

Synaptic activity-responsive element in the *Arc/Arg3.1* promoter essential for synapse-to-nucleus signaling in activated neurons

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The neuronal immediate early gene *Arc/Arg-3.1* is widely used as one of the most reliable molecular markers for intense synaptic activity in vivo. However, the *cis*-acting elements responsible for such stringent activity dependence have not been firmly identified. Here we combined luciferase reporter assays in cultured cortical neurons and comparative genome mapping to identify the critical synaptic activity-responsive elements (SARE) of the *Arc/Arg-3.1* gene. A major SARE was found as a unique \approx 100-bp element located at >5 kb upstream of the *Arc/Arg-3.1* transcription initiation site in the mouse genome. This single element, when positioned immediately upstream of a minimal promoter, was necessary and sufficient to replicate crucial properties of endogenous *Arc/Arg-3.1*'s transcriptional regulation, including rapid onset of transcription triggered by synaptic activity and low basal expression during synaptic inactivity. We identified the major determinants of SARE as a unique cluster of neuronal activity-dependent *cis*-regulatory elements consisting of closely localized binding sites for CREB, MEF2, and SRF. Consistently, a SARE reporter could readily trace and mark an ensemble of cells that have experienced intense activity in the recent past in vivo. Taken together, our work uncovers a novel transcriptional mechanism by which a critical 100-bp element, SARE, mediates a predominant component of the synapse-to-nucleus signaling in ensembles of *Arc/Arg-3.1*-positive activated neurons.

immediate-early genes | MEF2 | SRF | calcium | CREB

Fine tuning of gene expression and protein translation in mature neurons is of vital importance to brain function (1). Aberrance of adaptive responses such as long-term memory or late-phase synaptic plasticity has indeed been the hallmark of several genetically engineered mouse mutants in which the expression or the function of key transcription factors have been altered (2, 3). Thus it appears that the supply of neuronal proteins must be tightly matched to the cellular demand at any given time, to maintain proper neuronal circuit function.

One critical determinant of neuronal gene expression is the neuron's own activity. Dramatic changes in gene expression have been reported upon robust reorganization of sensory information processing (such as during critical period plasticity of the visual cortex), or through characteristic cognitive processes (such as establishment of long-term memory) (4–6). Barrages of synaptic activity that alters synaptic strength can directly up- or downregulate various constituents of the synaptic machinery and many signaling molecules (7–9). Studies of the transcriptional activation mechanisms leading to upregulation of the immediate early genes *c-fos*, *zif268*, and of the brain-derived neurotrophic factor (BDNF) indicated that transcription factors such as the Ca^{2+} /cAMP-response element binding protein (CREB) and the serum response factor (SRF) may play a privileged role as key neuronal activity sensors in the nucleus (10–12).

An activity-regulated cytoskeleton-associated protein (*Arc/Arg-3.1*) has recently emerged as an attractive candidate effector molecule/immediate early gene product (13, 14), whose induction

may correlate, at least in part, with the induced synaptic alteration phenotype (15–18). Furthermore, *Arc/Arg-3.1* transcription is induced extremely rapidly and *Arc/Arg-3.1* mRNA detection, by fluorescence in situ hybridization, has now been validated as a reliable trace of intense synaptic activity within a neuronal ensemble in the hippocampus (e.g., during novelty exposure), in the amygdala (e.g., during acquisition of long-term fear memory), or in the sensory cortices (e.g., after intense sensory experience following sensory deprivation) (19–22). Despite the growing interest in *Arc/Arg-3.1* function, however, surprisingly little is yet known about the genomic mechanism by which acute delivery of synaptic information can be reliably encoded into *Arc/Arg-3.1* transcriptional events in the nucleus. To address this question, we investigated the *cis*-acting enhancer elements responsible for such stringent neuronal activity dependence.

Results

Presence of a Strong Synaptic Activity-Responsive Element in a Distal Portion of the Mouse *Arc/Arg-3.1* Promoter. We performed transcriptional reporter assays using primary culture neurons in which endogenous *Arc/Arg-3.1* is rapidly and transiently induced by 4AP/BIC stimulation (Fig. 1A). In the mouse genome, 7 conserved regions exceeding 65% identity per 100 bp with humans were found between 0 and -5 kb (from the transcription initiation site), and 1 distal region was mapped between -6 kb and -7 kb (Fig. 1B). No further conserved regions were found between -7 kb and -10 kb.

Prior studies have demonstrated the role of a proximal region (<2 kb) of the *Arc/Arg-3.1* promoter in its activity dependence (23, 24). Consistent with this, proximal regions [Arc1000 (-996 to $+198$) and Arc2000 (-1996 to $+198$)] showed 2- to 3-fold induction after stimulation in our assays (Fig. 1C). Genomic regions up to 3 kb (Arc3000, -2996 to $+198$) conferred an 8-fold induction (Fig. 1C). The activated levels of 4-kb and 5-kb fragments [Arc4000 (-3996 to $+198$) and Arc5000 (-4996 to $+198$)] were similar to that of Arc3000, but their basal levels in TTX were significantly lower than those of Arc1000, Arc2000, and Arc3000 ($P < 0.01$), resulting in an \approx 20-fold induction (Fig. 1C). Unexpectedly, extension of the genomic sequence up to 7 kb (Arc7000, -7065 to $+198$) resulted in further increase in its induction ability, of >150 -fold, in

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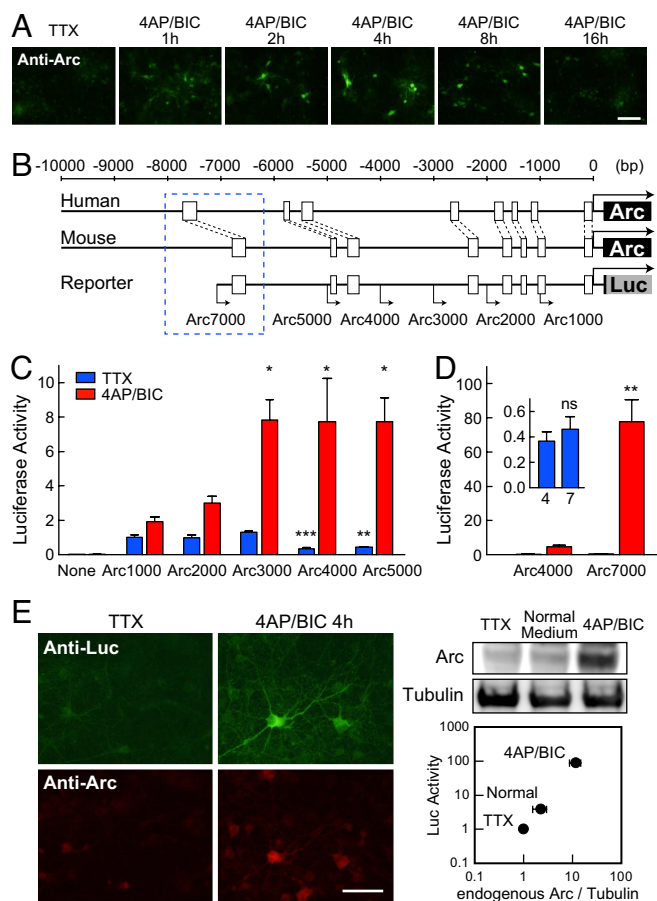


Fig. 1. Multiple regulatory elements in the mouse *Arc/Arg-3.1* promoter. (A) Time course of endogenous *Arc/Arg-3.1* protein induction by 4AP/BIC stimulation. Cultured cortical neurons pretreated with TTX were stimulated with 4AP/BIC for indicated hours. (Scale bar, 100 μ m.) (B) Evolutionarily conserved genomic regions in the upstream of the *Arc/Arg-3.1* gene. Regions conserved between mice and humans are shown as white boxes. A destabilized luciferase (Luc-PEST) gene was used as a reporter. (C) Transcriptional regulatory activities of Arc1000–5000. Cultured cortical neurons were stimulated with 4AP/BIC for 4 h. Luciferase activities are normalized relative to the activity of Arc1000 under the TTX treatment. This normalization applies to all luciferase assay data in this study. Statistical analyses were performed separately for the TTX and 4AP/BIC data sets. $n = 5$ independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with the TTX or the 4AP/BIC value of Arc1000 (1-way ANOVA with Tukey's test). (D) Strong activation ability of Arc7000. The y axis for the basal levels were expanded and shown in the inset for clarity. $n = 7$ independent experiments. **, $P < 0.01$; ns, not significant (paired t test) compared with the 4AP/BIC or the TTX value of Arc4000. (E) Arc7000-driven luciferase correlates with endogenous Arc induction. *Left*, coexpression of luciferase and endogenous Arc protein after stimulation. (Scale bar, 50 μ m.) *Right*, simultaneous quantification of luciferase activities and endogenous Arc protein levels in the same samples. Cell lysates were prepared from neurons with no treatment (normal medium), with TTX, or with 4AP/BIC for 4 h. Arc protein levels were quantified by Western blotting and plotted against the reporter luciferase activities. Duplicated samples were analyzed and shown.

response to stimulation ($P < 0.01$) (Fig. 1D), indicating the presence of a very potent enhancer in the most distal conserved region.

We also examined the relative potency of Arc7000 at single-cell resolution, using a destabilized GFP as a fluorescent reporter. As expected from the results of luciferase assays, GFP signals in individual neurons were more elevated downstream of Arc7000 than of Arc4000 ($P < 0.001$) [supporting information (SI) Fig. S1]. Additionally, no GFP signals were detected in glial cells (data not shown), indicating that Arc7000 may also determine the neuron-specific expression pattern of the *Arc/Arg-3.1* gene.

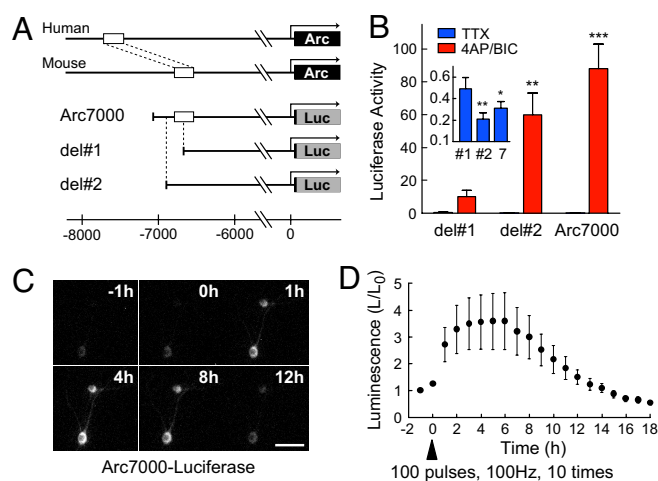


Fig. 2. The distal conserved region is crucial for the Arc7000 promoter activity. (A) Arc7000 deletion mutants. The genomic locus framed by a dashed box in Fig. 1B is expanded and shown. (B) Presence of a potent enhancer element between Arc7000-del no. 1 and Arc7000-del no. 2. $n = 6$ independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with the TTX or the 4AP/BIC value of Arc7000-del no. 1 (1-way ANOVA with Tukey's test). (C and D) Rapid and transient induction of luciferase luminescence by Arc7000 after high-frequency electrical stimulation. Hippocampal neurons transfected with a click beetle luciferase (ELuc) vector was electrically stimulated at 0 h (triangle), and luminescence in the soma was monitored. Representative images were shown in C. $n = 9$ neurons. (Scale bar, 50 μ m.)

The Arc7000 activity correlated well with the endogenous *Arc/Arg-3.1* expression, because the reporter luciferase after stimulation was mostly coexpressed in the same cells as endogenously induced *Arc/Arg-3.1* (Fig. 1E), and Western blots showed a linear relation between luciferase activity and endogenous Arc protein levels (Fig. 1E).

Identification of a Synaptic Activity-Responsive Element (SARE) in Arc7000 That Is Critical for Activity-Regulated Gene Expression. To pin down the critical enhancer element, we next examined a genomic deletion (del) mutant Arc7000-del no. 1 (–6667 to +198), in which the most distal conserved region was truncated (Fig. 2A). This mutant revealed a striking decrease in activity dependence compared with Arc7000 ($P < 0.01$) (Fig. 2B). In contrast, Arc7000-del no. 2 (–6893 to +198), in which the conserved region was spared, possessed an activity dependence indistinguishable from Arc7000 (Fig. 2A and B). Intriguingly, the basal level of Arc7000-del no. 2 in TTX was indistinguishable from that of Arc7000, but was significantly lower than that of Arc7000-del no. 1 ($P < 0.05$) (Fig. 2B).

We then tested whether robust high-frequency synaptic activity triggered by electrical stimulation could induce reporter gene expression downstream of Arc7000 in single neurons. Hippocampal neurons transfected with an Arc7000-ELuc-PEST plasmid were stimulated with field electrical pulses. In responding neurons, luminescence started to increase immediately after the stimulation, reached plateau after 4–6 h, and attenuated gradually after 8 h (Fig. 2C and D). This activation time course closely resembled the induction time course of endogenous *Arc/Arg-3.1* following electrical stimulation in vivo (25).

These findings were most consistent with the presence of a potent enhancer, which we termed SARE, in a region between Arc7000-del no. 1 and Arc7000-del no. 2 (Fig. 2A and B). Close examination of the evolutionarily conserved region present in del no. 2 but truncated in del no. 1 revealed a series of short conserved sequence stretches, of ≈ 100 bp (Fig. 3A).

To directly test the role of these sequence stretches, we fused a 104-bp fragment (–6793 to –6690) to a TATA-containing short

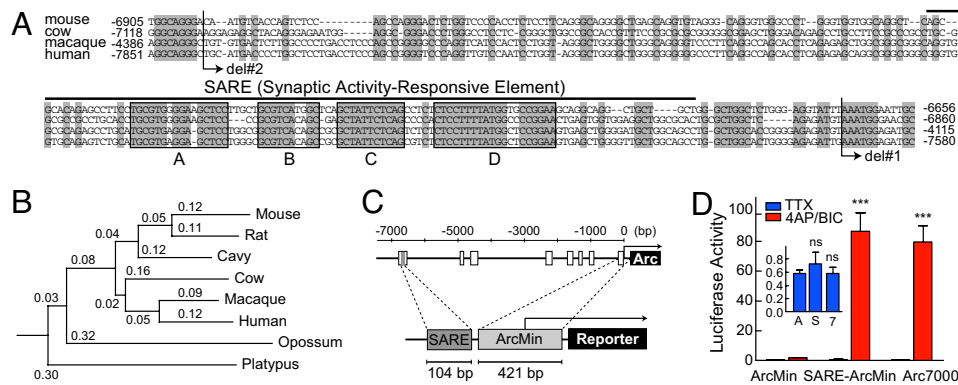


Fig. 3. *Arc/Arg-3.1* SARE replicates Arc7000 promoter activity. (A) Comparison across multiple mammalian species. Conserved sequences were highlighted in gray. A short stretch of sequences (thick line) consisted of 4 highly conserved boxes (boxes A–D) was termed a synaptic activity responsive element (SARE). (B) Dendrogram showing the divergence of SARE sequences across various mammalian species. The numbers represent the branch length, which indicates the degree of nucleotide differences. Detailed nucleotide information for this analysis is shown in Fig. S3. (C) SARE-ArcMin reporter vector. SARE was fused directly upstream of ArcMin, a TATA-containing sequence around the transcription initiation site of the *Arc/Arg-3.1* gene. (D) SARE-ArcMin replicates the activation ability of Arc7000. $n = 5$ independent experiments. ***, $P < 0.001$; ns, not significant, compared with the TTX or the 4AP/BIC value of ArcMin (1-way ANOVA with Tukey's test).

promoter (−222 to +198) of the *Arc/Arg-3.1* gene (SARE-ArcMin) (Fig. 3A and C). Luciferase activity driven by SARE-ArcMin was increased >100-fold in response to stimulation (Fig. 3D). The basal levels did not differ between SARE-ArcMin and ArcMin (Fig. 3D). Importantly, the activation level of SARE-ArcMin was indistinguishable from that of Arc7000 (Fig. 3D). The fluorescent protein reporter assay also revealed a dramatic increase in GFP signal positivity in SARE-ArcMin reporter-transfected neurons, following synaptic stimulation in individual cells (Fig. S2). None of the other conserved genomic regions showed such strong enhancer activities on their own (data not shown).

Taken together, these results indicate the existence of a novel SARE in the distal *Arc/Arg-3.1* promoter, which appeared to be critical for replicating the amplitude of activity-induced transcriptional response of the full promoter in the absence of any other adjacent genomic regions, when placed immediately upstream of the minimal *Arc/Arg-3.1* promoter.

Contribution of 3 Activity-Regulated Transcription Factors, CREB, MEF2, and SRF, in SARE Activation. SARE contained 4 conserved sequence stretches (Fig. 3A). This raised the possibility that perhaps distinct transcription factors could in principle bind to SARE, and thus, these putative binding sites were tentatively called boxes A–D (Fig. 3A). All 4 boxes were conserved in most mammals, except some species such as the opossum and the platypus (Fig. 3B and Fig. S3). To address their requirements, we introduced mutations in each of these boxes, and tested them either in the upstream of a TATA-containing minimal CMV promoter (minCMV, which has virtually no transcriptional activity per se), or in the context of the full Arc7000 promoter (Fig. 4A and B). Luciferase activity of SARE-minCMV was increased >80-fold in response to stimulation, and this induction was dramatically diminished by single mutations in boxes B, C, and D ($P < 0.001$). In contrast, a mutation in box A had no significant effect (Fig. 4A). Combined mutations in both boxes B and D almost completely blocked the induction (Fig. 4A). Similar results were obtained in the context of the full Arc7000 promoter (Fig. 4B). These data suggested that boxes B, C, and D were required for the enhancer activity of SARE, and some cooperativity might exist between them. Additionally, the basal level of SARE-minCMV was significantly elevated by mutating box D ($P < 0.001$) (Fig. 4A), indicating that box D might also contribute to attenuating transcription during synaptic inactivity.

We next sought to identify the transcription factors that bound to these sites. Box B contained a sequence matching a half site of the consensus binding sequence for CREB (Fig. 4C). Box C matched

the consensus for myocyte enhancer factor 2 (MEF2) with the exception of 2 nucleotides (Fig. 4C). The 5'-end of box D perfectly matched the consensus for SRF, while the 3'-end perfectly matched the consensus for the ternary complex factors (TCFs), the well-characterized accessory proteins of SRF (Fig. 4C). An electrophoretic mobility-shift assay (EMSA) revealed the formation of each specific protein-DNA complex for boxes B, C, and D in nuclear extracts obtained from electroconvulsive shock-treated rat cortices (Fig. 4C). Consistent with the consensus sequence matches, each complex was disrupted by adding excess amounts of competitor DNA, and gel supershifts were detected using CREB-, MEF2-, and SRF-specific antibodies. Furthermore, chromatin immunoprecipitation (ChIP) assay using neuronal culture confirmed physical binding of CREB, MEF2, and SRF to SARE in cultured neurons (Fig. 4D and Fig. S4). Together, these data strongly suggest that CREB, MEF2, and SRF play critical roles in SARE activation via binding to boxes B, C, and D, respectively (Fig. 4C).

We further investigated the intracellular signaling pathways upstream of these transcription factors. The SARE activation in the context of Arc7000 by 4AP/BIC treatment was almost completely suppressed when both AMPA-R and NMDA-R were blocked, confirming that excitatory synaptic inputs trigger the activation (Fig. S5A). In addition, the activation was reduced 80% when only NMDA-R was blocked, indicating that synaptic Ca^{2+} influx through NMDA-R is critical for the SARE activation (Fig. S5A). The activation was also significantly diminished by KN-93, a Ca^{2+} /calmodulin-dependent protein kinase (CaMK) inhibitor, and U0126, a mitogen-activated protein kinase kinase (MEK) inhibitor (Fig. S5B). An essentially similar pharmacological profile was obtained when the SARE activity was tested in the upstream of the minimal CMV promoter (Fig. S5C). These results confirmed that SARE was crucial for a large part of the transcriptional response of the *Arc/Arg-3.1* gene that lied downstream of activity-regulated signaling mechanisms, such as CaMK and MAPK cascades, both of which were previously shown to underlie many aspects of neuronal adaptive responses (8, 26).

Visualization of Activated Cells in a Neuronal Circuit Using a SARE Reporter Lentivirus Vector. One potential caveat of the above experiments was that these in vitro assays did not involve genomic integration of the reporter genes. Therefore, we designed a lentivirus-based gene delivery system to introduce SARE-driven reporters into the neuronal genome (Fig. 5). We constructed a lentivirus vector containing 2 expression cassettes placed in opposite directions and separated by an insulator sequence. In 1 cassette,

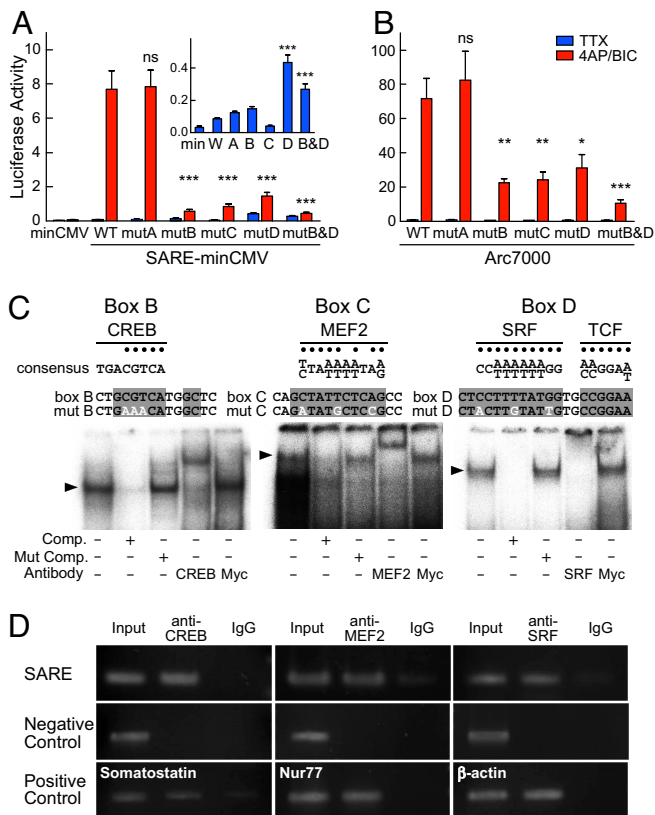


Fig. 4. Involvement of coclustered CREB, MEF2, and SRF in the SARE activation. (A) Loss of the SARE activity by mutations in the boxes B, C, and D, but not A, in the context of a minimal CMV promoter (SARE-minCMV). Note that the basal level (TTX) was elevated by a mutation in the box D (inset). WT, wild-type; ***, $P < 0.001$; ns, not significant, compared with the TTX or the 4AP/BIC value of WT (1-way ANOVA with Tukey's test). (B) Loss of the Arc7000 activity by mutations in the boxes B, C, and D. WT, wild-type; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant, compared with the 4AP/BIC value of WT (1-way ANOVA with Tukey's test). (C) EMSA revealed binding of CREB, MEF2, and SRF to the boxes B, C, and D. *Top*, the matched nucleotides are indicated by dots. The evolutionarily conserved nucleotides are highlighted in gray. The mutated nucleotides are shown with white letters. *Bottom*, representative results of EMSA. The brain nuclear extracts were added with probes of the boxes B, C, and D. Specific DNA-protein complexes (arrows) were observed, which disappeared by adding excessive amounts of unlabeled competitors (Comp), but not by mutated competitors (Mut Comp). Antibodies against CREB, MEF2, and SRF disrupted or supershifted the complexes, whereas a control antibody (anti-Myc) had no effects. (D) Chromatin immunoprecipitation (ChIP) assays revealed physical binding of CREB, MEF2, and SRF to SARE in the genome. Target genomic sequences were amplified with specific primer sets by qPCR. *Left*, the genomic region adjacent to the SARE sequence (within 200 bp) was detected in the immunoprecipitates obtained using an anti-CREB antibody, while a negative control sequence 10 kb downstream of SARE was not. The Somatostatin promoter was used as a positive control. *Middle*, detection of SARE in the immunoprecipitates obtained using an anti-MEF2 antibody. The Nur77 promoter was used as a positive control. *Right*, detection of SARE in the immunoprecipitates obtained using an anti-SRF antibody. The β -actin promoter was used as a positive control.

an RFP marker (TurboFP635) was driven under the control of a constitutive *pgk* promoter, while in the other, a d2EGFP reporter was expressed under the control of SARE-ArcMin. A control virus vector contained all these elements except for SARE (Fig. 5A).

BDNF is known to effectively induce endogenous Arc/Arg-3.1 expression in neurons. When SARE-ArcMin lentivirus-infected neurons were stimulated with BDNF, GFP signals became detectable in individual neurons (Fig. 5B). We further examined, by time-lapse live-cell fluorescence imaging, the activation time course of the genome-integrated SARE, upon high-frequency electrical

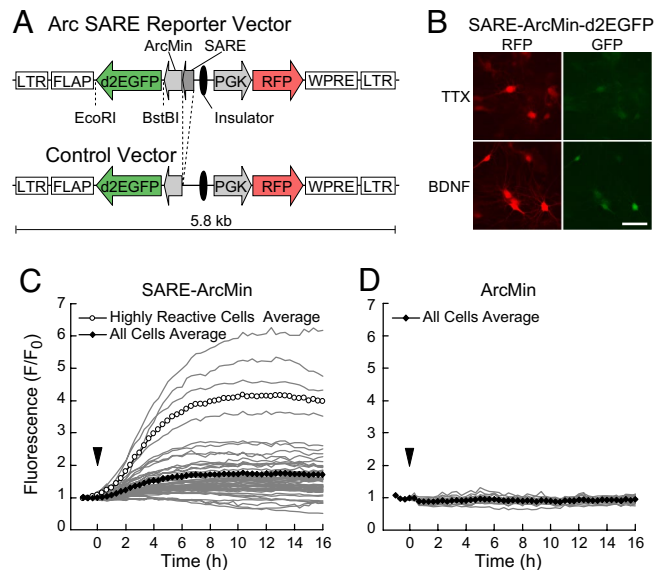


Fig. 5. Lentivirus-based genomic integration of the SARE reporter. (A) Design of lentiviral vectors. *Top*, the SARE reporter vector encodes an inducible reporter GFP (d2EGFP) under the control of SARE-ArcMin and a constitutively expressed infection marker RFP (TurboFP635) under the control of a *pgk* promoter. *Bottom*, a control vector lacking SARE. (B) Hippocampal neurons infected with the SARE reporter lentivirus were stimulated with BDNF for 6 h at 14 days postinfection. (Scale bar, 50 μ m.) (C) GFP live-cell imaging of SARE-virus-infected neurons stimulated with high-frequency electrical pulses. Hippocampal neurons infected with the SARE reporter lentivirus were stimulated with field electrical pulses at time 0 h (triangle, 100 pulses at 100 Hz, 9 times), and GFP fluorescence was monitored. Gray lines, traces of individual neurons. Filled squares, the average trace of all neurons examined ($n = 59$). Open circles, the average trace of highly reactive neurons (top 10% of all neurons sorted by the F/F_0 value at the time of 8 h, $n = 6$). (D) No GFP fluorescence changes were observed in control-virus-infected neurons. Filled squares, the average trace of all neurons examined ($n = 18$).

stimulation (9 bursts of 100 Hz, 1 sec applied by field stimulation). In most of the SARE-ArcMin lentivirus-infected neurons, GFP fluorescence consistently increased immediately after the stimulation and started to saturate at ≈ 6 –8 h (Fig. 5C), while the control virus-infected neurons showed virtually no GFP responses (Fig. 5D). Some of the neurons were particularly responsive (Fig. 5C), indicative of high copy number expression from multiple and/or transcriptionally favorable genomic loci. Alternatively, these neurons might have been favored because of their highly excitable properties within the neuronal network in culture as previously reported (10).

The above results suggested that the SARE-ArcMin lentivirus might provide a way to visualize specific sets of activated neurons in a given neural circuit in the brain. As a model, we designed an *in vivo* SARE-ArcMin reporter assay by directly injecting into mouse brains a d2EGFP reporter lentivirus. Consistent with prior reports (27), viral injection into the lateral ventricles of E15 mouse embryos reproducibly resulted in selective integration of infected neurons into layers 2/3 throughout the neocortex, as identified by RFP fluorescence and morphological criteria. No systematic differences were observed in the numbers of infected neurons between the 2 hemispheres. In our conditions, the infection rate was ranging 2–5% of the total cells in layers 2/3.

Cortical activity in the primary visual cortex (V1) was then stimulated by direct manipulation of the visual sensory inputs. Virus-infected mice (3–4 week-old) were sensory-deprived on 1 eye by suture, dark-reared for 1–3 days, and the intact open eye was exposed to light (Fig. 6A). Such sensory manipulation produced unilateral activation of the V1, as visualized by high immunoreactivities of endogenous Arc/Arg-3.1 at layers 2/3 and 4 in the

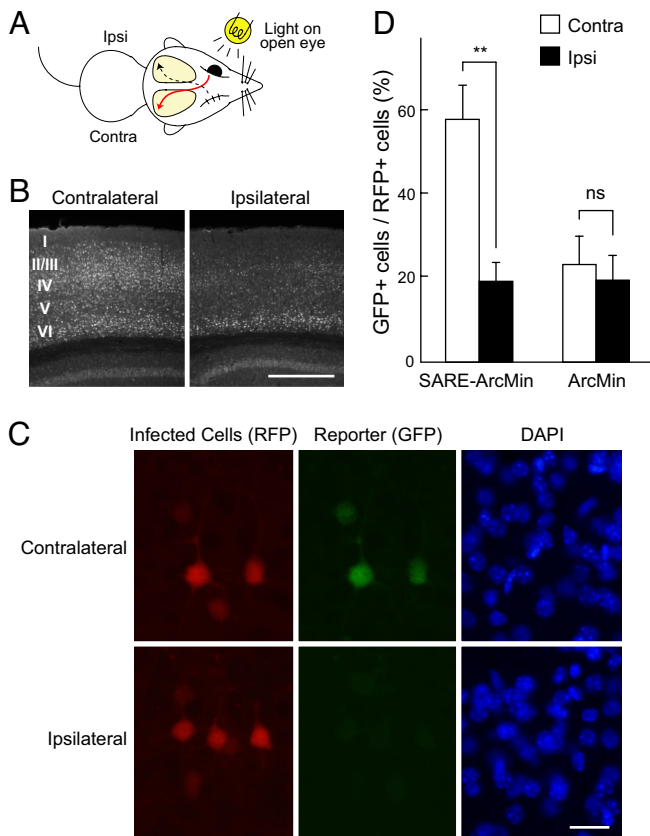


Fig. 6. Visualization of activated neurons in vivo by the SARE viral vector. (A) Manipulation of neuronal activity in the mouse visual cortex. The virus-infected mice were sensory-deprived on 1 eye by suture, dark reared for 2–3 days, and exposed to light on the intact open eye. (B) Endogenous *Arc/Arg-3.1* immunohistochemistry showing unilateral activation of the visual cortex. (Scale bar, 0.5 mm.) (C) The SARE reporter virus-infected neurons in layers 2/3 of the visual cortex. Activity-reporter GFP signals were detected in the contralateral side, but not in the ipsilateral side. (Scale bar, 20 μ m.) (D) The percentages of GFP-positive neurons over RFP-positive neurons in each hemisphere. $n = 6$ mice for SARE-ArcMin, $n = 5$ mice for ArcMin. **, $P < 0.01$; ns, not significant, compared to the ipsilateral side (paired t test).

contralateral, but not in the ipsilateral, visual cortex (Fig. 6B) (21). Consistent with this pattern, we found that the majority ($58.3 \pm 7.9\%$) of RFP-positive neurons were GFP-positive in the contralateral hemisphere that received visual inputs, whereas only a small number ($19.3 \pm 4.4\%$) of neurons were positive in the ipsilateral hemisphere (Fig. 6C and D). The percentage of GFP- and RFP-double positive neurons in the contralateral hemisphere were comparable with that of *Arc/Arg-3.1*- and RFP-double positive neurons ($57.8 \pm 4.6\%$) (Fig. S6), consistent with coexpression of reporter GFP and endogenous *Arc/Arg-3.1*. The control ArcMin virus-infected neurons showed low GFP positivity (about 20%) and did not show imbalances between hemispheres (Fig. 6D). Taken together, these results indicate that SARE-ArcMin lentivirus could reliably mark activated neurons within a targeted neuronal circuit.

Discussion

Elucidation of a Novel Transcriptional Switch Mechanism That Links Synaptic Activity with Nuclear Transcription: Participation of 3 Major Activity-Regulated Transcription Factors CREB, SRF, and MEF2 in *Arc/Arg-3.1* Transcriptional Response. Our work indicates that the *Arc7000* promoter essentially replicates the transcriptional profile of endogenous *Arc/Arg-3.1*, and that a relatively short DNA sequence of ≈ 100 bp provides a key regulatory mechanism for synapse-to-nucleus signaling. This novel *cis*-acting element contains

binding sites for 3 major activity-dependent transcription factors, CREB, MEF2, and SRF, and drove as much reporter induction as *Arc7000*, in response to synaptic stimuli, when placed upstream of the *Arc/Arg-3.1* minimal promoter. Mutations in either one of the binding sequences resulted in a severe block of transcriptional activity. Thus, intact promoter occupancy by all 3 factors appears to be essential for proper transcriptional activation upon synaptic activity. Pharmacological analyses revealed that synaptic Ca^{2+} influx through NMDA-R was critical for its activation. On the basis of these findings, we named this main activity-sensor for *Arc/Arg-3.1* transcription “synaptic activity-responsive element (SARE).”

A functional CRE is present on many activity-regulated neuronal genes, and CREB has repeatedly been demonstrated to be a critical factor in establishment of long-term plasticity and long-term memory (1, 2, 20). Similarly, a critical role for SRE and SRF in activity-dependent gene expression has extensively been documented (3). Recent evidence also supports a role for MEF2 upstream of various activity-induced genes which directly regulate synaptic functions (28). However, the discovery of a physical clustering, corequirement and sufficiency of the 3 activity-dependent *cis*-regulatory elements located in such close proximity (≈ 100 bp) in SARE is particularly striking, suggesting that SARE may have a unique role as a coincidence detector for the 3 activity-dependent transcription factors. Intriguingly, we observed that mutations in the SRF-binding site not only abolished SARE activation, but also significantly augmented basal activity under silenced conditions, suggesting an additional role of SRF for transcriptional repression as previously reported in certain contexts in nonneuronal cells (29).

Although distally located SARE likely acts as a major element for activity-dependent *Arc/Arg-3.1* induction, other proximal elements may further cooperate with SARE. Our own data suggested that a proximal region possessed a 2- to 3-fold transcriptional activation ability, consistent with a previous study that showed the contribution of cAMP/MAPK pathways in the activity-dependent response mediated by this region (23). Furthermore, *Egr-1/3* may also mediate the late, protein-synthesis dependent phase of *Arc/Arg-3.1* induction via binding to this proximal region (24).

Mapping of Active Ensembles Within a Neuronal Circuit Can Be Achieved Using SARE-Based Reporters. Detailed analyses of *Arc/Arg-3.1* expression using in situ hybridization have revealed that *Arc/Arg-3.1* transcription is specifically triggered by task- or sensory input-related information processing in several brain areas, including the hippocampus, amygdala, and cerebral cortex (19–21). The shortness of SARE sequence allows designing a virus-based tool to monitor *Arc/Arg-3.1* expression for both in vitro and in vivo imaging. We here performed an experiment where we injected a SARE-reporter lentivirus into embryonic mouse cerebral ventricles in utero, at embryonic day 15 (E15). Prior work established that only the neuronal progenitor cells, present in the ventricular zone (VZ) on the day of injection, are susceptible to viral infection. VZ cells at E15 are destined to become layers 2/3 neurons in the cerebral cortex (27). More than 20 days after lentiviral infection, when the viral integration into the genome has been completed, we quantified the extent of visual activation in layers 2/3 of the primary visual cortex. The virus-injected mice were first dark reared for more than 24 h, which suppressed the reporter expression to the baseline. Following light exposure to 1 eye for 2–3 h, we found that $\approx 60\%$ of the infected neurons of layers 2/3 were GFP reporter positive, which is consistent with prior functional mapping/electrophysiological reports (30), indicating that the majority of neurons indeed experienced an intense period of input activity, presumably emanating from layer 4 neurons.

Taken together, our work demonstrates that a SARE reporter, both in vitro and in vivo, can readily trace and mark an ensemble of cells that have experienced intense activity in the recent past. Further works are ongoing to design novel pharmacogenetic and

optogenetical experiments using SARE as a driver for conditional Cre recombinase such as hormone-activated CreER^{T2} (31), or for channelrhodopsins/halorhodopsins (32), with a view to manipulating specific gene expression or neuronal excitability in an activated ensemble of neurons within a circuit.

Materials and Methods

Plasmids, Lentiviruses, and Reagents. Reporter plasmid construction, lentiviral vector preparation, and reagents are described in *SI Materials and Methods*.

Neuronal Culture Preparation, Luciferase Reporter Assay, Fluorescent Protein Reporter Assay, and Luciferase Live-Cell Imaging. Dissociated neuronal culture was prepared from rat embryonic neocortex or postnatal hippocampus as described previously (10, 33). For luciferase assay, cortical neurons were transfected with a firefly luciferase reporter plasmid and an internal control Renilla luciferase plasmid by electroporation. At 10 days in vitro (div), the cells were silenced with TTX for 12–24 h, stimulated with a 4-aminopyridine/bicuculline (4AP/BIC) mixture for 4 h, and lysed. Luciferase activities were measured using the Dual Luciferase assay system (Promega).

For lentiviral reporter assay, hippocampal neurons cultured on coverslips were infected with lentiviruses at 2 div. At 15 div, neurons were silenced with TTX and then stimulated with BDNF (a gift from Dainippon Sumitomo Pharma, Osaka, Japan). For luciferase live-cell imaging, hippocampal neurons transfected with the Arc7000-ELuc-PEST plasmid were imaged in a culture medium containing d-luciferin (0.5 mM, Toyobo) at 14–18 div.

All animal experiments were carried out in accordance with regulations and guidelines of the University of Tokyo and approved by the institutional review committee of University of Tokyo Graduate School of Medicine. More information is provided in *SI Materials and Methods*.

Electrophoretic Mobility Shift Assay (EMSA) and Chromatin Immunoprecipitation (ChIP) Assay. Preparation of brain nuclear extracts is described in *SI Materials and Methods*. The nuclear extracts (2 μ g for CREB and SRF and 10 μ g for MEF2) were reacted with ³²P-radiolabeled DNA probes. Excess amounts (300-fold) of unlabeled probes were added to the reaction mixture for competition assay.

Protein-DNA complexes were separated on acrylamide gels and analyzed by an image analyzer (BAS2500, Fujifilm).

Chromatin immunoprecipitation was performed using a kit (Active Motif) essentially following the manufacturer's instructions. Precipitates were analyzed using a real-time PCR system (Roche) and gel electrophoresis. More information is provided in *SI Materials and Methods*.

Viral Reporter Assay in Mouse Visual Cortex. Production of lentivirus-infected mice is described in *SI Materials and Methods*. After 3–4 postnatal weeks, the infected mice were subjected to monocular deprivation, dark reared for 1–3 days, exposed to a bright environment for 2–3 h, and then perfused for immunohistochemical analysis. For quantification, infected neurons were identified by native RFP signals and TSA-enhanced GFP-immunoreactive cells were counted. More information is provided in *SI Materials and Methods*.

Statistical Analysis. Statistical analyses were performed using Prism 4.0 (Graphpad Software). Student's paired *t* test and 1-way analysis of variance with repeated measures (ANOVA) followed by Tukey's post hoc test were used for comparisons between 2 groups and 3 groups, respectively. Mann-Whitney *U* test was used for fluorescence reporter assay. All data are shown as mean \pm standard error of the mean (SEM), unless otherwise stated.

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