attempted to rescue the late phase of LTD by co-transfecting $Arc^{-/-}$ Purkinje cells with both the SRE 6.9 mutant BAC-Arc and a plasmid encoding a Gal4-SRF fusion protein[29]. The Gal4-SRF fusion protein can bind the Gal4-binding domains that we used to disrupt SRE 6.9 and should reconstitute the SRF-Arc interaction. Indeed, although transfection of $Arc^{-/-}$ Purkinje neurons with the SRE 6.9 mutant BAC failed to rescue glutamate and DHPG induction of Arc, co-transfection of the SRE 6.9 mutant BAC with the Gal4-SRF fusion protein plasmid yielded ~14-fold larger Arc induction (Supplementary Fig. 5). This co-transfection strategy rescued the late phase of both glutamate and depolarization pairing LTD (47 ± 8.9% of baseline at *t* = 40 min, 45 ± 7.9% of baseline at *t* = 120 min, *n* = 9) and chemical LTD (33 ± 4.7 pA at *t* = 0 min, 17 ± 7.0 pA at *t* = 30 min, 16 ± 8.4 pA at *t* = 90 min; Fig. 4). A control condition, in which the Gal4-SRF plasmid was introduced in the absence of the SRE 6.9 mutant BAC, failed to rescue the late phase of either pairing-induced LTD (54 ± 7.4% of baseline at *t* = 40 min, 100 ± 8.1% of baseline at *t* = 120 min, *n* = 8) or chemically induced LTD (34 ± 4.0 pA at *t* = 0 min; 17 ± 8.6 pA at *t* = 30 min, 34 ± 7.1 pA at *t* = 90 min, *n* = 10). Similarly, a second control condition in which a plasmid encoding the Gal4 DNA binding domain alone (Gal4) was co-transfected with the SRE 6.9 mutant BAC also failed to rescue the late phase of pairing-induced LTD (54 ± 7.5% of baseline at *t* = 40 min, 103 ± 8.6% of baseline at *t* = 120 min, *n* = 7) and chemically induced LTD (34 ± 5.6 pA at *t* = 0 min, 17 ± 6.8 pA at *t* = 30 min, 33 ± 7.8 pA at *t* = 90 min).

What is the biochemical cascade that links LTD induction to SRF activation at SRE 6.9? There are two potential signaling pathways that control the activity of distinct SRF coactivators. One pathway employs the Ras, Raf and ERK signaling cascade and leads to activation of the SRF coactivator $p62^{TCF}$ (ref. [30]), whereas the second uses an actin polymerization pathway leading to activation of the coactivator, MAL. This pathway, first defined in non-neuronal cells[31–33], controls nuclear accumulation and activation of MAL following F-actin polymerization. In contrast, G-actin binds and represses MAL in a transcriptionally inactive complex. Recently, it has been shown that this actin, MAL and SRF pathway also functions in cultured hippocampal neurons, where it is involved in regulation of neurite elongation and growth cone motility[13,34].

To test the hypothesis that actin, MAL and SRF signaling is required for the late phase of LTD, we used two strategies (Fig. 5). First, we transfected Purkinje cells with an EGFP marker plasmid and a dominant-negative form of MAL (DN-MAL) lacking the C-terminal region (amino acids 1–630), in which the transactivation domain was mutated, but SRF binding remained intact[35]. Control Purkinje cells transfected with EGFP alone showed robust early- and late-phase LTD using either glutamate and depolarization pairing (57 ± 9.0% of baseline at *t* = 40 min, 53 ± 8.4% of baseline at *t* = 120 min, *n* = 9) or chemical induction (28 ± 5.3 pA at *t* = 0 min, 14 ± 5.6 pA at *t* = 30 min, 14 ± 6.3 pA at *t* = 90 min, *n* = 10). However, DN-MAL– and EGFP-transfected cells showed a suppression of the late phase in both pairing (53 ± 7.4% of baseline at *t* = 40 min, 98 ± 7.7% of baseline at *t* = 120 min, *n* = 9) and chemical induction protocols (30 ± 5.0 pA at *t* = 0 min, 18 ± 6.6 pA at *t* = 30 min, 30 ± 5.4 pA at *t* = 90 min, *n* = 10). Second, we overexpressed a mutant β-actin (R62D) that fails to polymerize and form F-actin. This mutant was coupled to a nuclear localization sequence (R62D-NLS–β-actin)[34]. Purkinje cells overexpressing R62D-NLS–β-actin and an

EGFP marker showed a suppression of late-phase LTD similar to that seen with DN-MAL: pairing (53 ± 7.4% of baseline at t = 40 min, 86 ± 7.1% of baseline at t = 120 min, n = 8) and chemical induction (32 ± 4.5 pA at t = 0 min, 16 ± 5.9 pA at t = 30 min, 27 ± 6.0 pA at t = 90 min, n = 10). Overexpression of wild-type β-actin resulted in normal early- and late-phase LTD for both pairing (54 ± 7.5% of baseline at t = 40 min, 53 ± 8.9% of baseline at t = 120 min, n = 7) and chemical induction (35 ± 4.3 pA at t = 0 min; 15 ± 6.9 pA at t = 30 min, 15 ± 6.4 pA at t = 90 min, n = 10). These results suggest that the late phase of LTD in cultured Purkinje cells requires β-actin polymerization and the consequent release of MAL from G-actin binding that allows it to bind to SRF.

This model predicts that a chemical manipulation that prevents F-actin assembly, but does not dissociate the actin-MAL complex, such as that produced by latrunculin-B, which has been shown to block MAL activation[31–33] and Arc induction by serum in fibroblasts (A. Descot and G.P., unpublished observations), would also block the late phase of LTD. Unfortunately, in our hands, pretreatment with this drug rendered the cells unstable, precluding long-term recording. Notably, none of the treatments and transfections altered the initial signaling processes required to activate PKC during LTD (Supplementary Fig. 6). We assessed this using bis–Fura-2 micro-fluorimetric imaging of Purkinje cell dendrites to measure the Ca^{2+} transient evoked by a depolarizing step (an index of voltage-gated Ca^{2+} channel function) and the Ca^{2+} transient evoked by application of an mGluR1/5 agonist (DHPG, 100 μM), an index of mGluR1 function. Other basal electrophysiological properties of Purkinje cells, such as mEPSC kinetics and R_{input} were also unaltered by these treatments (data not shown).

DISCUSSION

Our results indicate that SRF binding to SRE 6.9 in the *Arc* promoter and SRF-dependent Arc expression are essential for establishing a late phase of cerebellar LTD in cultured Purkinje cells. To the best of our knowledge, this is the first time that a specific interaction between a transcription factor and a target gene has been implicated in any form of use-dependent alteration of synaptic strength. Although SRF binding to SRE 6.9 is necessary for the late phase, it is not clear whether it is sufficient. It is worth noting that SRE 6.9 lies in a 100-bp region that also includes functional binding sites for the activity-driven transcription factors CREB and MEF2 (ref. [17]), raising the possibility of combinatorial control of Arc expression.

These findings also suggest that actin-regulated MAL signaling is necessary for the late phase of cerebellar LTD in cultured Purkinje cells (Fig. 5). It is likely that MAL is acting through SRF, although we do not have direct proof of this. It also remains unclear precisely how LTD induction engages the actin-polymerization MAL signaling cascade. In addition, the role in LTD of another SRF activation pathway, TCF cofactor signaling, remains unexplored.

We propose that Arc, normally found at low basal levels in Purkinje cells, is induced in response to LTD stimuli and acts to maintain depression of synaptic transmission through its interaction with the endocytic machinery. This suggests that mechanisms of long-term plasticity that require protein synthesis are at least partially conserved in different brain regions, as Arc is also important for an early phase of hippocampal LTD[21,23,24]. However, our data do suggest some important differences between hippocampal and cerebellar LTD. Protein synthesis–dependent hippocampal LTD requires group 1 mGluRs for induction, but its maintenance is not affected by transcriptional inhibitors[36], whereas maintenance of cerebellar LTD in cultured Purkinje cells is disrupted by both translation and transcription inhibitors[6,37]. Our data suggest that both Arc mRNA and protein levels are low in Purkinje

neurons in basal conditions. Introduction of the BAC-Arc alone into Purkinje cells had no effect on synaptic transmission, presumably because the protein could only be induced by neuronal activity. We propose that cerebellar LTD in cultured Purkinje cells requires the transcription and translation of Arc to maintain LTD and that Arc acts only in the later stages of LTD. In contrast, hippocampal LTD requires the rapid translation of Arc from existing *Arc* mRNA in dendrites, but does not require *Arc* transcription[23,24]. A similar mechanism has been proposed for the role of fragile X mental retardation protein in mGluR-dependent hippocampal LTD[24,38].

The ability of particular patterns of synaptic activity to drive transcriptional events in neurons is likely to comprise at least one mechanism by which memories are consolidated in the brain. We found that SRF binding to SRE 6.9 in the *Arc* promoter is crucial for the late phase of cerebellar LTD in cultured Purkinje cells. This information allows for very explicit behavioral predictions: if the late phase of cerebellar LTD in cultured Purkinje cells reflects processes *in vivo* and if LTD *in vivo* underlies particular forms of motor learning, such as associative eyelid conditioning or vestibulo-ocular reflex adaptation, then mutant mice harboring inactivating mutations in SRE 6.9 in their Purkinje cells should show deficits in the consolidation of these particular memories.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank D. VanNess for excellent technical assistance and to members of the Linden and Worley laboratories for helpful suggestions. M. Greenberg's laboratory (Harvard University) provided the Gal4-SRF fusion construct plasmid. The authors thank B. Knöll (University of Tübingen) for sharing β-actin constructs and for helpful suggestions. The FLAG-tagged dominant-negative MAL was a generous gift from R. Prywes (Columbia University). This work supported by grants from the US Public Health Service (NIH R37 MH51106 to D.J.L., P50 MH084020 to D.J.L., D.D.G., P.F.W. and R.L.H., and NIH 5K12 NS001696-07 to C.S.-H.), the National Basic Research Program of China (2004CB518800 to B.X. and 2004CB518800 to X.Z.) and the Science Fund for Creative Research Group, China (B.X.).

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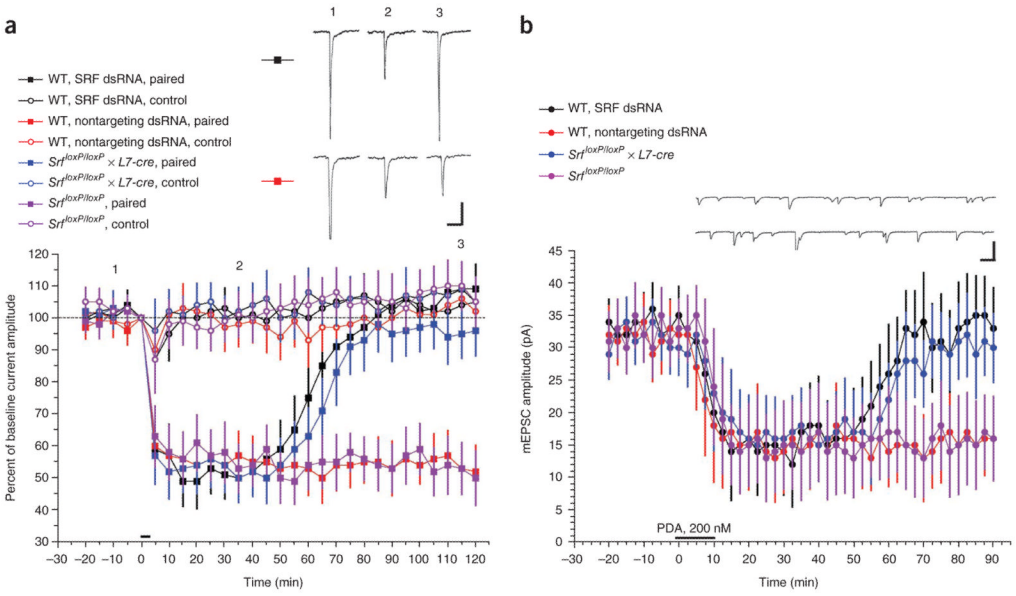

Figure 1.

Interfering with SRF blocks the late phase of cerebellar LTD in cultured Purkinje cells. (**a**) Test pulses of glutamate were applied to two non-overlapping sites on the Purkinje cell dendrite. Pulses were delivered to alternate sites at 10-s intervals. To induce LTD, we delivered 6 3-s-long depolarizing commands to 0 mV, coupled with glutamate pulses, to the 'paired' pathways at $t = 0$ min, as indicated by the horizontal bar. The 'control pathways' received only somatic step depolarization. Alternate test pulses were then resumed for the duration of the experiment. Exemplar traces are single (unaveraged) responses and they correspond to the points indicated on the time-course graph. Data are mean ± s.e.m. Scale bars represent 2 s and 50 pA. Wild-type (WT) SRF dsRNA ($n = 8$), wild-type nontargeting dsRNA ($n = 7$), $Srf^{loxP/loxP} \times L7\text{-}cre$ ($n = 7$), $Srf^{loxP/loxP}$ ($n = 7$). Statistical comparisons at $t = 120$ min for the paired pathway: wild-type SRF dsRNA versus wild-type nontargeting dsRNA, $P < 0.001$; $Srf^{loxP/loxP} \times L7\text{-}cre$ versus $Srf^{loxP/loxP}$, $P < 0.001$. (**b**) Tetrodotoxin was added to the bath and mEPSCs were measured. Chemical LTD was produced by bath application of the PKC activator PDA at $t = 0$–10 min, as indicated by the horizontal bar ($n = 12$ cells per group). Statistical comparisons at $t = 90$ min: wild-type SRF dsRNA versus wild-type nontargeting dsRNA, $P < 0.01$; $Srf^{loxP/loxP} \times L7\text{-}cre$ versus $Srf^{loxP/loxP}$, $P < 0.02$. Raw exemplar traces are from the group in the corresponding legend label. Scale bars represent 20 ms and 60 pA. Error bars represent the s.e.m.

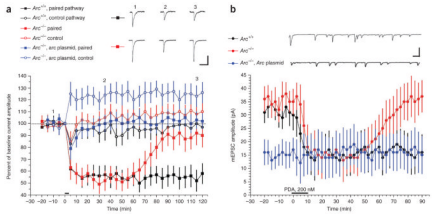

Figure 2.

The late phase of cerebellar LTD is blocked in $Arc^{-/-}$ cultured Purkinje cells. (**a**) LTD induced by glutamate and depolarization pairing. Scale bars represent 2 s and 100 pA. $Arc^{+/+}$ ($n = 8$), $Arc^{-/-}$ ($n = 9$), $Arc^{-/-}$ and *Arc* plasmid ($n = 7$). Statistical comparisons at $t = 120$ min for the paired pathway: $Arc^{+/+}$ versus $Arc^{-/-}$, $P < 0.01$; $Arc^{-/-}$ versus $Arc^{-/-}$ and *Arc* plasmid, $P > 0.20$. (**b**) Chemical LTD induced by bath application of PDA ($n = 10$ cells per group). Statistical comparisons at $t = 90$ min: $Arc^{+/+}$ versus $Arc^{-/-}$, $P < 0.02$; $Arc^{-/-}$ versus $Arc^{-/-}$ and *Arc* plasmid, $P > 0.20$. Raw exemplar traces are from the group in the corresponding legend label. Scale bars represent 20 ms and 40 pA. Error bars represent the s.e.m.

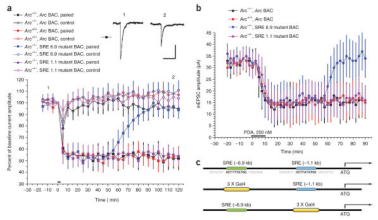

Figure 3.

Expression of a BAC construct containing *Arc* and its genomic regulatory regions rescues early- and late-phase LTD in $Arc^{-/-}$ neurons. (**a**) LTD induced by glutamate and depolarization pairing. Scale bars represent 2 s and 40 pA. $Arc^{-/-}$ and Arc-BAC ($n = 8$), $Arc^{+/+}$ and Arc-BAC ($n = 7$), $Arc^{-/-}$ and SRE 6.9 mutant BAC ($n = 8$), $Arc^{-/-}$ and SRE 1.1 mutant BAC ($n = 7$). Statistical comparisons at $t = 120$ min for the paired pathway: $Arc^{-/-}$ and Arc-BAC versus $Arc^{-/-}$ and SRE 6.9 mutant BAC, $P < 0.005$; $Arc^{-/-}$ and SRE 6.9 mutant BAC versus $Arc^{-/-}$ and SRE 1.1 mutant BAC, $P < 0.01$. (**b**) Chemical LTD induced by PDA ($n = 10$ cells per group). Statistical comparisons at $t = 90$ min: $Arc^{-/-}$ and Arc-BAC versus $Arc^{-/-}$ and SRE 6.9 mutant BAC, $P < 0.02$; $Arc^{-/-}$ and SRE 6.9 mutant BAC versus $Arc^{-/-}$ and SRE 1.1 mutant BAC, $P < 0.02$. Error bars represent the s.e.m. (**c**) Schematic diagram of the *Arc* promoter showing two SREs, where SRF can bind, and the ATG start codon.

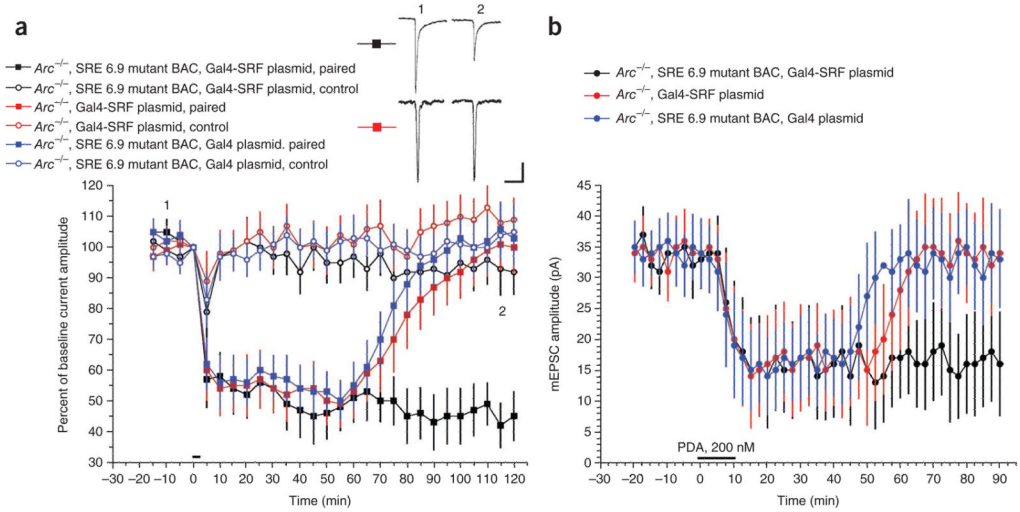

Figure 4.

The failure of the SRE 6.9 mutant Arc-BAC to rescue late-phase LTD is reversed by co-transfection with a plasmid encoding a Gal4–SRF fusion protein. (**a**) LTD induced by glutamate and depolarization pairing. Scale bars represent 2 s and 40 pA. $Arc^{-/-}$, SRE 6.9 mutant BAC and Gal4-SRF plasmid (n = 9). $Arc^{-/-}$ and Gal4-SRF plasmid (n = 8). $Arc^{-/-}$, SRE 6.9 mutant BAC and Gal4 plasmid (n = 7). Statistical comparisons at t = 120 min for the paired pathway: $Arc^{-/-}$, SRE 6.9 mutant BAC and Gal4-SRF plasmid versus $Arc^{-/-}$ and Gal4-SRF plasmid, $P < 0.005$; $Arc^{-/-}$, SRE 6.9 mutant BAC and Gal4–SRF plasmid versus $Arc^{-/-}$, SRE 6.9 mutant BAC and Gal4 plasmid, $P < 0.001$. (**b**) Chemical LTD induced by PDA (n = 10 cells per group). Statistical comparisons at t = 90 min: $Arc^{-/-}$, SRE 6.9 mutant BAC and Gal4-SRF plasmid versus $Arc^{-/-}$, Gal4-SRF plasmid, $P < 0.05$; $Arc^{-/-}$, SRE 6.9 mutant BAC and Gal4-SRF plasmid versus $Arc^{-/-}$, SRE 6.9 mutant BAC and Gal4 plasmid, $P < 0.05$. Error bars represent the s.e.m.

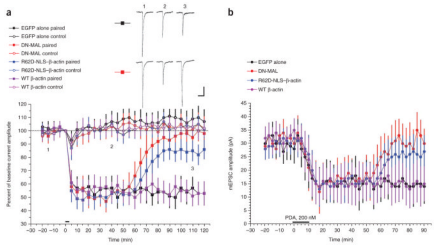

Figure 5.

Manipulations that inhibit actin-MAL signaling block the late phase of cerebellar LTD in cultured Purkinje cells. (**a**) LTD induced by glutamate and depolarization pairing. Scale bars represent 2 s and 30 pA. EGFP alone (n = 9), DN-MAL (n = 9), R62D-NLS–β-actin (n = 8), wild-type β-actin (n = 7). Statistical comparisons at t = 120 min for the paired pathway: EGFP alone versus DN MAL, $P < 0.005$; R62D-NLS–β-actin versus wild-type β-actin, $P < 0.02$. (**b**) Chemical LTD induced by PDA (n = 10 cells per group). Statistical comparisons at t = 90 min: EGFP alone versus DN MAL, $P < 0.02$; R62D-NLS–β-actin versus wild-type β-actin, $P < 0.05$. Error bars represent the s.e.m.