Enrichment of Conserved Synaptic Activity-Responsive Element in Neuronal Genes Predicts a Coordinated Response of MEF2, CREB and SRF

Fernanda M. Rodríguez-Tornos¹, Iñigo San Aniceto², Beatriz Cubelos³, Marta Nieto¹*  
¹Centro Nacional de Biotecnología (CNB-CSIC), Darwin 3, Campus de Cantoblanco, Madrid, Spain, ²Facultad de Informática, Universidad Complutense de Madrid, Profesor García Santestmases s/n, Madrid, Spain, ³Centro de Biología Molecular 'Severo Ochoa', Universidad Autónoma de Madrid, Madrid, Spain

Abstract

A unique synaptic activity-responsive element (SARE) sequence, composed of the consensus binding sites for SRF, MEF2 and CREB, is necessary for control of transcriptional upregulation of the Arc gene in response to synaptic activity. We hypothesize that this sequence is a broad mechanism that regulates gene expression in response to synaptic activation and during plasticity; and that analysis of SARE-containing genes could identify molecular mechanisms involved in brain disorders. To search for conserved SARE sequences in the mammalian genome, we used the SynoR in silico tool, and found the SARE cluster predominantly in the regulatory regions of genes expressed specifically in the nervous system; most were related to neural development and homeostatic maintenance. Two of these SARE sequences were tested in luciferase assays and proved to promote transcription in response to neuronal activation. Supporting the predictive capacity of our candidate list, up-regulation of several SARE containing genes in response to neuronal activity was validated using external data and also experimentally using primary cortical neurons and quantitative real time RT-PCR. The list of SARE-containing genes includes several linked to mental retardation and cognitive disorders, and is significantly enriched in genes that encode mRNA targeted by FMRP (fragile X mental retardation protein). Our study thus supports the idea that SARE sequences are relevant transcriptional regulatory elements that participate in plasticity. In addition, it offers a comprehensive view of how activity-responsive transcription factors coordinate their actions and increase the selectivity of their targets. Our data suggest that analysis of SARE-containing genes will reveal yet-undiscovered pathways of synaptic plasticity and additional candidate genes disrupted in mental disease.

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* E-mail: mnlopez@cnb.csic.es

Introduction

Neuronal plasticity and memory formation require changes in gene expression that are triggered by synaptic activity. The nature and organization of this response is the subject of intense research, and a number of transcription factors (TF) have been identified in recent years as necessary for long-term memory consolidation and storage. The Ca2+/cAMP response element-binding protein (CREB) was initially identified as the main interlocutor in the dialogue between the synapse and the nucleus [1]. Later studies revealed the complexity of this process and implicated other transcription factors, including the serum response factor SRF [2], MEF2 [3] and Npas4 [4]. The availability of efficient methods for gene expression analysis has also contributed with a large collection of mRNAs, possible targets of these TF, whose gene expression analysis has also contributed with a large collection of mRNAs, possible targets of these TF, whose expression is modulated by activity and experience [5,6].

The large number of potential targets for these factors does not facilitate a model that clarifies how TF establish a coordinated response and regulate transcription for efficient remodeling of neuronal connections. The description of a 100 bp cis-regulatory enhancer element containing a cluster of CREB, MEF2 and SRF binding sites suggests a mechanism that might help to explain the selectivity and coordination of the activity-dependent transcriptional response. This sequence, termed SARE, was identified in the gene that encodes the activity-regulated cytoskeleton-associated protein (Arc) [7]. The SARE sequence is conserved in mammalian Arc regulatory regions; it is sufficient to drive a rapid transcriptional response following synaptic activation and to reproduce, both in vitro and in vivo, the endogenous Arc activation pattern [7]. Despite the novelty and potential repercussion of this finding, the study restricted the description of this sequence to the Arc gene and did not determine whether SARE appear in the regulatory regions of other genes, or the specificity of this sequence to the nervous system. We studied the broader implication of SARE sequences in the context of the response to neuronal activity, and validated SARE analysis as able to identify elements of synaptic plasticity. Using the in silico tool SynoR [8], we analyzed the SARE sequences conserved in the mammalian genome. Comparison of mouse and human genome sequences showed enrichment in conserved SARE clusters in the regulatory regions of genes that are expressed specifically in neural tissues, that are involved in neural development and homeostatic

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maintenance, and that encode mRNA targeted by FMRP. These data support the concept that SARE sequences are true transcriptional regulatory elements, responsible for the coordinat-
ed response of TF that convey information from the postsynaptic
compartment to the nucleus. These findings might contribute to
understanding the genetic causes of mental diseases linked to
neuronal plasticity.

Results and Discussion

We used SynoR to study the possible relationship between the
SARE regulatory region and genes related to the nervous system
[8], specifically those involved in synaptic activity and mental
processes. We sought sequence regions containing clusters of the
consensus binding sites for CREB, MEF2 and SRF (Fig. 1A) in the
human genome, and compared them to the mouse genome to
to identify conserved sequences. Based on these criteria, we identified
887 genetic regions containing SARE sequences (Table S1 and
data deposited in the SynoR tool, ID: s1219104005847). The
SARE regions are assigned to the gene(s) of which they form part
or to which they are proximal, and are classified as intergenic,
intronic, utr (untranslated), cds (coding sequence), or promoter,
depending on their position within the gene (Table S1 and Fig. 1B).
Control searches for clusters containing combinations of other
related TFBS yield significantly less number of regions and were
not enriched in neural biological functions (Fig. 1C and
Experimental Procedures). The original SARE sequence of the
Arc promoter is not identified in our search because it contains
only half of the CREB binding site, and its MEF2 binding
sequence shows 2 nucleotide mismatches compared to the
consensus [7]. Binding site predictions for individual TF using
TRANSFAC. We have validated the presence of the SARE
clusters in more than ten of our candidates manually using this tool.
These include ATFS, CUX1, CUX2, FOXP1, FOXP2, HOMER1,
LMPDH2, NRG1, NPAS4, NR4A1, PLXNA4 and SEMA6A. Simil-
arily, there might be additional SARE sequences not identified by
this search because of the analysis procedure: it first identifies
TFBS clusters in the human genome and subsequently searches for
homology to this specific sequence in mice.

The analysis showed that SARE clusters were most abundant in
intergenic and intronic regions (Fig. 1), potential areas for gene
expression control. The list of SARE-containing genes showed
genes with central roles in the nervous system such as NMDA
(Grin2a, essential for excitatory synapses), Robo2 (with major
functions in axon guidance) and Call/Cux2 (determinant for
cerebral cortex layer II-IV) (examples in Table 1). Classification of
genes containing SARE sequences at the GO categories using
Topfun application (http://toppgene.cchmc.org/ “Topfun”) in-
dicated that the processes potentially affected by SARE regulation
are clearly related to the nervous system (Table S2). This analysis
yielded several enriched GO categories, out of 112 significantly
enriched GO biological processes, 21 (18,75%) of them related to
neural functions (Table 2). All of these categories are specifically
related to nervous system development and maintenance, and
many showed significant greater enrichment than other categories
(Table 2 and Table S2). In accordance with our hypothesis, this
prevalence of neural functions supports potentially important,
selective action of SARE-mediated mechanisms in the nervous
system. Next, SARE containing genes were grouped into two main
categories representing potential distal and proximal regulatory
sequences: intergenic; and intragenic, cds, promoter and utr
regions (Table S2) and GO analysis was performed separately with
these two groups. Both groups showed similar enrichment in
neural functions; therefore it did not favor proximal or distal
regulatory regions as more relevant to plasticity.

The analysis of genes containing the SARE cluster appeared an
appropriate approach for identification of mechanisms of homeo-
Stasis, plasticity and activity-dependent remodeling in the nervous
system. This study disclosed a large number of genes known to
participate in plasticity and synaptogenesis (examples in Table 1);
Homer1 is an example in this category. Homer genes encode
scaffolding proteins that bind Ca2+ signaling proteins and target
them to their correct subcellular localization [9,10]; they are
essential for dynamic regulation of the synapse, synaptic plasticity,
and spatial learning [11,12]. Coincident expression of experience-
triggered Homer and Arc proteins is found in hippocampal and
cortical neurons [13], which supports simultaneous activation, as
predicted by our analysis. We also identified axonal guidance
molecules (Table 1 and Table S1), including PlxnA4 and its ligand
Semad6 [14] as molecules potentially regulated by SARE. The
semaphorin and plexin receptor families, together with neuropi-
lins, are crucial during nervous system development and homeo-
stasis, and mark the pathway for axon growth [14]. These proteins
also control synaptogenesis, axon pruning, the density and
maturation of dendritic spines and are implicated in a number of
developmental, psychiatric and neurodegenerative disorders
[15]. As for the axon guidance cues, we found a number of genes
that encode cytoskeletal remodeling molecules at the synapse
(Table 1). For example, ankyyrin link integral membrane proteins
to the underlying spectrin-actin cytoskeleton; they have key roles
in activities such as cell motility, activation, proliferation, contact,
and maintenance of specialized membrane domains. They might
be involved in bipolar disorder and other mental alterations [16].

Less anticipated were SARE-containing genes not previously
implicated in plasticity or structural maintenance of the synapse; in
this category, we found neuronal subtype-specific TF such as Cux1
and Cux2 [17], Zic2 [18] and Sox6 [19,20] (Table 1 and Table S1).
Cux TF expression is restricted to neurons of layers II-III and IV
of the cerebral cortex. During development, Cux regulate
dendritic branching, spine morphogenesis and synapse maturation
[17]. Cux expression is maintained through adulthood, but
nothing is yet known of their function in mature neurons.
Whereas Cux functions could be associated with plasticity at the
postsynaptic site [17], Zic2 might act on the presynaptic terminal,
as it is associated with axon development in retinal ganglion cells;
Sox6, in turn, is described as essential for neuronal differentiation
[19,20]. These observations suggest that activity-dependent
mechanisms act on pathways specific to neuronal subtypes.

To test the relevance of our findings and the predictive
capability of our gene set to identify genes up-regulated upon
neuronal activation we searched for experimental confirmation.
Several studies of gene expression changes induced by neuronal
activation have been reported and made useful available
contributions. Many of them analyze the effects of the gabaergic
inhibitor bicuculline to trigger neuronal excitatory response. We
therefore compare the list of SARE containing genes with those of
genes which expression was modify in studies analyzing the in vitro
effects of infusion of bicuculline into the accessory olfactory bulb
[21]; in vitro bicuculline treatment of cortical cells [22]; and
hippocampal neuronal cultures [23]. This allowed us to extend
our validation to several neuronal types. In all three cases, the
comparison revealed a highly significant enrichment between the
SARE containing genes and those up-regulated upon neuronal
activation, but none or of lower statistical significance, when
compared to the list of genes that are down-regulated (Table 3).
Several of the SARE genes, such as Homer, Atf3, Klf6 and Bclaf1
are common to two or the three studies, and may represent a general
pan-neuronal response, while unique ones might represent tissue-specific responses. These significant overlapping validate our results with external independent data. We next took the reverse approach and tested the predictive capability of our study by testing the expression of genes picked from our list upon neuronal activation. Cells from E18 mouse cortex were dissociated, neurons were cultivated and neuronal activity triggered using bicuculline [7]. RNA was obtained and transcript expression of twelve SARE containing genes, including Arc, analyzed by using quantitative real time RT-PCR (Q-PCR) (Fig. 2A). Up-regulation of Arc gene demonstrated efficient neuronal activation and, also expected, the levels of the S-isoform of Homer1 were increased [24,25]. Six more genes showed up-regulation when neurons were activated. \textit{Atf3}, \textit{Impdh2}, and \textit{Npas4} up-regulation in cortical cells was in agreement with our own analysis of the raw data obtained from gene expression arrays reported by other investigators [22], and further confirmed our comparison with external sources (Table 3). Interestingly, up-regulation of \textit{Cas1}, \textit{Cas2}, and \textit{PlxnA4}, genes not suspected to be regulated by activity, again confirmed the predictive capacity of our study. Four genes, \textit{Lmo4}, \textit{Robo1}, \textit{Robo2} and \textit{Klf6} did not show significant changes. This can be ascribed to the almost certain possibility of a number of false positive in our list, to the fact that other splicing variants might be affected, or to the possibility that subsets of genes may respond differently depending on the stimulus that triggers neuronal response.

Next, the sequence corresponding to two of these SARE sequences were cloned upstream of a minimal promoter into vectors containing luciferase reporters to test their ability to activate transcription in response to neuronal activity. Cells from E18 mouse cortex were transfected with reporter constructs, neuronal activity triggered using bicuculline [7], and luciferase activity compared to control tetrodotoxin (TTX) treated neurons.

**Figure 1. SARE sequences are found in intergenic and intronic genetic regions.** Scheme showing consensus TFBS for SARE sequence and the SARE sequenced found in the promoter of \textit{SOX14} (top). For the SynoR search, random relative position of the three TFBS was permitted. Table shows the number and percentages of SARE sequences classified according to their position within the genes and the diagram represents the distribution of each category.

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These experiments demonstrated that these novel identified SARE sequences replicate the promoter activity of the SARE sequence corresponding to the Arc gene and significantly increase transcription upon depolarization (Fig. 2B).

Our analyses thus point to overlooked pathways that might participate in activity-dependent regulatory mechanisms and, by extension, suggests the identification of genes potentially linked to mental diseases caused by plasticity defects [26,27,28]. This is the case of genes reported as candidates for autism in which we found

Table 1. SARE regulation affects several aspects of neuron function.

| Gene symbol | Name | Human gene ID | Type |
|-------------|------|---------------|------|
| Synaptogenesis-Plasticity | | | |
| SYNCRIP | synaptotagmin binding, cytoplasmic RNA interacting protein | 10492 | utr |
| HDAC5 | histone deacetylase 5 | 10014 | utr |
| HOMER1 | homer homolog 1 (Drosophila) | 9456 | promoter |
| KALRN | kalrin, RhoGEF kinase | 8997 | utr |
| NCAM1 | neural cell adhesion molecule 1 | 4684 | intron |
| NRG1 | neuregulin 1 | 3084 | intron |
| NRXN1 | neurexin 1 | 9378 | intron |
| SCN3A | sodium channel, voltage-gated, type III, alpha subunit | 6328 | utr |
| SEPT7 | septin 7 | 989 | utr |
| Citoskeletal-synaptic proteins | | | |
| ANK3 | ankyrin 3, node of Ranvier (ankyrin G) | 288 | utr |
| CAMK1D | calcium/calmodulin-dependent protein kinase ID | 57118 | intron |
| MAP2K5 | mitogen-activated protein kinase kinase 5 | 5607 | intron |
| NR4A1 | nuclear receptor subfamily 4, group A, member 1 | 3164 | intron |
| RABGAP1L | RAB GTPase activating protein 1-like | 9910 | intron |
| RAPGEF2 | Rap guanine nucleotide exchange factor (GEF) 2 | 9693 | intron |
| RAPGEF6 | Rap guanine nucleotide exchange factor (GEF) 6 | 51735 | intron |
| RASSF8 | Ras association (RaLGDS/AF-6) domain family (N-terminal) member 8 | 11228 | utr |
| RASGEF1C | RasGEF domain family, member 1C | 255426 | intron |
| RHQ | ras homolog gene family, member Q | 23433 | utr |
| Axon guidance and maintenance | | | |
| CRIM1 | cysteine rich transmembrane BMP regulator 1 (chordin-like) | 51232 | intron |
| DCC | deleted in colorectal carcinoma | 1630 | intron |
| EPHB1 | EPH receptor B1 | 2047 | promoter |
| NRG1 | neuregulin 1 | 3084 | intron |
| PLCXD3 | phosphatidylinositol-specific phospholipase C, X domain containing 3 | 345557 | intron |
| PLXNC1 | plexin C1 | 10154 | intron |
| PLXNA4 | plexin A4 | 91584 | intron |
| RGMA | RGM domain family, member A | 56963 | intergenic |
| ROBO1 | roundabout, axon guidance receptor, homolog 1 (Drosophila) | 6091 | intergenic |
| SEMA6A | sema domain, TM and cytoplasmic domain, (semaphorin) 6A | 57556 | promoter |
| Transcription factors | | | |
| CUX1 | cut-like homeobox 1 | 1523 | intron |
| CUX2 | cut-like homeobox 2 | 23316 | intron |
| FOXP1 | forkhead box P1 | 27086 | intron |
| FOXP2 | forkhead box P2 | 93986 | intron |
| PAX6 | paired box 6 | 5080 | intron |
| PHOX2B | paired-like homeobox 2b | 8929 | utr |
| RUNX2 | runt-related transcription factor 2 | 860 | utr |
| SOX6 | SRY (sex determining region Y)-box 6 | 55553 | intron |
| ZIC2 | Zic family member 2 | 7546 | utr |
| ZNF292 | zinc finger protein 292 | 23036 | utr |

Examples of genes containing SARE sequences according to function, extracted from the list of 827 genes identified.
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SARE Coordinate Transcription during Plasticity

Table 2. Classification of SARE-containing genes at GO indicated significant enrichment of processes related to the nervous system.

| GO: Biological Process | Rank | ID | Name | P-value |
|-----------------------|------|----|------|---------|
| 2                     | GO:0030182 | neuron differentiation | 1.76E-11 |
| 4                     | GO:0048699 | generation of neurons | 1.52E-10 |
| 5                     | GO:002008 | neurogenesis | 2.28E-10 |
| 9                     | GO:0048812 | neuron projection morphogenesis | 2.87E-09 |
| 10                    | GO:007409 | axonogenesis | 3.40E-09 |
| 13                    | GO:0048666 | neuron development | 6.43E-09 |
| 16                    | GO:0031175 | neuron projection development | 2.28E-08 |
| 28                    | GO:0007411 | axon guidance | 2.75E-07 |
| 31                    | GO:007417 | central nervous system development | 3.49E-07 |
| 45                    | GO:0035295 | tube development | 9.04E-06 |
| 58                    | GO:0045664 | regulation of neuron differentiation | 1.27E-04 |
| 73                    | GO:0030900 | forebrain development | 1.74E-03 |
| 74                    | GO:0007420 | brain development | 1.75E-03 |
| 83                    | GO:0021772 | olfactory bulb development | 5.05E-03 |
| 84                    | GO:0031290 | retinal ganglion cell axon guidance | 5.46E-03 |
| 87                    | GO:0021889 | olfactory bulb interneuron differentiation | 6.39E-03 |
| 91                    | GO:0021988 | olfactory lobe development | 7.48E-03 |
| 96                    | GO:007423 | sensory organ development | 1.29E-02 |
| 97                    | GO:001537 | telencephalon development | 1.30E-02 |
| 111                   | GO:0001654 | eye development | 4.50E-02 |
| 112                   | GO:0021891 | olfactory bulb interneuron development | 4.54E-02 |

The analysis yielded 112 significantly enriched GO biological processes. The table shows the 21 categories with significantly high fold enrichment that are related to nervous system development and maintenance. Rank indicates the position within the list of the total 112 when ordered from higher to lower enrichment. Neural functions are ranked in the higher positions.

SARE sequences, such as NRXN1 and 2 [29], FOXP1 [30], FOXP2 [30,31], GRID2 [32], KCNMA1 [33] and others (http://gene.sari.org) [34] (see Table S1).

Validation of our prediction nonetheless required evaluation of true enrichment of genes involved in cognitive dysfunction. Fragile X syndrome (FXS) is a well-characterized form of autism, caused by loss of function of the Fragile X mental retardation protein (FMRP), which regulates local translation and plasticity at pre- and postsynaptic sites [28,35]. Based on a recent extensive list of genes targeted by FMRP, from which the authors extract a stringent list of SARE-regulated genes at intergenic locations) with the stringent list of FMRP targets resulted in 70 genes common to both (0.5% overlap), an enrichment of biological relevance (p = 4.3909–13; see Methods) (Table 4). The relationship between the SARE-containing genes and FMRP targets thus strongly supports SARE involvement in activity-dependent regulation. In addition, it suggests that mutations in SARE or SARE-containing genes and pathways can contribute to mental retardation, autism spectrum disorders and other psychiatric diseases.

Correct function of nervous system networks and subnetworks is possible thanks to the extraordinary spatial and temporal coordination of gene expression that is guided by the TF subset expressed by each neuronal population. Our findings suggest that cooperation between CREB, SRF, and MEF2 transcription factors at the SARE region is one of the precisely regulated mechanisms that govern the transcriptional program of activated neurons. This transcriptional cooperation might also apply to other TF to initiate an appropriate, specific transcriptional response in other biological processes. This study also highlights the value of the development and use of computational tools and databases for the comprehensive analysis of biological events. We identified a subset of genes whose transcription is potentially regulated by the SARE cluster after synaptic activation. Most of these genes are directly related to nervous system development and maintenance; several of them are reported at the synapse, some are mutated in human mental disorders, and many form part of FMRP-regulated mechanisms. The identification and functional analysis of SARE-containing genes provided here is thus a useful for implicating new candidate genes in plasticity, memory, and mental retardation, and suggests new approaches to the study of mental disorders in which synaptic activity might have a central role.

Methods

Genome Sequence Analysis

In silico analyses were performed using SynoR (Identifying synonymous regulatory elements in vertebrate genomes), a tool described by Ovcharenko and Nobrega [8]. SynoR is available at the National Center for Biotechnology Information (NCBI) DCODE.org Comparative Genomics Developments (http://syncr.dcode.org/), and performs de novo identification of synonymous regulatory elements (SRE) using known patterns of transcription factor binding sites (TFBS) in active regulatory elements (RE) as seeds for genome scans. The search was performed on the human genome assembly (hg18; July 2007 NCBI Build 36.1) and compared to the mouse genome assembly (mm9; July 2007NCBI Build 37). ECBR Browser performs whole genome Blastz-based alignments using the TFBS data of the transcription factors under study from the TRANSFAC Professional database. The TFBS studied were those of CREB (CREB_01, CREBP1_01, CREBP1CJUN_01, CREB_02, CREB_Q2, CREB_Q4, CREBP1_Q2, CREB_Q3, CREB_Q2_01, CREB_Q4_01, CREBATF_Q6), MEF2 (MEF2_01, MEF2_02, MEF2_03, MEF2_04, MEF2_Q6_01) and SRF (SRF_01, SRF_Q6, SRF_C, SRF_Q4, SRF_Q5_01, SRF_Q5_02). The maximum distance between two adjacent TFBS was set at 125 base pairs. Random relative position of TFBS was allowed. To test for the significance of the results, we performed analysis of several TFBS combinations. A search for clusters combining TFBS for ZIC2, BRCA and PAX1 yields only 1 cluster indentified by SynoR, combination of ACAAT, Bach1, Lhpl1 gives 30 clusters. Combination of TF related to the nervous system, EGR1, MEF2, NF-kB results on 244 clusters identified. Substitution of any of the TFBS from our particular search of MEF2, CREB and SRF significantly decreased the number of identified clusters. For example, substitution of CREB for ZIC2, i.e. search for MEF2, SRF and ZIC2 gives 142, compared to 842.

Gene Annotation and Analysis of Gene Ontology

The list of SARE genes obtained from SynoR was updated using Topfun application from Toppgene suit Cincinnati Children’s Hospital Medical Center (http://toppgene.cchmc.org/“TopFun”) and manually curated. A small number of genes were not annotated in NCBI but appeared annotated in other data.
Figure 2. Up-regulation of the mRNA of SARE containing genes and promoter activation in response to neuronal activity. A) Upregulation of SARE identified candidates in primary cortical neurons. Primary neurons from E18 cortex were cultivated for 16 days before triggering neuronal activation with 4AP/bicuculline. RNA was obtained and transcript expression of SARE containing genes analyzed by quantitative real time RT-PCR (Q-PCR). Expression of each gene is normalized to the expression in control cells. Arc up-regulation demonstrated efficient neuronal activation. * indicates p < 0.05, ** p < 0.01, *** p < 0.005 (t-Student test). B) Novel SARE sequences activate transcription in response to neuronal activity. Mouse genomic sequence containing SARE regulatory regions (see below) corresponding to those identified for the Cux1 and Cux2 genes were cloned into the pGL4.23 luciferase vector (Promega). Neurons obtained from E18.5 cortex were co-transfected with the indicated firefly luciferase reporter construct and internal control Renilla luciferase plasmid at a ratio of 4:1. Neuronal activity was triggered with 4AP/bicuculline before measuring transcription of the reporter. Relative expression of each reporter constructs was determined by normalizing the activity of each reporter to its activity on TTX treated neurons. Data represent mean and standar deviation of results obtained in three different experiments. * indicates p < 0.01, ** p < 0.001 (t-Student test). doi:10.1371/journal.pone.0053848.g002
bases [Shaded genes on STableI]. GO analysis was performed using Toppfun shown in table S2. Similar significant enrichment of nervous system related functions of GO categories were obtained from the SynoR application.

Gene Expression Data Sets of Bicuculline Treatments
Experimental datasets were obtained from [23–24] as processed datasets. Unprocessed data from [22] was analyzed using GeoR2 (http://www.ncbi.nlm.nih.gov/geo/geo2r/). Gene annotation was updated using Toppfun application for all three datasets.

Primary Neurons Culture and Bicuculline/4-aminopyridine Induction

Neurons from E18 embryo cortex were trypsinized using 0.25 µg/ml trypsin (SIGMA-Aldrich) in EBSS (Gibco, Invitrogen, Carlsbad, CA) 3.8% MgSO4 (Sigma-Aldrich, St. Louis, MO), penicillin/streptomycin (Gibco, Invitrogen, Carlsbad, CA). The reaction was stopped and cells were mechanical dissociated in EBSS media complemented with 0.26 mg/ml Trypsin inhibitor, 0.08 mg/ml DNase, and 3.8% MgSO4 heptahydrate (all from Sigma-Aldrich, St. Louis, MO). Dissociated cells were seeded onto 24 well Poly-D-Lys (Sigma-Aldrich, St. Louis, MO) coated plates in neurobasal media supplemented with B27 complement 1 6, glutamax 1 6 and penicillin/streptomycin (Gibco, Invitrogen, Carlsbad, CA). 500 µl media were replaced every 2 days until 16 days (DIV 16). 12 h before inducing neuronal activity cells were incubated with 2 mM tetrodotoxin (TTX) (Alomone-labs, Jerusalem, Israel). Then media was replaced with media containing 4-aminopyridine (4AP) 100 µM, strychnine 1 µM, glycine 100 µM and bicuculline 30 µM (Sigma-Aldrich, St. Louis, MO), and RNA was extracted after 5 h using the Qiagen RNeasy Kit as described in the manufacture handbook (Qiagen).

Luciferase Reporter Assays

Mouse genomic sequence containing SARE regulatory regions (see below) corresponding to those identified for the Cux1 and Cux2 genes were cloned into the pGL4.23 luciferase vector.
Table 4. FMRP targets containing SARE sequence genes.

| Gene Symbol | mm9 Symbol | Entrez Gene ID | RefSeq ID | Description |
|-------------|------------|----------------|-----------|-------------|
| ANK3        | Ank3       | 11735          | NM_146005.3 | ankyrin 3, node of Ranvier (ankyrin G) |
| APP         | App        | 11820          | NM_007471.2 | amyloid beta (A4) precursor protein |
| ARID1B      | Arid1b     | 239985         | NM_001085355.1 | AT rich interactive domain 1B (SWI1-like) |
| ATN1        | Atn1       | 13498          | NM_007881.4 | atrophin 1 |
| ATP2A2      | Atp2a2     | 11938          | NM_001110140.2 | ATPase, Ca++ transporting, cardiac muscle, slow twitch 2 |
| ATXN1       | Atxn1      | 20238          | NM_009124.5 | ataxin 1 |
| BAI1        | Bai1       | 107831         | NM_174991.3 | brain-specific angiogenesis inhibitor 1 |
| BCAN        | Bcan       | 12032          | NM_007529.2 | brevican |
| BIRC6       | Birc6      | 12211          | NM_007566.2 | baculoviral IAP repeat-containing 6 |
| CAPD5       | Cadps      | 27062          | NM_012061.3 | Ca++-dependent secretion activator |
| CTNND2      | Ctnnd2     | 18163          | NM_008729.2 | catenin (cadherin-associated protein), delta 2 |
| CUX1        | Cux1       | 13047          | NM_009896.3 | cut-like homeobox 1 |
| CUX2        | Cux2       | 13048          | NM_007804.2 | cut-like homeobox 2 |
| DIP2B       | Dip2b      | 239667         | NM_172819.2 | DIP2 disco-interacting protein 2 homolog B (Drosophila) |
| EPB41L1     | Epb4.1l1   | 13821          | NM_001003815.2 | erythrocyte membrane protein band 4.1-like 1 |
| FAM5B       | 6430517E21Rik | 240843     | NM_207583.1 | family with sequence similarity 5, member B |
| FOX2X       | Fox2x      | 68837          | NM_001080932.1 | forkhead box K2 |
| GABBR1      | Gabbr1     | 54393          | NM_019439.3 | gamma-aminobutyric acid (GABA) B receptor, 1 |
| GNB1        | Gnb1       | 14688          | NM_008142.3 | guanine nucleotide binding protein (G protein), beta polypeptide 1 |
| GRIK3       | Grik3      | 14807          | NM_001081097.2 | glutamate receptor, ionotropic, kainate 3 |
| HDAC5       | Hdac5      | 15184          | NM_001077696.1 | histone deacetylase 5 |
| HIPK2       | Hipk2      | 15258          | NM_001136065.1 | homeodomain interacting protein kinase 2 |
| HIPK3       | Hipk3      | 15259          | NM_010434.1 | homeodomain interacting protein kinase 3 |
| IDS         | Ids        | 15931          | NM_010498.2 | iduronate 2-sulfatase |
| KALRN       | Kalm       | 545156         | NM_001164268.1 | kalirin, RhoGEF kinase |
| KCNDO2      | Kcdn2      | 16508          | NM_019697.3 | potassium voltage-gated channel, Shal-related subfamily, member 2 |
| KCN7H7      | Kch7       | 170738         | NM_133207.2 | potassium voltage-gated channel, subfamily H (eag-related), member 7 |
| KCNMA1      | Kcam1      | 16531          | NM_010610.2 | potassium large conductance calcium-activated channel |
| LINGO1      | Lingo1     | 235402         | NM_181074.4 | leucine rich repeat and Ig domain containing 1 |
| LBRCC7      | Lrc7       | 242274         | NM_001081358.1 | leucine rich repeat containing 7 |
| MAGI2       | Magi2      | 50791          | NM_015823.2 | membrane associated guanylate kinase, WW and PDZ domain containing 2 |
| MHHAS1      | Mhhas1     | 52065          | NM_001081279.1 | malignant fibrous histiocytoma amplified sequence 1 |
| MIB1        | Mib1       | 225164         | NM_144860.2 | mindbomb homolog 1 (Drosophila) |
| MYT1L       | Myt1l      | 17933          | NM_001093775.1 | myelin transcription factor 1-like |
| NCAM1       | Ncam1      | 17967          | NM_001081455.1 | neural cell adhesion molecule 1 |
| NFIX        | Nfix       | 18032          | NM_001081981.1 | nuclear factor I/X (CCAAAT-binding transcription factor) |
| Npas2       | Npas2      | 18143          | NM_008719.2 | neuronal PAS domain protein 2 |
| Nrx1        | Nrx1       | 18189          | NM_020252.2 | neuroexin 1 |
| Nrx2        | Nrx2       | 18190          | NM_020253.2 | neuroexin 2 |
| Nrx3        | Nrx3       | 18191          | NM_172544.3 | neuroexin 3 |
| Ntrk3       | Ntrk3      | 18213          | NM_182809.2 | neurotrophic tyrosine kinase, receptor, type 3 |
| ODZ2        | Odz2       | 23964          | NM_011856.3 | odz, odd Oz/ten-m homolog 2 (Drosophila) |
| OGDH        | Ogdh       | 18293          | NM_010956.3 | oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide) |
| PCDH7       | Pcdh7      | 54216          | NM_001122758.1 | protocadherin 7 |
| PCDH9       | Pcdh9      | 211712         | NM_001081377.1 | protocadherin 9 |
| PHACTR1     | Phactr1    | 218194         | NM_198419.3 | phosphatase and actin regulator 1 |
| PLEXA4      | Plaxa4     | 243743         | NM_175750.3 | plexin A4 |
| PPARGC1A    | Ppargc1a   | 19017          | NM_008904.1 | peroxisome proliferator-activated receptor gamma, coactivator 1 alpha |
The SARE sequence for Cux1 is CTGGCCGAATCTGGCTGCCTGGCGTCCTGTGAGTAATTATAGCTCTGTTAACAGAGCAGGGAACAGGGAA-CACTTGCAAGTGACGGAGAT. The sequence for Cux2 TTAAATAAAGCTGTCACGACTCTTCCATCAGGAGG-GATGGGCTCCAAACATGAGAGTTTCCAGAGCCGT-GACTATAACAGGAGTGGAAATTTATCCCTTCTAAT-TATGTAATTGCATAATTTTAGGTAGCATTGGAAAT-TATGTA.

E18.5 neuronal cells cultured for 8 days were co-transfected with the corresponding firefly luciferase reporter constructs and internal control Renilla luciferase plasmid, at a ratio of 4:1 using lipofectamine 2000 (Invitrogen). Neuronal response was triggered using bicuculline/4-amino-pirydine as described above. Control cells Luciferase and Renilla activity was measured using the Dual-Luciferase Reporter Assay System (Promega) and following the manufacturer protocol. Relative expression of each reporter construct was determined by normalizing the ratio of reporter activity to the activity on TTX treated neurons.

Q-PCR Analysis

1 μg of total RNA was reverse transcribed with random primers (Invitrogen-Life Technologies, Carlsbad CA) and the superscript reverse transcriptase (New England BioLabs, Beverly, MA). PCR reaction mixtures containing DNA Master Sybr green I mix (Applied Biosystems, Foster City, CA) were incubated at 95°C for 5 min followed by 40 PCR cycles (5 s at 95°C, 45 s at 60°C, 90 s at 68°C) in an Abi-prism 7000 detector (Applied Byosystems). Primers for Robo 1 (forward GACCTGATCGTCTCAAAGGA; reverse TTGTCGGTCTCCACTCTTTCC); Robo2 (forward TGATGGATCTCGTCTTCGTCA; reverse GTCGGCCCTCTGCTTTACAG); Cux1 (forward GGGGCTTTTTATCTGCCATC; reverse CCCCCCTTCTCTCTTTACATTTGCAATATTGCGTCTGTTTAAGAAG); Cux2 (forward TCTGTCCTTCATTGCACAACC; reverse TTCGGAGGTGA-CTTGAAAC); Atf3 (forward TCCTGGGTCACTGG-TATTTG; reverse ATGGCGAATCTCAGCTCTTC); Impdh2 (forward CTCCAAAGATGCCAAGAAGC; reverse TGGGGAGTGACCCAAATTCACATTTCCCTTCTACATTTGCAATATTGCGTCTGTTTAAGAAG); Arc (forward TCGTCCCTCTATGGCACCAACC; reverse TTCCCGGGGTCACTGAATC); Homer1-S (forward CTCCAAAGATGCCAAGAAGC; reverse TGGGGAGTGACCCAAATTCACATTTCCCTTCTACATTTGCAATATTGCGTCTGTTTAAGAAG); Lmo4 (forward ACATTGGGCAATCGTCTGTTAC; reverse TCGGCGGGCTCTGCTTTTACAG); Plox A (forward TGGGAGGTGA-CTTGAAAC); and Plox B were tested. The results were normalized as indicated by the parallel amplification of β-actin (forward GGCTGTATTCCCCTCCATCG; reverse CCAGTTGGTAACTGTTCCACATTTCCCTTCTACATTTGCAATATTGCGTCTGTTTAAGAAG).

Table 4. Cont.

| Gene Symbol | mm9 Symbol | Entrez Gene ID | RefSeq ID | Description |
|-------------|------------|----------------|-----------|-------------|
| PRICKLE2    | Prickle2   | 243548         | NM_001081146.1 | prickle homolog 2 (Drosophila) |
| PTC1H       | Ptc1       | 19206          | NM_008957.2 | patched homolog 1 (Drosophila) |
| PTTPR6      | Ptp6       | 19270          | NM_008981.3 | protein tyrosine phosphatase, receptor type, G |
| PUM2        | Pum2       | 80913          | NM_030723.1 | pumilio homolog 2 (Drosophila) |
| R3HDMI      | R3hdm1     | 71750          | NM_027900.3 | R3H domain containing 2 |
| RAPGEF2     | Rapge2     | 76089          | NM_001099624.2 | Rap guanine nucleotide exchange factor (GEF) 2 |
| SASH1       | Sash1      | 70097          | NM_175155.4 | SAM and SH3 domain containing 1 |
| SLTRK5      | Slitrk5    | 75409          | NM_19865.1  | SLIT and NTRK-like family, member 5 |
| SMARCA2     | Smarca2    | 67155          | NM_011416.2 | SWI/SNF related, matrix associated, actin dependent regulator of chromatin |
| SMG1        | SMG1       | 26102070SRik   | 233789     | SMG1 homolog, phosphorylphosphatidinositol 3-kinase-related kinase (C. elegans) |
| SPRED1      | Spred1     | 114715         | NM_033524.2 | sprouty-related, EVH1 domain containing 1 |
| TANC2       | Tanc2      | 77097          | NM_181071.3 | tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 2 |
| TCF4        | Tcf4       | 21413          | NM_013685.2 | transcription factor 4 |
| TRD         | Tri       | 223435         | NM_001081302.1 | triple functional domain (PTPRF interacting) |
| TRIP12      | Trip12     | 14897          | NM_133975.4 | thyroid hormone receptor interactor 12 |
| VPS13D      | Vps13d     | 230895         | NM_001128198.1 | vacuolar protein sorting 13 homolog D (S. cerevisiae) |
| ZEB2        | Zeb2       | 24136          | NM_015753.3 | zinc finger E-box binding homeobox 2 |
| ZNF365      | Zfp365     | 216049         | NM_178679.2 | zinc finger protein 365 |
| ZNF462      | Zfp462     | 242466         | NM_172867.3 | zinc finger protein 462 |
| ZNF521      | Zfp521     | 225207         | NM_145492.2 | zinc finger protein 521 |
| ZNF536      | Zfp536     | 243937         | NM_172385.2 | zinc finger protein 536 |
| ZNF827      | Zfp827     | 622675         | NM_178267.3 | zinc finger protein 827 |

The list of genes identified as containing SARE sequences was compared to a list of 842 reliable FMRP targets. This resulted in an overlap of 70 genes common to both lists. This represents a significant enrichment of p = 4.39096.93e-13, far from the expected random distribution of coincidences between the genome and the mouse nervous system transcriptome (see Experimental Procedures).

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Statistical Analysis
Probability of overlap between the FMRP target gene, and the SARE-containing gene list was based on a binomial function, considering the size of the human genome as $28,000$ genes; the mouse nervous system transcriptome as $12,000$ transcripts and the total number of all genes associated to one or more SARE cluster is $827$. We calculated the density function that describes the probability of having a number of genes within the transcriptome, with the binomial $X = B(n, p = 827, p = 12/27)$. We then obtained the density function $Y$, which describes the probability of having a number of coincidences between both lists, with a new binomial

$$Y = \sum_{i=0}^{n} B(n_2 = 842, p = \frac{i}{12000}) \times X(i) \quad (n = \text{total number of FMRP targets}).$$

This probability distribution yielded a $p$ value of $4.3909 \times 10^{-13}$ for $Y(70)$ (for 70 coincidences). Equal analysis was performed to calculate the significance of overlapping between datasets from experimental data of bicuculline modulated genes and the SARE containing genes list.

Supporting Information
Table S1 SARE sequences conserved in human and mouse assigned to genes according to proximity. Using SynoR, we searched for regions containing clusters of the consensus binding sequences for SRF, MEF2 and CREB in the human genome and compared it to the mouse genome to identify conserved sequences. Based on these criteria, we identified 887 genetic regions with conserved SARE sequences that are assigned to the proximal genes: 530 clusters were found on intragenic regions and 357 in intergenic. (data deposited in the SynoR tool, ID n° s1219104005847). Additional tab (OtherTFclusters) show results from the analysis of other TFBs combinations and graphics showing the relative low number of clusters identified and their lower relation to neuronal functions.

Table S2 Gene Ontology analysis of SARE-containing genes. GO analysis of all genes containing the SARE cluster performed by Toppfun application. Additional tab on table shows the 21 GO categories out of the total 112 enriched GO categories of biological functions related to nervous system and the same analysis performed on subsets of SARE containing genes grouped as intergenic (distant regulated genes) or intronic, promoter, CDS and utr (closely regulated genes). Most of the functions seem to be regulated by both distant and close SARE, but some of them are specific to each category (light shaded blue).

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Author Contributions
Conceived and designed the experiments: MN FRT. Performed the experiments: FRT BC ISA. Analyzed the data: FRT ISA BC MN. Wrote the paper: FRT MN.

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