

# Molecular Network and Chromosomal Clustering of Genes Involved in Synaptic Plasticity in the Hippocampus<sup>\*[S]</sup>

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Chang Sin Park<sup>‡</sup>, Ruomu Gong<sup>‡</sup>, Joshua Stuart<sup>§</sup>, and Shao-Jun Tang<sup>‡1</sup>

From the <sup>‡</sup>Department of Neurobiology and Behavior, Center for Neurobiology of Learning and Memory, University of California, Irvine, California 92697-3800 and the <sup>§</sup>Department of Biomolecular Engineering, University of California, Santa Cruz, California 95064

Gene transcription is required for establishing and maintaining the enduring form of long term potentiation (LTP). However, the transcriptome and its associated molecular programs that support LTP are not well understood. The purpose of this study was to identify activity-regulated genes (ARGs) and their molecular pathways that are modulated by LTP induction and to investigate the genomic mechanism for coordinating the transcription of ARGs. We performed time course DNA microarray analyses on the mouse dentate gyrus to determine the temporal genomic expression profiles of ARGs in response to LTP-inducing tetanic stimulation. Our studies uncovered ARGs that regulate various cellular processes, including the structure and function of the synapse, and offered an overview of the dynamic molecular programs that are probably important for LTP. Surprisingly, we found that ARGs are clustered on chromosomes, and ARG clusters are conserved during evolution. Although ARGs in the same cluster have apparently different molecular properties, they are functionally correlated by regulating LTP. In addition, ARGs in specific clusters are co-regulated by the cAMP-response element-binding protein. We propose that chromosomal clustering provides a genomic mechanism for coordinating the transcription of ARGs involved in LTP.

Synaptic plasticity is a fundamental property of the nervous systems and is essential for animals to cope with dynamic environments. One of the best characterized forms of synaptic plasticity is long term potentiation (LTP)<sup>2</sup> (1). LTP is thought as a leading cellular substrate for long term storage of information in the brain (2–6). In addition to learning and memory, a vari-

ety of other experience-induced behavioral changes in different brain regions are also mediated by LTP-like mechanisms (7).

There are temporally distinguished forms of LTP that require distinct mechanisms for their maintenance (8, 9). One is the short term LTP (early LTP) that usually lasts for less than 1 h and results from modifications of pre-existing synaptic proteins. Another is the longer lasting LTP (late LTP) that requires activity-induced protein synthesis and gene transcription (8). At the cellular level, the maintenance of late LTP is associated with structural remodeling of synapses that can lock in the change of synaptic strength (8–10). Activity-induced protein synthesis and gene expression are probably necessary to support such longer lasting structural changes of the synapse. Therefore, identifying the genes that are regulated during LTP is essential for understanding the molecular mechanism underlying long lasting LTP.

Previous studies reported individual ARGs that are up-regulated by LTP-inducing tetanic stimulations (9, 11–15). More recently, a number of microarray analyses have been carried out to identify ARGs regulated by various experimental stimuli, including electroconvulsive seizures (16, 17), KCl-mediated membrane depolarization (18), *N*-methyl-D-aspartate stimulations (19), and learning tasks (20–23). Many of the identified ARGs belong to the group of immediate early genes (IEGs) (24). Functional characterizations of IEGs such as *Zif 268* (25), *Homer 1* (26), tissue-plasminogen activator (27), and *Arc/Arg3.1* (28, 29) suggest their critical roles in LTP and memory (24, 26, 30, 31). Despite the important progress, we still lack a genomic overview of ARGs during LTP. We also do not know how ARGs are organized in the genome.

Here we present data from a time course DNA microarray analysis of the genomic expression after LTP-inducing tetanic stimulations in the mouse dentate gyrus (DG). Our studies identify a large number of ARGs responding to LTP induction and reveal temporal molecular processes that accompany the progress of LTP expression and maintenance. Interestingly, ARGs are clustered on chromosomes. Many ARG clusters are located in chromosomal domains enriched with CREB-binding loci and display CREB-dependent transcription.

## EXPERIMENTAL PROCEDURES

**Electrophysiology**—For all electrophysiology experiments, hippocampal slices (400  $\mu$ m) were prepared from 21- to 28-day-old male mice (C57/BL6). Prior to recording, slices were allowed to recover first for 30 min at room temperature in oxygenated (95% O<sub>2</sub>; 5% CO<sub>2</sub>) artificial cerebrospinal fluid (ACSF;

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[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. 1 and supplemental Tables 1–4.

<sup>1</sup> Supported by Whitehall Foundation, American Heart Association, EJLB Foundation, and United States Army Medical Research and Materiel Command. To whom correspondence should be addressed: 303 Qureshey Research Laboratory, Center for Neurobiology of Learning and Memory, University of California, Irvine, CA 92697-3800. Tel.: 949-824-9580; Fax: 949-824-9762; E-mail: stang@uci.edu.

<sup>2</sup> The abbreviations used are: LTP, long term potentiation; ARG, activity-regulated genes; LG, LTP-related genes; CREB, cAMP-response element-binding protein; RT, reverse transcription; IEGs, immediate early genes; siRNA, short interfering RNA; ACSF, artificial cerebrospinal fluid; MAPK, mitogen-activated protein kinase; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase; DG, dentate gyrus; GO, gene ontology; EST, expressed sequence tag.

## Clusters of LTP Regulatory Genes on Chromosomes

119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 1.0 mM NaH<sub>2</sub>PO<sub>4</sub>, 26.2 mM NaHCO<sub>3</sub>, 11.0 mM glucose), and then for 60 min at 27 °C in the submerged recording chamber that was continuously perfused with oxygenated ACSF. Field excitatory postsynaptic potentials of perforant path-dentate gyrus synapses were evoked with concentric bipolar tungsten-stimulating electrodes and recorded with low resistance glass microelectrodes filled with 3 M NaCl. Both stimulating and recording electrodes were visually placed in the middle molecular layer of the dentate gyrus. Long term potentiation (LTP) was induced by high frequency stimulations (four trains of 1-s 100-Hz stimulations spaced by 30-s intervals). To determine input-output curves, increasing stimulation strengths were applied (from 0 to 15 mA in 0.5-mA steps), and three field excitatory postsynaptic potentials slopes were averaged per stimulation strength value. For electrophysiology experiments pertaining to microarray analysis, hippocampal slices were microdissected to isolate the dentate gyrus prior to recovery in the oxygenated chamber. From each animal, five dentate gyrus mini slices were prepared; this set of slices from the same animal was processed, recovered, and recorded under the same conditions. One of the slices received test stimulations but not LTP induction and was used as a control; the other four slices received LTP induction and were collected for RNA purification at 30, 60, 90, and 120 min post-LTP induction. No stimulations were applied after test and tetanic stimulations (Fig. 1D). Electrophysiology on all five slices was conducted in the same recording chamber at the same time in series to maintain the consistency of experimental conditions. Ten sets of mini slices from independent experiments were collected to purify RNA for each microarray analysis, which was repeated four times using independently prepared RNA samples. To ensure the RNA integrity necessary for microarray and real time RT-PCR experiments, all electrophysiology experiments were conducted within 4 h following slice preparations after which all slices were flash-frozen for subsequent RNA extraction. Drugs and antibodies in oxygenated ACSF were perfused into the recording chamber. Cystamine was obtained from Sigma; NSC663284 was from Dr. Schultz (NCI, National Institutes of Health), and anti-PHPS1 was from Santa Cruz Biotechnology (Santa Cruz, CA).

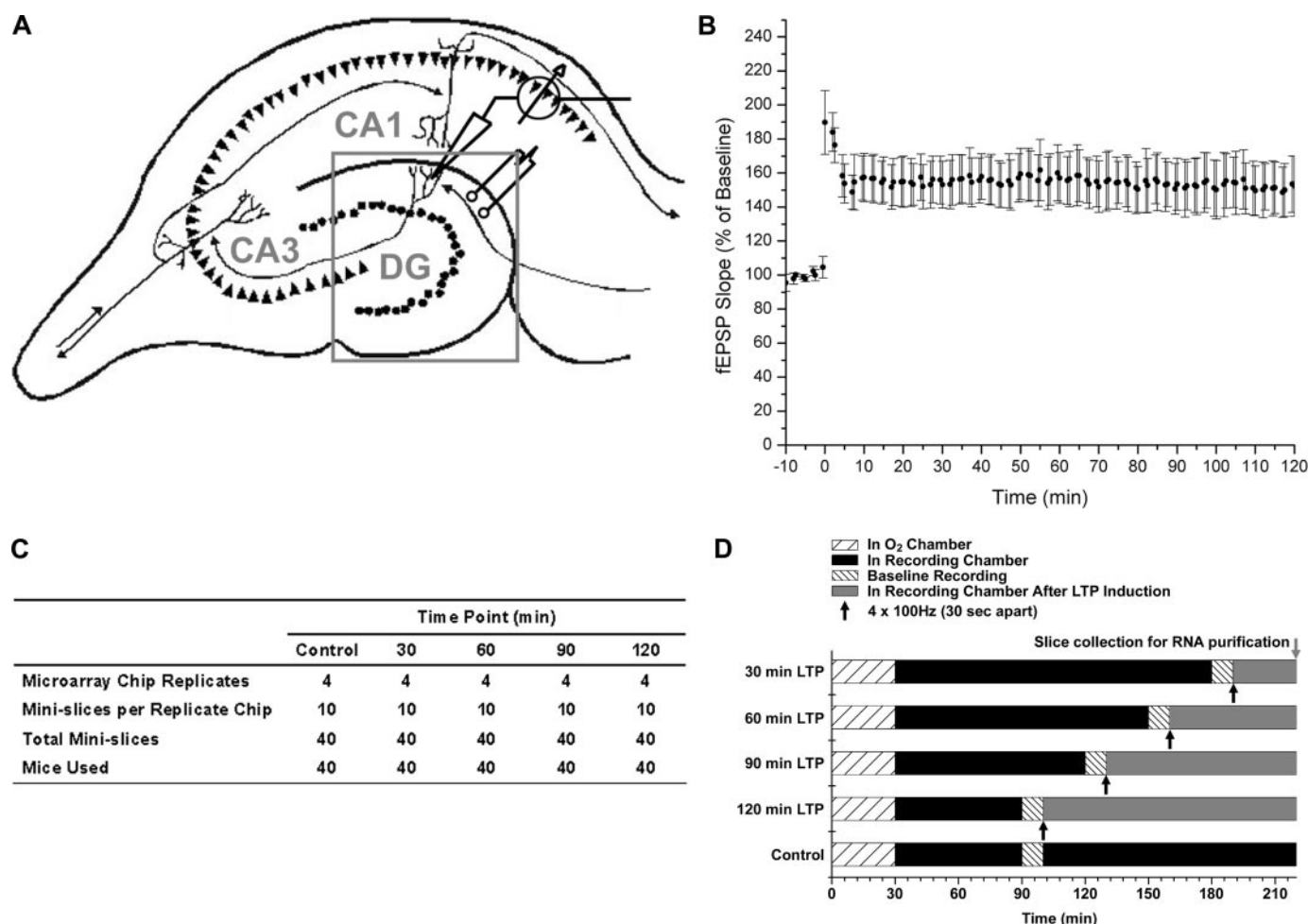
**Microarray Analysis**—Total RNA was extracted from frozen (−80 °C) dentate gyrus mini slices collected following the electrophysiology experiments. For control and each LTP time point, four sets of 10 dentate gyrus mini slices were pooled together and homogenized, and total RNA was extracted using Trizol reagent (Invitrogen). Prior to microarray hybridization, the quality of each RNA sample was verified with the Agilent 2100 Bioanalyzer. Total RNA was reverse-transcribed into cDNA, converted to biotin-labeled cRNA, and then hybridized onto individual Affymetrix *Mus musculus* MGU74Av2 GeneChip arrays following the manufacturer's instructions. We used a statistical strategy of pairwise comparisons suggested for time course microarray experiments (32) to identify genes that have significantly changed their expression levels at various time points after LTP induction, compared with controls. Two sets of normalized gene expression values were obtained using MAS 5.0 and dCHIP. Although the MAS 5.0 algorithm is suitable for determining high level expression values, for low level expres-

sion values, the model-based expression index analysis performed in dCHIP improves the accuracy of the expression values by reducing the variability of low expressing targets. Pairwise statistical comparisons of individual LTP time points with controls was performed using CyberT, which performs *t* tests that incorporate a Bayesian estimate of the variance of the microarray expression data to compensate for a low number of experimental replicates (33). Statistically significant genes were combined to generate a list of activity-regulated genes (ARGs) for further analysis. Hierarchical clustering of ARG expression profiles was performed using GeneSpring (Silicon Genetics).

**Chromosomal Gene Clustering Analysis**—Analysis of chromosomal clustering of ARGs and LGs was performed using a statistical approach described previously (34). This statistical strategy was chosen because it allows us to compare the ARG distribution pattern with patterns computed from the same number of randomly profiled genes from the gene pool on the U74Av2 gene chip. Only those ARGs and LGs that had chromosomal position information in the University of California, Santa Cruz, genome data base were used in this analysis. After comparing to 1000 random sampling profiles of the same number of MUG74Av2 genes with expression values higher than the background and positional information, we found that ARGs were significantly clustered on chromosomes at an intergenic distance of 500 and 1000 kb, whereas LGs were significantly clustered at 100 and 250 kb. Hypergeometric cumulative distribution calculations were performed to determine the statistical significance of the association of LGs with ARG clusters, based on the probability of the association of LGs with ARG clusters. Conservation of mouse ARG clusters in the different vertebrate and invertebrate species was determined using gene homology information obtained from the Affymetrix NetAffx Analysis Center data base. Of the ARGs that formed clusters, only those that had identifiable homologs and chromosomal position information were included for the conservation analysis. A mouse ARG cluster was considered conserved in a different genome if 75% of ARG homologs appeared together on a chromosome locus in the same order.

**Quantitative Real Time RT-PCR**—Real time RT-PCR was performed with an ABI Prism 7700 sequence detection system and LUX fluorogenic primers (Invitrogen). For each target gene, the fluorophore-labeled LUX forward primers and their corresponding unlabeled reverse primers were designed using LUX Designer. From each sample of total RNA used for microarray hybridization, ~0.5 mg of total RNA was converted to cDNA using SuperScript First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. PCR cycles were as follows: 2 min at 50 °C, 2 min at 95 °C, and then 45 cycles of 15 s at 95 °C and 30 s at 60 °C. The relative difference of expression levels between control and LTP time points for each gene was calculated by a relative cycle threshold method using  $\beta$ -actin as a reference.

**siRNA Transfection and Western Blot Analysis**—Primary cortical neurons were isolated from rat embryos (E18) according to Banker and Goslin (35). For siRNA transfection, 5 × 10<sup>6</sup> cells in 100  $\mu$ l of the rat-specific nucleofactor solution (Amaxa) were transfected with 2.5  $\mu$ M siRNA (Upstate), using the nucleofactor device from Amaxa. Immediately after transfec-



**FIGURE 1. Microarray analysis of ARGs in the hippocampus.** *A*, schematic diagram of the hippocampus. DG mini slices (indicated by the box) from the mouse hippocampus were used for microarray experiments after LTP induction in the perforant pathway. *B*, LTP could be reliably induced in the perforant pathway of DG mini slices by tetanic stimulations ( $n = 5$ ). *fEPSP*, field excitatory postsynaptic potential. *C*, time course experimental designs for the microarray analysis. Microarray analyses were performed on four time points after tetanus. Mini slices that received test but not tetanic stimulations were used as controls. Four independent array repeats were included for each time point and control. Ten mini slices from different mice and independent LTP experiments were collected for preparation of RNA for one replicate. *D*, strategy for preparing control and experimental mini slices. See text for details.

tion, cells were plated in 6-well plate coated with polylysine (Sigma). With fluorescently labeled siRNA, we detected that all cells were transfected. Cells were harvested at day 6 post-transfection for Western blot analysis with the ECL detection system (Amersham Biosciences). The antibodies used were anti-CREB antibodies (1:1000) from Cell Signaling and peroxidase-conjugated donkey anti-rabbit antibodies (1:5000) from Amersham Biosciences.

## RESULTS

**Characterization of Synaptic ARGs by DNA Microarray Analysis**—Previous studies identified a number of genes, especially IEGs, regulated by neuronal activities, and some of them are implicated in synaptic plasticity (24, 30, 36). We reasoned that a systematic characterization of ARGs modulated by synaptic activities that are relevant to LTP induction would allow us to identify molecular pathways involved in LTP expression. Therefore, we sought to profile ARGs that were up- or down-regulated at various time points after tetanic stimulations that induce LTP. We thought that a time course profiling of ARG expression would provide a means to uncover the dynamics of molecular processes during LTP expression.

In this study, we focused on profiling ARGs in the DG because many previously characterized ARGs were identified in this hippocampal region. To minimize potential masking effects from other hippocampal regions without LTP induction, we prepared mouse DG mini slices (Fig. 1*A*). We reliably induced LTP in the perforant pathway of mini slices, using the protocol of tetanic stimulations (four trains of 100 Hz spaced by 30 s) (Fig. 1*B*). Mini slices at 30, 60, 90, and 120 min after LTP induction were collected for microarray analysis, whereas mini slices receiving only test stimulations but without LTP induction were used as controls. The mouse U74Av2 gene chips from Affymetrix were used to determine the expression profiles of 12,000 genes and ESTs. As LTP induction may not occur in all neurons in the dentate gyrus and slicing processes can induce transcription of ARGs (37), we expected that the magnitude of ARG expression changes in response to tetanus would be partially masked and thus small. To obtain sufficient statistical power to detect LTP-regulated ARGs, we included four independent microarray replicates for controls and experiments at each time point (Fig. 1*C*). RNA for each replicate was prepared from pooled 10 mini slices from independent experiments. To



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control the variability between slices from different time points, we designed the following strategy for mini slice preparation (Fig. 1D). Each set of mini slices included control and 30-, 60-, 90-, and 120-min samples and were generated from the same animal. The same set of mini slices was treated exactly the same. They were recovered in the O<sub>2</sub> chamber for 30 min and then simultaneously transferred to the same recording chamber (27 °C). After 60 min in the recording chamber, LTP mini slices sequentially received 10 min of test stimulation following by tetanus (four times at 100 Hz with 30-s intervals) as indicated in Fig. 1D, whereas control mini slices only received test stimulations. Following LTP induction, slices were kept in the recording chamber without stimulation until all slices were collected and frozen at the same time for RNA purification. Therefore, the source and treatments of control and experimental mini slices prepared with this procedure were exactly the same

**TABLE 1**  
Pearson correlation (*r* value) between replicates of the same time point

Time point	No. of replicates	Range of <i>r</i> values	Average <i>r</i> value
<i>min</i>			
Control	4	0.957–0.993	0.978
30	4	0.961–0.993	0.978
60	4	0.926–0.992	0.965
90	4	0.956–0.993	0.974
120	4	0.951–0.989	0.976

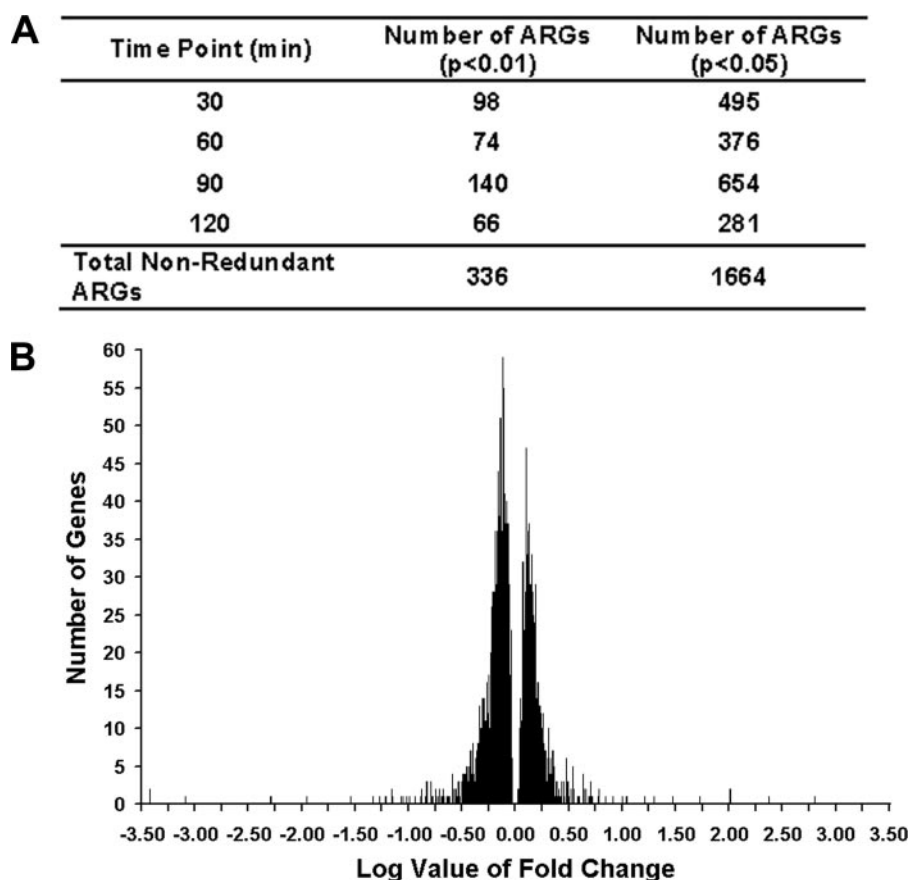
except for stimulation; slices with LTP induction at different time points had different period of time prior to LTP induction (Fig. 1D).

We first tested the reproducibility of microarray data between different replicates. The overall similarity in gene expression profiles between replicates of the same time point was assessed by determining their Pearson correlation (*r* value), which ranged from 0.965 to 0.978 with a mean of 0.9742 (Table 1). These high *r* values indicated a high degree of reproducibility in sample collection, RNA preparation, and array hybridization.

To identify ARGs that significantly changed their expression levels at specific time points after LTP induction, we used a statistical analysis strategy of multiple comparisons suggested for time course microarray experiments (32). Using the Bayesian-based *t* test platform CyberT (33), we performed statistical comparisons of genomic expression between a specific experimental time point and the control. This statistical strategy was chosen because the goal was to identify genes that were changed at specific time points after LTP induction. Genes with significant alterations in their expression levels at one or more experimental time points were counted as ARGs. We found a total of 336 nonredundant ARGs at the *p* < 0.01 level and 1664 ARGs at the *p* < 0.05 level (Fig. 2A; supplemental Table 4; note that supplemental Table 4 contains a small number of redundant genes represented by different probe sets). The magnitude

of ARG (*p* < 0.05) expression changes was indicated in Fig. 2B and supplemental Table 4. Of all ARGs at the *p* < 0.05 level, 39% were up-regulated and 61% were down-regulated. The identified ARGs included *fos* and *homer 1* (Fig. 3; supplemental Table 4) that are known to be regulated by neuronal activities (24). To validate the microarray data further, we performed quantitative real time RT-PCR to profile the dynamic expression of a group of ARGs (Fig. 3). Results showed that 89% (32/36) of microarray expression data were confirmed by real time RT-PCR (Fig. 3). Using a recently developed statistical approach (38), we estimated that the overall false positive rates at the level of *p* < 0.01 and *p* < 0.05 were 14 and 23%, respectively.

**Enrichment of LGs in ARGs**—As we profiled ARGs that responded to LTP-inducing synaptic stimulations, we expected that some of the identified ARGs should be implicated in LTP by previous studies. To evaluate this possibility, we compared our list of ARGs with LGs identified through a search of the biomedical literature in the PubMed data base

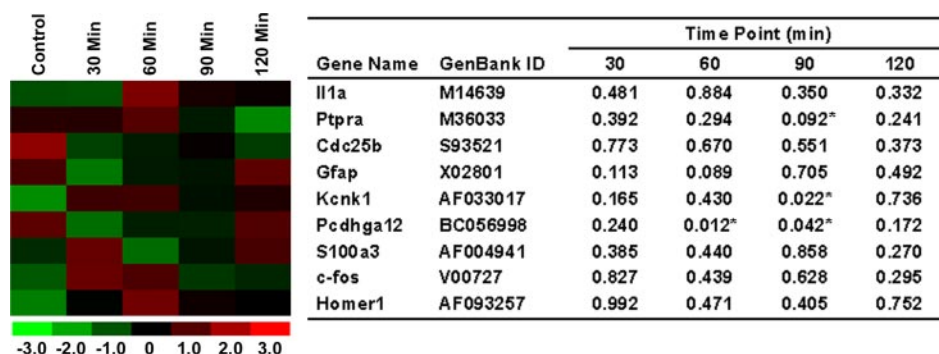


**FIGURE 2.** A, summary of ARGs that significantly changed their expression level at individual time points after LTP induction in comparison with control. B, histogram for the distribution of fold changes (log<sub>2</sub> transformed) of ARG (*p* < 0.05). Up-regulated ARGs are indicated by plus values and down-regulated by minus values.

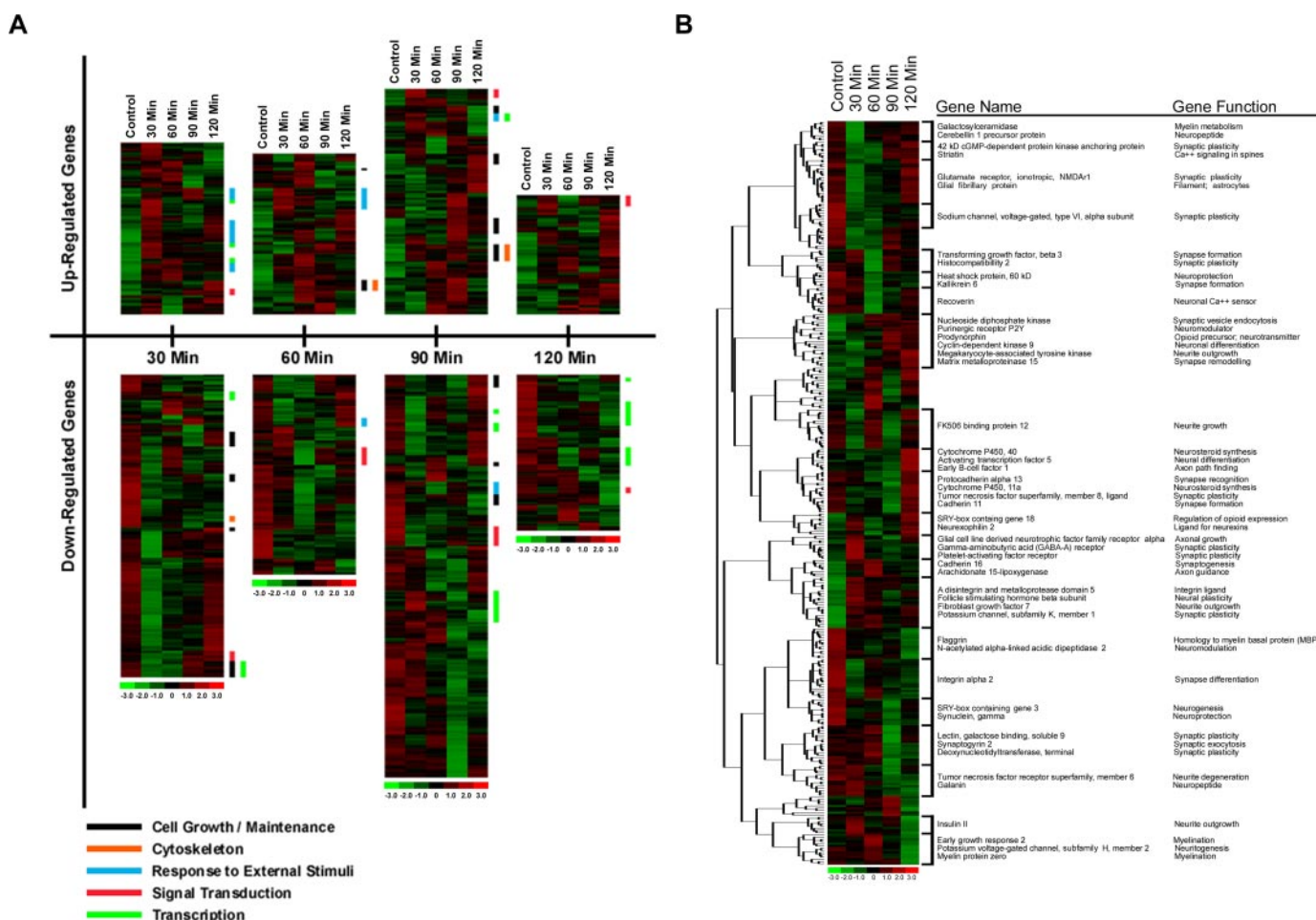
(supplemental Table 2). 55 ARGs ( $p < 0.05$ ) were LGs, which is significantly higher than the number computed from randomly sampled profiles ( $p = 1.3 \times 10^{-51}$ ). These observations indicate that the ARGs are substantially enriched for LGs.

**Global Molecular Changes after LTP Induction**—ARGs identified by the microarray analysis provide an unbiased way to assess the global molecular alterations evoked by LTP induction. We employed two approaches to assign functional categories for ARGs. In the first approach, we performed Gene

Ontology (GO) analysis on all ARGs at the  $p < 0.05$  level using the NetAffx data base. This analysis revealed that the GO functional groups of ARGs covered a wide range of cellular processes, including responses to external stimuli ( $p = 2 \times 10^{-6}$ ), signal transduction ( $p = 3.9 \times 10^{-5}$ ), transcription ( $p = 10^{-2}$ ), and regulation of cytoskeleton ( $6.3 \times 10^{-3}$ ) (Fig. 4A; supplemental Table 1). This observation indicates that tetanus-mediated LTP induction evokes complex transcriptional cascades that may ultimately lead to changes of cellu-



**FIGURE 3. Validation of microarray expression data by real time RT-PCR.** Nine ARGs at the level of  $p < 0.05$  were selected for real time RT-PCR experiments. Normalized relative expression values from four microarray and six real time RT-PCR experiments were statistically compared by  $t$  tests at the same time point.  $p$  values from  $t$  tests are given in the table. Of the 36 experimental data points, only 4 (indicated by asterisks) show significant differences between microarray and real time RT-PCR data ( $p < 0.05$ ), indicating that ~89% (32/36) of microarray data points were reproduced by real time RT-PCR. The dynamic expression profiles of individual genes from the results of microarray analysis are shown on the left. Low expression is indicated by green; high expression is indicated by red.



**FIGURE 4. Hierarchical clustering of temporal expression profiles of ARGs.** A, organization of ARGs at the  $p < 0.05$  level by hierarchical clustering. Up- or down-regulated ARGs from individual time points were organized by hierarchical clustering separately to reveal GO groups that are up- or down-regulated during LTP expression. Temporal expression clusters with over 50% of genes in the same GO groups were considered to be enriched with genes of this GO group and are indicated by colored bars. B, organization of ARGs at the  $p < 0.01$  level by hierarchical clustering. Clusters containing genes with known neural and synaptic functions are indicated by brackets.

lar and synaptic functions accompanying the expression of LTP.

To rigorously investigate the functionalities of ARGs, we also performed an extensive PubMed search to extract functional information for individual ARGs as reported in the biomedical literature. For this analysis, we focused on 336 ARGs at the  $p < 0.01$  level. Functional categories were not assigned for ARGs that were only ESTs or that had limited published information regarding their functions. By using this approach, we were able to assign functions for 261 ARGs (Fig. 4B; Table 2). Several salient features were observed through this analysis. First, many ARGs are involved in the regulation of cell surface and adhesion, extracellular matrix, cytoskeleton, cytokine and growth factor signaling, and transcription. These observations suggest that tetanus-mediated LTP induction leads to alterations in multiple molecular processes that modulate cell-cell and cell-extracellular matrix interactions, cell morphologies, and gene expression. These pathways likely work together to regulate LTP expression. Second, although certain categories (*e.g.* the cell surface/adhesion and extracellular matrix categories) were found to be both up- and down-regulated at different time points, ARGs in the same category that were up-regulated were different from those that were down-regulated (Table 2), indicating that different aspects of the same cellular processes were deliberately modulated during LTP expression. Third, ARGs of some categories were biased to be up- or down-regulated. For instance, ARGs involved in the regulation of chromatin structure were only found to be up-regulated. On the other hand, ARGs that are implicated in translation, mitochondrial energy production, and myelin metabolism and that encode proteases and their inhibitors were mainly down-regulated (Table 2). Finally, many ARGs are known to play roles in synaptogenesis, synapse differentiation, neurite outgrowth, and synaptic plasticity (Fig. 4B), supporting the notion that formation and remodeling of synapses are involved in LTP expression (39–42).

**Molecular Dynamics during LTP Expression**—Global analyses do not provide information about expression changes of individual genes associated with the temporal evolution of LTP. For example, “how do expression changes of ARGs at various time points correlate with underlying LTP-related cellular alterations?” Such a question has to be addressed by examining the functions and temporal behaviors of individual ARGs. We attempted to address such a problem by two approaches. First, we sought to organize temporal profiles for individual ARGs ( $p < 0.05$ ) by hierarchical clustering. To uncover the dynamic molecular changes during LTP expression, we performed separate hierarchical clustering analyses for up- and down-regulated ARGs at each time point (Fig. 4A). We intended to identify specific GO groups that are up- or down-regulated at different time points to help us assess the temporal molecular changes associated with LTP expression. We found that many ARGs in the same GO groups shared similar temporal expression profiles and thus were in the same expression clusters (Fig. 4A), supporting the notion that temporally clustered genes are likely involved in the same biological functions (43). Each time point was characteristically marked by specific GO clusters (Fig. 4A),

indicating distinct temporal molecular changes during LTP expression. For instance, several expression clusters of ARGs that are involved in responses to external stimuli were up-regulated at 30 and 60 min after LTP induction; these ARGs include matrix metalloproteinase, immunoglobulins, and histocompatibility proteins (supplemental Table 1). These results suggest that LTP-inducing synaptic activities turn on cascades of molecular responses for intercellular and cell-matrix interactions. These processes may play an important role in interactions between pre- and post-synaptic components during the early phase of LTP expression. ARG clusters involved in cell growth and maintenance were found to be down-regulated at 30 and 90 min and up-regulated at 60 and 90 min (Fig. 4A; supplemental Table 1), indicating an extensive regulation of these processes during the course of LTP expression. Cell growth and maintenance-related ARGs are largely different at different time points (supplemental Table 1), suggesting a temporal dynamic change of different aspects of this biological process during the evolution of LTP expression. Cytoskeleton-related clusters were found down-regulated at 30 min but up-regulated at 60 and 90 min (Fig. 4A). Not surprisingly, given the importance of the cytoskeleton in cell growth and maintenance, some cytoskeleton-, cell growth-, and maintenance-related ARG clusters were overlapped (Fig. 4A). The cytoskeleton-related ARGs may be involved in structural changes of the synapse that are associated with LTP expression. Many ARG expression clusters related to transcriptional regulation were down-regulated throughout the course of LTP expression, although a few were up-regulated at 30 min (Fig. 4A). These observations indicate that ARG-mediated transcriptional regulation is implicated in various time points through LTP expression, supporting the important role of gene expression in long lasting synaptic plasticity.

How would ARGs regulate structural and functional changes of the synapse during LTP expression? To address this problem, we analyzed the function of individual ARGs ( $p < 0.01$ ) at each time point. At 30 min, many ARGs were found to be involved in cell-cell interactions, neurite outgrowth, synapse formation, and remodeling. Among the up-regulated ARGs neuromodulin 2, matrix metalloproteinase 15, a disintegrin, metalloproteinase 5, and fibroblast growth factor 7 are included; among the down-regulated ARG CD34 and protocadherin  $\gamma$  subfamily A 10 are included. These observations indicate that at this stage of synapse formation and remodeling is a major cellular process that is extensively regulated by up- and down-regulation of specific ARGs. At 60, 90, and 120 min, many ARGs implicated in regulation of the cytoskeleton, including actin network and microtubules, were up-regulated; these include Wiskott-Aldrich syndrome homolog, myosin heavy chain 8, annexin A8, kinesin-like 1, pericentrin 2, and carcinoembryonic antigen-related cell adhesion molecule 10. Some of them are known to be involved in neurite morphogenesis and synapse formation. Other cytoskeletal regulators, including dynein heavy chain 8, erythrocyte protein band 4.2, annexin A1, and tubulin  $\alpha 4$ , were down-regulated. Given the importance of the cytoskeleton in synapse differentiation and stabilization, the regulation of these genes may be important for the structural changes of synapses during the expression of LTP. Numerous ARGs that were dif-



TABLE 2

Functional groups of ARGs ( $p < 0.01$ )

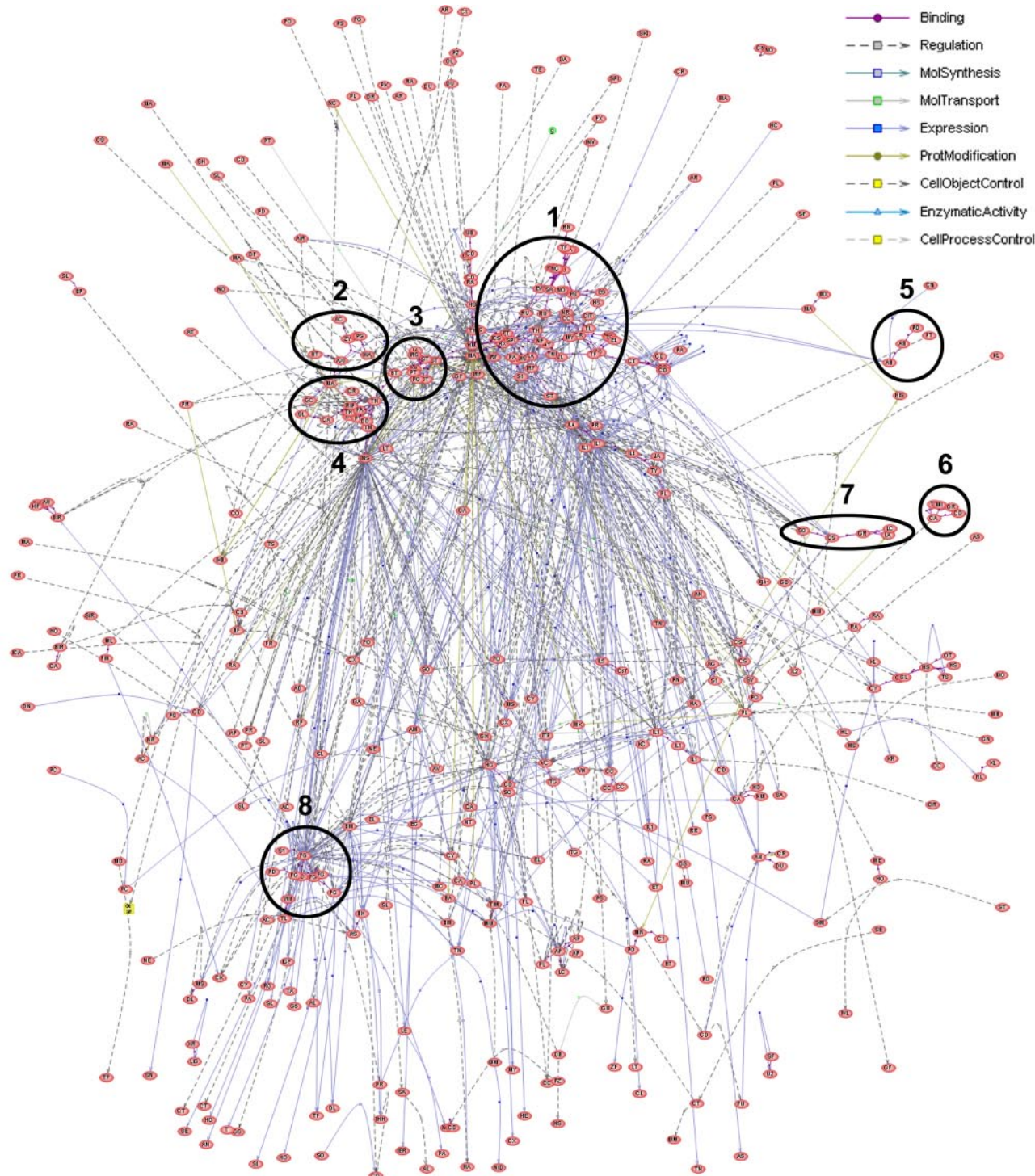
Functional groups of ARGs are shown ( $p < 0.01$ ). Microarray expression values were used to determine up- or down-regulation of the 336 statistically significant ARGs ( $p < 0.01$ ) at times 30, 60, 90, and 120 min compared with control. Gene functions were assigned based on PubMed literature searches. Total number of genes (including those not listed) in each functional group is given in parentheses. GenBank™ accession numbers are given in the parentheses in front of each gene name.

A. Up Regulated	B. Down Regulated
<b>ECM and its Regulation (6)</b>	<b>ECM and its Regulation (10)</b>
(D86332) Matrix metalloproteinase 15	(AF011450) Procollagen, type XV
(U43298) Laminin, beta 3	(X75427) Integrin alpha 2
	(M82831) Matrix metalloproteinase 12
<b>Membrane Protein / Cell Surface / Adhesion Molecule (10)</b>	<b>Membrane Protein / Cell Surface / Adhesion Molecule (17)</b>
(U22059) Disintegrin and metalloprotease domain 5	(AV276689) GPI-anchored membrane protein 1
(U56650) Neurexophilin 2	(AF016271) Cadherin 16
(L38422) CEA-related cell adhesion molecule 10	(AV346841) Cadherin 11
	(AI156095) CEA-related cell adhesion molecule 11
	(X75427) Integrin alpha 2
<b>Metabolism of Neurosteroid Hormone (0)</b>	<b>Metabolism of Neurosteroid Hormone (5)</b>
	(AB006034) Cytochrome P450, 40
	(U49861) Deiodinase, iodothyronine, type I
<b>Cytokine / Growth Factor / Receptor (16)</b>	<b>Cytokine / Growth Factor / Receptor (8)</b>
(Z22703) Fibroblast growth factor 7	(L41352) Amphiregulin E5F receptor ligand
(U81451) Estrogen receptor 2 (beta)	(X75337) Interleukin 2 receptor
(M86672) Interleukin 12a	
<b>Other Receptors / Signaling (13)</b>	<b>Other Receptors / Signaling (15)</b>
(AF108020) Edg5 G-protein coupled receptor	(AF011421) Vomeranase 2, receptor, 11
(AF011424) Vomeranase 2, receptor, 14	(U47281) Bdkrb G-protein coupled receptor
<b>Ion Channel (4)</b>	<b>Ion Channel (4)</b>
(AF033017) Potassium channel, subfamily K, member 1	(AF042066) R-type calcium channel alpha 1 subunit
(AJ002390) Annexin A8	(L36179) Sodium channel, voltage-gated, type VI
<b>Transcription Factor / Regulation (13)</b>	<b>Transcription Factor / Regulation (21)</b>
(L35032) SRY-box containing gene 18	(AL009226) Ring finger protein 3
(M87802) Homeo box D1	(U03873) Paired related homeobox 1
(AB000096) GATA binding protein 2	(AA866668) SRY-box containing gene 3
	(X69019) Homeo box C4
<b>Translation (1)</b>	<b>Translation (5)</b>
(AW048363) Ribosomal protein L31	(M20632) Ribosomal protein S2
	(AV160244) Eukaryotic translation initiation factor 4A1
<b>Neurotransmitter Receptor / Neuromodulator (3)</b>	<b>Neurotransmitter Receptor / Neuromodulator (5)</b>
(AV382264) Prodynorphin	(AW123901) Glutamate receptor, ionotropic, NMDA-like 1A
	(X61448) Cerebellin 1 precursor protein
<b>Regulation of Cytoskeleton (15)</b>	<b>Regulation of Cytoskeleton (9)</b>
(Aj223293) Kinesin-like 1	(AV291613) Tubulin, alpha 4
(U42471) Wiskott-Aldrich syndrome homolog (human)	(AA789879) Dynein heavy chain 8
(AV242220) Myosin, heavy polypeptide 8	
<b>Mitochondrial / Energy Production (2)</b>	<b>Mitochondrial / Energy Production (8)</b>
(M96265) Galactose-1-phosphate uridyl transferase	(L11163) Acetyl-Coenzyme A dehydrogenase, short chain
	(AF080469) Glucose-6-phosphatase
<b>Proteases and their Inhibitors (1)</b>	<b>Proteases and their Inhibitors (9)</b>
(M57626) Mast cell protease 6	(AA762212) Serine (or cysteine) proteinase inhibitor
	(L00653) Mast cell protease 7
	(M68898) Mast cell protease 5
<b>Immuno-response Proteins / Oxidative Stress / Neuroprotection / Cell Death (13)</b>	<b>Immuno-response Proteins / Oxidative Stress / Neuroprotection / Cell Death (13)</b>
(V00793) Immunoglobulin heavy chain 4	(AI173145) CD34 antigen
(AV224353) Histocompatibility 2	(AB016589) Inhibitor of kappaB kinase epsilon
(AF030001) Major histocompatibility locus	(AF017255) Synuclein gamma
<b>Myelin-related Proteins (0)</b>	<b>Myelin-related Proteins (6)</b>
	(D38557) Galactosylceramidase
	(M62860) Myelin protein zero
<b>Chromatin Structure (4)</b>	<b>Chromatin Structure (0)</b>
(AJ132771) Chromatin assembly factor 1, subunit A	
(AA097203) Actin dependent regulator of chromatin	

ferentially up- or down-regulated at various time points are involved in transcription regulation and signaling transduction. Their roles in regulation of synaptic structures and functions are less clear and most likely indirect.

*Molecular Pathways with Enriched ARGs*—One goal of this study was to characterize the synaptic activity-modulated molecular pathways that may be implicated in LTP regulation. We therefore investigated the genetic networks and pathway

Clusters of LTP Regulatory Genes on Chromosomes



	Cluster number							
	1	2	3	4	5	6	7	8
Function	Transcription regulation	Cytoskeletal adaptor proteins	Signal transduction	Cell apoptosis	Cellular transport	Assembly of MHC class I complex	Cytokine signaling	FGF signaling
P-value	0.015131	0.050441	0.000007	0.040260	0.013733	0.026816	0.001293	0.006661
Examples of Cluster Members	CREB1	ACTN1	BTK	CASP2	ABCB1	CALR	CSF1R	FGF2
	CREBBP	AXIN1	JAK2	CFLAR	ABCC2	MICB	GRAP2	FGFR2
	CITED1	WAS	PRLR	CRADD	PTAFR	TAPBP	LAT	S100A4
		ZYX	STAT3	FADD			SOCS1	SDC1
				TNFRSF1A				



structures of ARGs using the GenMAPP data base. If genes of a pathway are significantly enriched as ARGs, it is likely that this pathway is involved in the regulation of LTP expression. For example, we found that genes in the MAPK signaling pathway, including *R-Ras*, *v-Raf*, *MEKK1*, and *MEKK2*, were enriched in the ARG list ( $p = 10^{-6}$ ). This observation is consistent with the importance of the MAPK signaling pathway in LTP (44). In addition, we observed that genes the Wnt signaling pathway were also enriched ( $p = 10^{-6}$ ). Guided by this finding, we specifically tested the potential role of Wnt signaling in synaptic plasticity, and we found that Wnt signaling plays a critical role in regulating LTP (45). These results together support a functional significance of the molecular pathways with enriched ARGs in the regulation of LTP.

We also used the PathwayAssist data base (Stratagene) to determine the physical and functional interactions among ARG proteins to identify ARGs that are involved in the same biological processes. This analysis revealed many physically or functionally interacting clusters of ARGs implicated in specific biological processes (Fig. 5). For example, ARGs in cluster 2 in Fig. 5 encode cytoskeletal adaptor proteins that are involved in the regulation of the cytoskeleton, Wnt signaling, and anchoring of the CaMKII protein and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors in the postsynaptic density. On the other hand, many ARGs in cluster 1 are transcription factors, including CREB1, CREB-binding protein (CREBBP), and CBP/p300 (CITED1) that are known to play important roles in long term synaptic plasticity. Some of other ARG clusters are related to cellular transport, cell signaling, fibroblast growth factor signaling, lipid metabolism, mitochondrial oxidative metabolism, and assembly of major histocompatibility complex class I complex. One cluster (cluster 4) is related to cell apoptosis (Fig. 5); ARGs in this cluster may mediate the toxic effects of excessive synaptic activation that results in neuronal damages.

**Chromosomal Clustering of ARGs**—The above results indicated that the LTP induction evokes dramatic genomic responses by altering the expression of a large number of ARGs, which may be functionally related to adaptive changes of the neuron, including LTP. To initiate the understanding of the chromatin mechanism underlying activity-regulated ARG expression, we sought to determine how ARGs are organized in the mouse genome. Toward this end, we examined the chromosomal distribution patterns of ARGs. After mapping ARGs ( $p < 0.05$ ) that had available positional information, we observed that ARGs were concentrated at specific chromosomal regions rather than a random distribution (Fig. 6A). To rigorously confirm this observation, we used a statistical method described previously to test the significance of ARG clustering (34). All tandemly duplicated genes were excluded in the clustering analysis to avoid their contributions to the formation of gene clusters. Statistical comparisons of the ARG patterns with 1000 patterns from randomly profiled genes showed that, at an inter-gene interval of 500 and 1000 kb, ARGs displayed a strong clustering pattern, which was significantly

different from the random profiles ( $p = 5.27 \times 10^{-4}$ ; Fig. 6A). 79.4% (927/1167) of ARGs with known position information were clustered (red bars in Fig. 6A). Although some ARGs formed small clusters with two to four genes, many ARGs formed clusters with more than five genes (Fig. 6B). These observations suggest that there are activity-responsive chromatin domains that host ARGs.

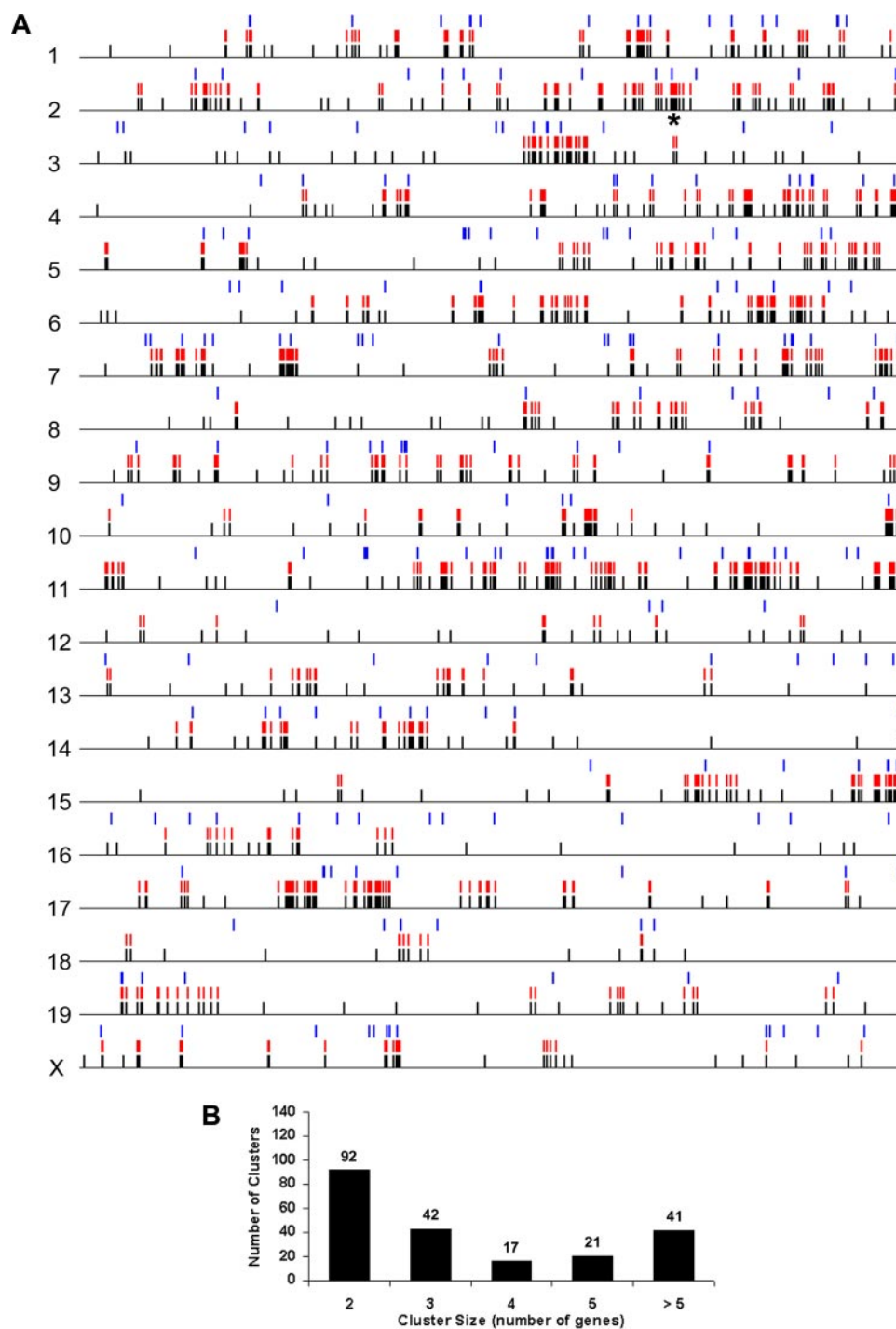
**Functional Correlations of Clustered ARGs in LTP**—Results described above revealed a chromosomal clustering of LTP-regulated ARGs, although we did not observe structural homologies among ARGs in the same clusters. We next sought to investigate whether clustered ARGs are functionally correlated in cellular processes regulated by synaptic activation. GO analysis of all clustered ARGs revealed that they are significantly enriched in a number of GO terms, including signal transducer activity ( $p = 7 \times 10^{-6}$ ), response to external stimulus ( $p = 1.6 \times 10^{-5}$ ), and transcription factor activity ( $p = 4.2 \times 10^{-3}$ ). However, examination of proteins encoded by individual ARGs in the same clusters failed to detect obviously correlated molecular functions or activities. As ARGs are regulated by LTP induction, we hypothesized that clustered ARGs may be functionally correlated by coordinating LTP expression. To test this hypothesis, we used bioinformatics and experimental approaches to investigate whether individual ARGs of distinct clusters are involved in LTP. For the initial analysis, we focused on an ARG cluster on chromosome 2, which consists of seven known genes and one EST (Fig. 7A; see supplemental Table 4 for their expression changes after LTP induction). ARGs in this cluster do not show structural homologies, and their protein products have apparently different molecular functions.

A search of the PubMed data base indicated that four ARGs in this cluster had been shown to be implicated in LTP expression by previous studies (Fig. 7B). These include cytokine interleukin 1 (46–48), neuromodulators prodynorphin (49) and arginine vasopressin (50), and receptor protein-tyrosine phosphatase  $\alpha$  (51, 52). The potential involvement of other ARGs in this cluster, including transglutaminase 3, CDC25B, and Src homology 2 domain-containing protein-tyrosine phosphatase substrate 1 (SHPS-1; Ptpns1), in synaptic plasticity had not been studied. Therefore, we performed experiments to determine whether they are also involved in LTP.

**Transglutaminases**—Transglutaminases are  $\text{Ca}^{2+}$ -dependent enzymes that catalyze protein cross-linking by covalent bonds (53). This class of enzymes is widely distributed in many tissues, including the brain and is found in the nerve termini and synapses (54). In the nervous system, a variety of regulatory activities of transglutaminases has been suggested. These include stabilization of synapses, synapse formation and differentiation, neurotransmitter release, and modulation of adenylyl cyclase and CREB activation (54–58). To examine if the transglutaminase is involved in the regulation of LTP, we determined the effect of cystamine, a specific antagonist of the enzyme (59, 60), on LTP expression. The result showed that cystamine impaired the expression of both early and late phase

**FIGURE 5. Functional clustering of ARGs.** ARGs ( $p < 0.05$ ) are organized according to their functional relationship, using the PathwayAssist data base (Stratagene). Functionally related ARGs are placed in close proximity. The functional relationship is assessed based on the physical binding and regulatory and other molecular interactions between ARGs or their coding proteins. Several examples of functional clusters of ARGs are circled and further described in the table.

## Clusters of LTP Regulatory Genes on Chromosomes



**FIGURE 6. Chromosomal clustering of ARGs and LGs.** *A*, the distribution of ARGs identified from microarray experiments ( $p < 0.05$ ; black bars), statistically significantly clustered ARGs (red bars), and previously identified genes implicated in LTP regulation (LGs) (blue bars) in the mouse genome. Statistical analysis of chromosomal gene clustering was performed according to a previously described method (34). *B*, histogram of the cluster size of ARGs. Note: because of a limited resolution of the physical map in Fig. 3A, some clustered genes that are closely located on chromosomes may appear as single bars.

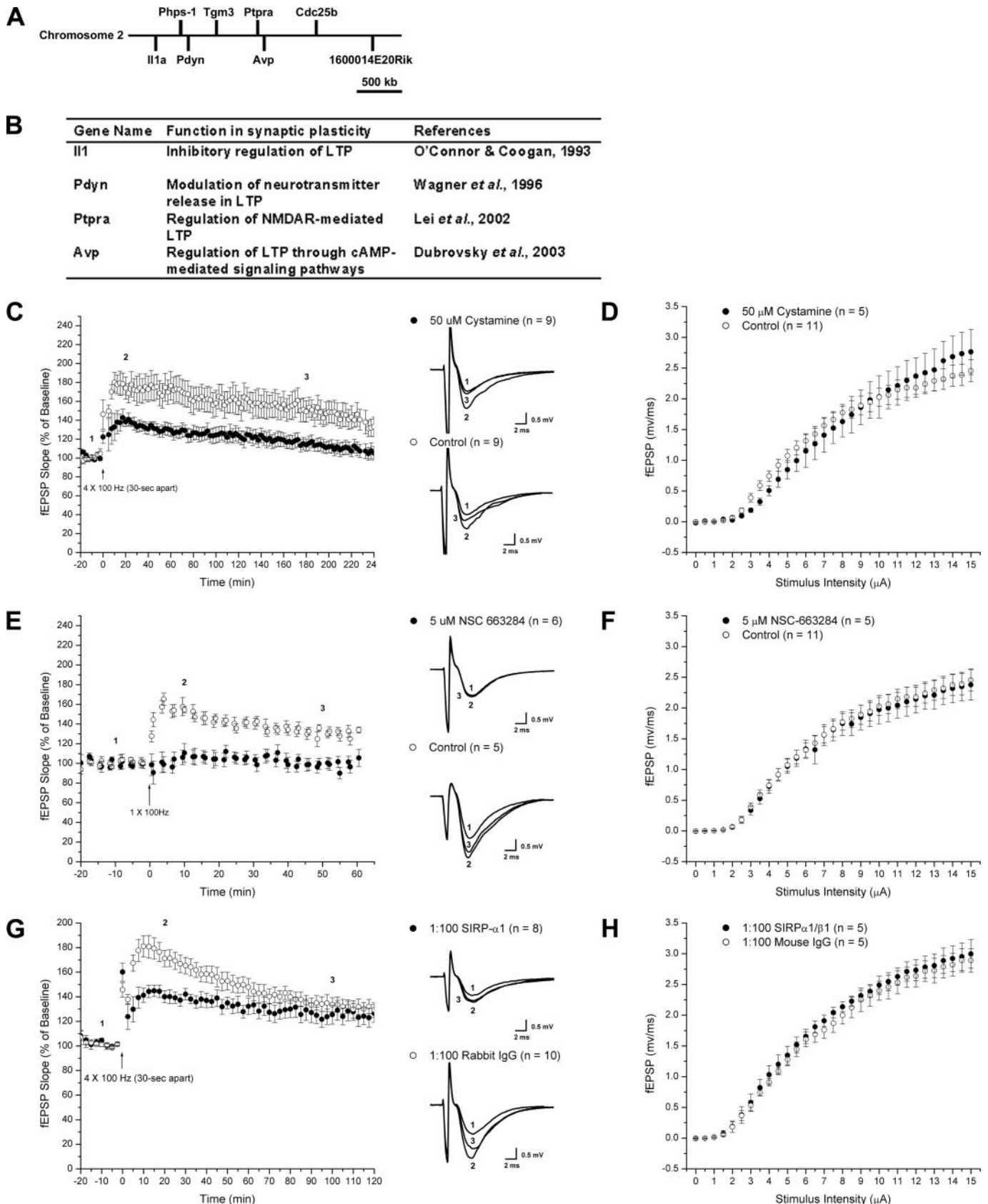
LTP induced in the perforant pathway (Fig. 7C). On the other hand, the basal synaptic transmission was not affected by cystamine (Fig. 7D). These observations indicate the activity of transglutaminases is essential for LTP expression. Consistent with this notion, previous studies suggested that activation of *N*-methyl-D-aspartate receptors led to the increase of transglutaminase activities and that LTP induction by tetanic

stimulations was accompanied with transglutaminase-catalyzed protein cross-linking (61, 62).

*CDC25B*—*CDC25B* is an oncogene encoding a tyrosine phosphatase that regulates cell cycles by controlling the activity of CDC2/cyclin B kinase (63). Previous studies had shown that the CDC25B protein is also expressed in terminally differentiated neurons of the adult brain (64). An abnormal up-regulation of CDC25B in post-mitotic neurons had been suggested to be involved in the development of Alzheimer disease (64). The fact that the expression of CDC25B was modulated by tetanus-evoked synaptic activities suggested that this cell cycle regulator may function in postmitotic neurons, possibly during synaptic plasticity. To determine the possible role of CDC25B in LTP, we used the NSC663284 compound, a specific inhibitor of CDC25B (65), to block its activities in hippocampal slices. Our results showed that NSC663284 was able to completely block LTP induction by one or four trains of tetanic stimulations (Fig. 7E; data not shown). We also observed that NSC663284 did not affect basal transmission (Fig. 7F). These results indicate that, in addition to its well known activities in cell cycle regulation, the CDC25B tyrosine phosphatase plays a critical role in LTP expression.

*SHPS-1*—SHPS-1 (Ptpns1) is a synaptic adhesive Ig-like transmembrane glycoprotein that belongs to the family of signal regulatory proteins and is involved in receptor tyrosine signaling (66). It has an extracellular domain containing three Ig-like repeats, a transmembrane domain, and an intracellular domain that can interact with multiple proteins to initiate signaling cascades, including the MAPK pathway (66). Various mitogens such as epidermal growth factor and platelet-derived growth factor can activate SHPS-1 signaling (66). Furthermore, SHPS-1 can regulate cell-cell interaction through bi-directional signaling by interacting with the integrin-associated protein (IAP/CD47), a transmembrane glycoprotein that is present at the synapse (67). PHPS-1 is associated with synaptic regions and can promote neurite outgrowth and filopodial extension in





**FIGURE 7. Involvement of clustered ARGs in LTP.** *A*, physical map of an ARG cluster on the mouse chromosome 2. *B*, several ARGs in this cluster were reported in previous studies to be involved in LTP. *C*, the transglutaminase-specific inhibitor cystamine impaired the early and late phase LTP. *D*, cystamine had no effects on the basal synaptic transmission as determined by the input-output curve. *E*, the CDC25B-specific inhibitor NSC663284 blocked the induction of early as well as late phase LTP. *F*, NSC663284 had no effect on the input-output curve. *G*, the specific anti-SHPS-1 antibody impaired early phase LTP but not late phase LTP. *H*, the anti-SHPS-1 antibody did not affect the input-output curve.

growth cones. Potential roles of PHPS-1 in synapse formation and synaptic stability and function have been postulated (68). We sought to determine the possible involvement of PHPS-1 in LTP expression. Toward this end, we used a specific anti-PHPS-1 antibody that binds the extracellular domain to block PHPS-1 functions, presumably by inhibiting the interaction with its ligands. Previous studies demonstrated that anti-PHPS-1 antibodies were able to specifically block PHPS-1-mediated neurite extension (69). Our results showed that incubation of hippocampal slices with anti-PHPS-1 antibody attenuated early phase LTP but had no obvious effects on late phase LTP (Fig. 7G). This antibody also did not affect basal synaptic transmission (Fig. 7H). These observations suggest that PHPS-1 plays a specific role during the expression of early phase LTP.

Results from our LTP experiments and previous studies from others described above (46–52) indicate that all known genes in this ARG cluster are involved in the regulation of LTP. It was of interest to note that inhibition of the transglutaminase, CDC25B, and PHPS-1 led to different LTP impairments that were characteristic for individual proteins (Fig. 7). Specifically, blocking transglutaminases resulted in decreased LTP during both early and late phases, whereas inhibition of PHPS-1 only affected early phase LTP. On the other hand, blocking CDC25B completely inhibited the induction of LTP. These differential effects suggest that individual ARGs in this cluster may regulate different physiological processes underlying LTP and together coordinate LTP expression. Therefore, although proteins encoded by ARGs in this cluster are different in their molecular properties, they all contribute to LTP expression.

Next, we sought to investigate if ARGs in other clusters are involved in LTP regulation. Toward this end, we analyzed another cluster on chromosome X. This cluster consists of two ARGs, v-Raf and tissue inhibitor of metalloproteinase 1 (TIMP1). v-Raf is an important regulator of the MAPK signaling pathway that is critical for LTP expression (44), although no LTP-related roles have been reported for TIMP1. We showed that applications of purified TIMP1 proteins potentiated early phase but not late phase LTP (supplemental Fig. 1), indicating a role of this ARG in LTP. Interestingly, this ARGs cluster is located close to Elk1, which is involved in LTP expression (supplemental Fig. 1) (70). Furthermore, we also performed a focused search of the published literature on ARGs in selected clusters and found that a number of clusters are enriched with genes that play a role in LTP expression (supplemental Table 3). Together, these observations suggest that clustered ARGs are involved in LTP.

**Clustering of LTP-related Genes on Chromosomes**—The fact that ARGs in multiple clusters described above are involved in LTP regulation suggests the possibility of chromosomal clustering of other LGs in the genome. To investigate this possibility, we searched the PubMed data base for genes that had been reported previously to be involved in LTP. All papers from this search were read to identify genes with experimental evidence for their roles in LTP. As a result, 290 homologs of LGs were found on the U74Av2 gene chip (supplemental Table 2). As we only used LTP and Gene as key words in the PubMed search, it is possible that there were other LGs that were missed. All iden-

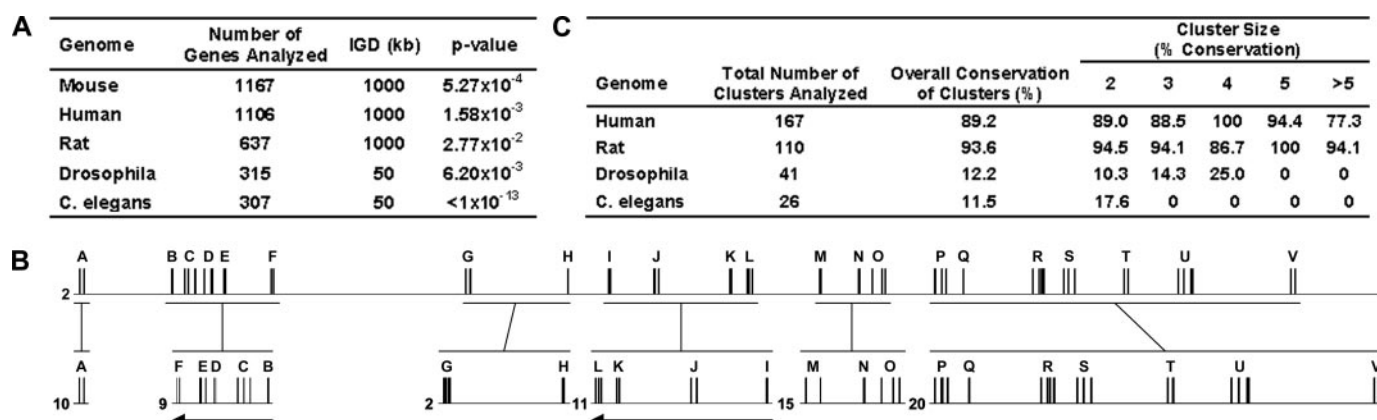
tified U74Av2 LGs were then mapped onto mouse chromosomes. Although LGs used in this genomic distribution analysis only represent 0.79% of total mouse genes, instead of a random distribution, they formed a clear pattern of clustering on chromosomes (*blue bars* in Fig. 6A). Statistical analysis showed that the clustering of LGs was significantly higher than that from 1000 random profiles ( $p = 1.69 \times 10^{-3}$ ). These observations support the idea that, similar to ARGs identified by the microarray experiments, LGs also tend to cluster on chromosomes.

**Association of LTP-related Genes with ARG Clusters**—To assess the involvement of other ARG clusters throughout the genome in LTP, we sought to determine whether ARG clusters are associated with LGs. We reasoned that associations between ARG clusters and LGs would provide suggestive evidence for the involvement of ARG clusters in LTP. A comparison of the mouse genomic map of LGs with that of ARGs indicated that many of the LGs were within or close to ARG clusters (Fig. 6A). Statistical analysis using the hypergeometric cumulative distribution method suggested that LGs were significantly associated with ARG clusters ( $p < 0.001$ ). Of 228 ARG clusters, 42.5% of them were associated with LGs. On the other hand, 60% of LGs with identified chromosomal positions were associated with ARG clusters. As our microarray analysis only covers one-third of the mouse genome, the rest of LGs that were not found to associate with the current ARG clusters may associate with other unknown clusters. The significant association between LGs and ARG clusters supports the idea that ARG clusters throughout the genome are potentially involved in LTP.

**Clustering of ARG Homologs in Other Genomes**—If chromosomal clustering of ARGs is important for synaptic plasticity, there would be a selective pressure during evolution to maintain ARG clusters. To test this hypothesis, we first performed clustering analysis of ARG homologs in other genomes to investigate if they also form clusters. ARG homologs were identified basing on Affymetrix NetAffx Analysis Center data base. When multiple homologs were found for the same mouse ARG, only the one that showed the highest homology was used for clustering analysis to avoid potential contributions of multiple homologs to clustering. Similar statistical methods as used above were used to determine the significance of clustering. We found that although only a portion of mouse ARGs have unambiguously identifiable homologs, these homologs were observed to significantly cluster in human, rat, *Drosophila*, and *Caenorhabditis elegans* genomes (Fig. 8A). These results indicate that clustering of ARGs on chromosomes is an evolutionarily conserved phenomenon.

We also investigated if mouse ARG clusters are maintained in other genomes as syntenic regions. Only ARGs that had identified homologs in other genomes and formed clusters in the mouse genome were used in this analysis. As exemplified in Fig. 8B, ARG clusters on mouse chromosome 2 are largely preserved in syntenic regions of human chromosomes. The relative positions of individual ARGs in a cluster in the mouse genome were also preserved in the corresponding human syntenic regions, although some syntenic regions were reversed in human chromosomes (Fig. 8B). The overall conservation rate of





**FIGURE 8. Clustering of ARG homologs and conservation of mouse ARG clusters as syntenic regions in other genomes.** *A*, clustering analysis of mouse ARGs and their homologs in other genomes. ARGs and their homologs are significantly ( $p < 0.05$ ) clustered in mouse and other genomes. *IGD*, intergenic distance. *B*, schematic diagram showing the ARGs clusters on the mouse chromosome 2 (upper panel) and the maintenance of these clusters in syntenic regions of the human genome (lower panel). Individual ARG clusters are labeled with letters A–V; ARG clusters on different human chromosomes are indicated by various colors and numbers (lower panel); reversed ARG clusters are indicated by arrows. *C*, conservation rates of mouse ARG clusters in the human, rat, *Drosophila*, and *C. elegans* genomes. The percentage of mouse ARG clusters maintained in other genomes was calculated. Only clustered mouse ARGs that had identifiable homologs in other genomes were used for this analysis. A mouse ARG cluster was considered conserved in another genome if 75% of the ARGs in the mouse cluster was found at the same chromosomal locus in the same topographic order.

mouse ARG clusters in the human genome is 89.2% (Fig. 8C). Similarly, mouse ARG clusters are also highly conserved in the rat genome (Fig. 8C). These observations indicate ARG clusters are evolutionarily conserved in mammalian genomes. Relatively low conservation rates for ARG clusters were observed in *Drosophila* and *C. elegans* genomes (Fig. 8C). Because only a small number of clustered ARG homologs were identified from these invertebrate genomes, these low conservation rates may partly due to underestimations.

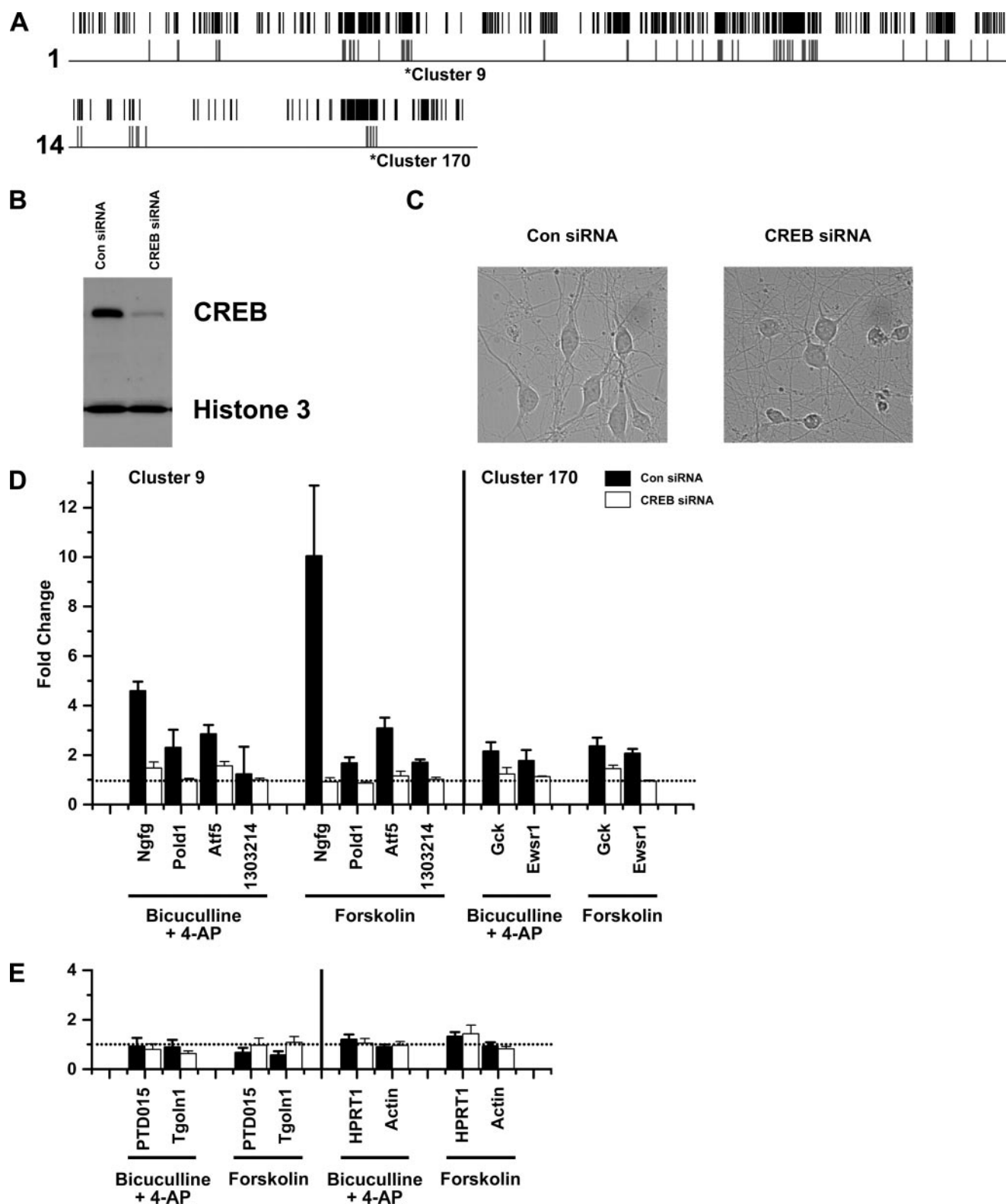
**CREB-regulated ARG Clusters**—Why are ARGs organized into clusters on chromosomes? One possibility is that chromosomal clustering of ARGs facilitates the co-regulation of these functionally related genes by synaptic activation. Therefore, we sought to investigate the mechanism by which synaptic activity co-regulates ARGs in the same cluster. We hypothesized that ARGs in a cluster could be co-regulated by sharing the same transcription factor and its regulatory elements. To test this hypothesis, we specifically focused on the potential role of CREB in coordinating the activity-dependent expression of clustered ARGs. Recently, the chromosomal pattern of CREB-binding loci in the rat genome was reported (71). We found that many rat ARG clusters are located in the same chromatin regions with enriched CREB-binding loci (Fig. 9A). This observation is consistent with the idea that ARGs in clusters in the chromatin domains enriched with CREB-binding sites may be co-regulated by CREB. To directly test this idea, we used the siRNA approach to knock down CREB in primary mouse cortical neurons and then determined the effect of down-regulation of CREB on activity-induced transcription of ARGs in two ARG clusters in chromatin domains enriched with CREB-binding sites (Fig. 9; see supplemental Table 4 for their expression changes after LTP induction). By using a CREB-specific siRNA, we successfully suppressed CREB protein expression in primary cortical neuron cultures (Fig. 9B). CREB knockdown did not affect neuron morphology (Fig. 9C). We then elicited synaptic activity of cultured neurons with bicuculline plus 4-aminopyridine (72). mRNA was extracted 1 h after bicuculline appli-

cation for quantitative real time RT-PCR analysis. Results indicated that bicuculline applications induced transcription from most ARGs of the clusters and that this transcriptional activation was abolished after CREB knockdown (Fig. 9D). To conclusively demonstrate that CREB siRNA blocked the activation of clustered ARGs via CREB down-regulation, we also determined the effect of CREB siRNA on forskolin-stimulated transcription, because forskolin activates gene transcription via the cAMP/cAMP-dependent protein kinase/CREB pathway. We found that CREB siRNA blocked forskolin-induced transcription of the clustered ARGs (Fig. 9D). In contrast, bicuculline and forskolin failed to activate ARGs at domains without CREB-binding loci and housekeeping genes, and CREB siRNA did not affect the basal transcription of these genes (Fig. 9E). These observations together suggest that the activity-induced transcription of ARGs in the clusters associated with CREB-binding sites is co-regulated by CREB.

## DISCUSSION

**ARGs and LTP-related Molecular Processes**—We have used DNA microarray techniques to characterize ARGs that alter their expression during the course of tetanus-induced LTP. Although numerous previous studies characterized genes that respond to neuronal activities, especially the group of IEGs (11, 12, 24), the time course microarray analysis reported here identified ARGs that are regulated by LTP induction and thus likely relevant to LTP maintenance. Indeed, the ARG list includes many previously reported LTP-related genes. The functional significance of the ARGs in LTP is also supported by the experiments on transglutaminase, CDC25B, SHPS-1, and TIMP1, whose roles in LTP were not known previously (Fig. 7). Collectively, ARGs identified in this study provide an overview of molecular processes that potentially underlie LTP expression. The ARG-associated processes are complicated, including signal transduction, transcription regulation, and modulation of synaptic structure and functions (Figs. 4 and 5; Table 2; supplemental Table 1). These findings indicate complex molecular

## Clusters of LTP Regulatory Genes on Chromosomes



**FIGURE 9. CREB and activity-induced co-activation of clustered ARGs.** *A*, distribution patterns of CREB-binding sites (*upper panel*) and clustered ARGs (*lower panel*) on rat chromosome 1 and 14. Two ARG clusters for analysis of activity-induced CREB-dependent transcription (*D*) are indicated by asterisks. *B*, Western blot of cultured cortical neurons transfected with control or CREB siRNAs. Note the CREB knockdown mediated by specific CREB siRNA. *C*, differential interference contrast images of cultured neurons transfected with control or CREB siRNAs. *D*, quantitative real time RT-PCR analysis of CREB-dependent transcription of ARG clusters (labeled in *A*) at domains with enriched CREB-binding loci. Presented are bicuculline- or forskolin-induced fold changes of ARG mRNAs in neurons transfected by control (black bars) or CREB (white bars) siRNAs. Fold changes were calculated by comparing stimulated and unstimulated neurons. Shown are summary data from six independent experiments. Note that bicuculline- and forskolin-induced mRNA up-regulation of clustered ARGs was diminished by CREB siRNA. *E*, effect of CREB siRNA on ARGs (PTD015, chromosome 7; Tgln1, chromosome 6) at domains not with enriched CREB-binding loci and on housekeeping genes (*HPRT1* and actin). Note that these genes were not activated by bicuculline and forskolin and that CREB siRNA did not affect their basal transcription.



cascades underlying LTP expression and maintenance. Consistent with this notion, LTP-related genes that were identified by previous studies are also involved in many different molecular processes (73, 74). We expect that some of ARG-associated processes are essential for LTP expression; many others may have regulatory functions.

ARGs provide molecular supports for proposed critical cellular changes associated with LTP. For instance, structural changes at the synapse and new synapse formation are thought to be critical for longer lasting LTP (10, 42). Consistent with this notion, a significant number of ARGs are involved in neurite outgrowth and synapse formation (Fig. 4B). In addition, many ARGs are implicated in the regulation of cytoskeletons and cell-cell or cell-extracellular matrix interactions (Figs. 4 and 5; Table 2). Activity-regulated expression of these genes may directly contribute to the synaptic remodeling or formation of the synapse, because some ARG proteins that regulate cell-cell/extracellular matrix interactions and cytoskeletons, such as the SHPS-1 and transglutaminase, are known to be enriched at the synaptic regions.

ARGs also suggest novel molecular processes underlying LTP. For example, CDC25B is an ARG that is a well known cell cycle regulator (63), but our results suggest that it plays a role in the regulation of synaptic plasticity (Fig. 7). In addition, we discovered previously unknown roles for other ARGs such as SHPS-1, transglutaminases, and TIMP1 in LTP (Fig. 7; supplemental Fig. 1). We expect that further investigation of the ARGs reported here will generate new knowledge about LTP-related molecular pathways.

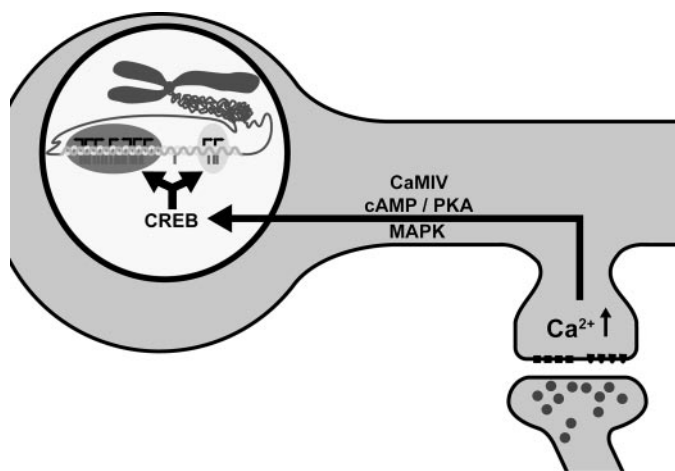
We investigated the network and pathway structure of ARGs to identify molecular pathways with enriched ARGs, because such ARG-rich pathways are likely critical for LTP. We found specific known LTP-related pathways, such as the MAPK signaling, were enriched with ARGs. In addition, we also identified novel ARG-rich molecular processes that potentially regulate synaptic plasticity. One of such novel pathways is the Wnt signaling pathway. Although the involvement of the Wnt signaling pathway in synaptic plasticity had not been known previously, many Wnt signaling-related genes were ARGs. Our subsequent analyses revealed a critical role of this signaling pathway in regulation of hippocampal LTP (45). Taken together, the above findings indicate that identification and characterization of ARG-rich pathways are fruitful approaches to obtain insights into the novel molecular mechanisms underlying LTP.

**Chromosomal Clustering and Transcriptional Regulation of ARGs**—One striking finding from this study is the chromosomal clustering of ARGs. Previous studies indicated that genes co-expressing in the same tissue tend to cluster at chromosomal domains (34, 75–77), but the biological significance of gene clustering was not clear. In prokaryotes, genes involved in the same biochemical pathway are often organized into operons, in which the clustered members are co-regulated by the same promoter (78). Similar organizations of functionally related genes are not found in higher organisms except *C. elegans* (79), although rare clusters of homologous genes that arose by duplication and divergence (e.g. the  $\beta$ -globin and Hox gene clusters) do exist (80). On the other hand, the lack of operon-like structures in mammals does not preclude the possibility

of a structured genomic organization related to biological functions. We found clustered ARGs are functionally correlated in LTP regulation, although they apparently do not share the same molecular functions. Furthermore, previously identified LTP-related genes are also clustered on chromosomes and associated with ARG clusters. These findings indicate that genes that respond to synaptic activities and are functionally correlated in synaptic plasticity are clustered in the genome. As suggested by the chromosomal clustering of ARG homologs and the conservation of ARG clusters in different genomes, there is a selection pressure to maintain these gene organizations during evolution. Together, these lines of evidence strongly argue for a biological relevance of ARG clustering. Interestingly, memory consolidation genes were reported to concentrate on specific chromosomes (20).

What is the functional significance of chromosomal ARG clustering for LTP-related transcriptional regulation? One possibility is that clustering of ARGs facilitates transcriptional coordination of the functionally related genes during LTP. Because LTP induction regulates the expression of numerous ARGs, the coordinated expression of ARGs is likely critical for LTP. For instance, the expression of some ARGs involved in the same molecular process of LTP may need to be temporally coordinated, whereas the level of certain ARG products that interact may need to be coordinated in a stoichiometric manner. Clustering of ARGs on chromosome may provide a genomic platform for the coordination of ARG transcription during LTP. We propose that chromosomal clustering may facilitate coordinated transcription of different ARGs from the following aspects. First, clustering provides a mechanism to reduce the burden of unpacking of DNA by decreasing the number of chromatin loci that need to be “opened” for transcription of ARGs during LTP. Given the extensive DNA compaction in the nucleus and the large number of LTP-related ARGs, such a strategy for simplifying DNA unpacking is probably important. Second, recent studies indicated that active genes in chromatin loops are dynamically organized into the transcription factories in the nucleus (81, 82). Compared with a dispersed distribution, clustering of functionally related ARGs on chromosomes would dramatically facilitate the formation of such transcriptional organization. In fact, given the large number of ARGs regulated by LTP induction, such a chromosomal compartmentalization of ARGs is likely necessary to avoid the package of numerous chromatin loops in transcription factories. Third, an open chromatin loop usually contains multiple genes that are potentiated for transcription. If there is only one ARG in an open chromatin loop, other potentiated genes may not be relevant to LTP. Even if the expression of these irrelevant genes is not harmful for LTP expression, it would be a waste to transcribe them. From this viewpoint, clustering of ARGs in chromatin domains may provide a genomic mechanism to reduce nonspecific transcription from genes that are irrelevant to LTP.

Previous studies postulated a role of chromatin structures in coordinating transcriptional co-regulation of clustered genes (34, 76, 83). We propose here a CREB-mediated mechanism by which activity co-regulates the transcription of ARGs in the same cluster. In support of such a mechanism, we find that



**FIGURE 10. Diagram showing the co-regulation of clustered ARGs by CREB in the open chromatin domain.** Synaptic activity activates CREB via MAPK, cAMP/cAMP-dependent protein kinase (PKA), or CaMKIV signaling pathways. Active CREBs are recruited to ARG clusters in open chromatin domains unpacked by mechanisms stimulated by synaptic activity (not shown). CREBs are concentrated on open chromatin domains harboring clustered ARGs and CREB-binding sites. In this model, we emphasize the cooperation of chromatin structure and CREB in co-regulation of clustered ARGs. Note the double helix, open chromatin. Curved arrows, ARGs; bars, CREB-binding sites; ovals, local CREB shared by clustered ARGs.

many ARG clusters are located in chromosomal domains enriched with CREB-binding loci and that the activity- and forskolin-induced transcription of the clustered ARGs depends on CREB (Fig. 9). One potential consequence for clustering CREB-regulated ARGs is that CREB and its regulatory elements can be shared by multiple ARGs in the same cluster. Furthermore, clustering CREB-regulated ARGs in chromosomal domains enriched with CREB-binding loci may increase local CREB concentration (Fig. 10). As such, the response sensitivity of ARGs in the cluster to CREB activation may be enhanced. We like to point out that the CREB-mediated co-regulation of clustered ARGs suggested here does not exclude the proposed mechanism mediated by chromatin domain structures (34, 76, 83). In fact, chromatin structure and CREB may cooperatively co-regulate the clustered ARGs (Fig. 10). In such a model, open chromatin domains provide a structural platform for co-regulation of clustered target genes, whereas CREB and other transcription factors are necessary to fine-tune the coordinated expression of individual genes in the open chromatin domain (Fig. 10). We propose that the cooperative changes of chromatin structures and activation of transcription factors such as CREB offers a sophisticated mechanism to coordinate the transcription of clustered ARGs during LTP.

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