Published in final edited form as:

Cell Calcium. 2011 May ; 49(5): 290–295. doi:10.1016/j.ceca.2011.04.003.

Biological Functions and Transcriptional Targets of CaRF in Neurons

Anne E. West

Department of Neurobiology, Box 3209, Duke University Medical Center, Durham, NC USA
27710, Phone: 919-681-1909, FAX: 919-668-6269

Anne E. West: west@neuro.duke.edu

Abstract

Calcium-response factor (CaRF) is a unique DNA-binding protein first recognized as a transcription factor for its role in modulating transcription of the gene encoding Brain-Derived Neurotrophic Factor (BDNF) in neurons. Here I review evidence for the biological functions and transcriptional targets of CaRF in the brain and discuss potential mechanisms by which calcium-activated signaling pathways may modulate CaRF-dependent transcription. These data paint an emerging picture of CaRF as a regulatory nexus for signaling pathways that control aspects of synaptic development and neuronal function.

Introduction

Stimulus-regulated transcription factors coordinate the induction of intracellular adaptations in response to changes in the extracellular environment. In neurons, transcription factors play an important role in transducing synaptic activity into the long-lasting changes in neuronal structure and function that underlie neural development, homeostasis, and synaptic plasticity [1–4]. Hundreds of genes show activity-dependent changes in transcription, and many of these gene products have specific functions at synapses [5, 6]. Over 1000 genes in the mammalian genome are annotated as transcription factors [7], however only a small fraction of these gene products are likely to be important for the regulation of activity-dependent neuronal plasticity [8]. Identifying these essential transcription factors and dissecting their specific functions in neurons are important steps toward understanding how the brain adapts to its ever-changing environment.

Discovery of CaRF as a transcription factor

At excitatory synapses in the CNS, neurotransmitter reception triggers calcium influx across the plasma membrane through a number of ligand- and voltage-gated calcium channels [9]. This increase in calcium activates intracellular signaling cascades, including those mediated by kinases of the calcium-calmodulin kinase (CaMK) and Ras/mitogen-activated protein kinase (MAPK) signaling pathways as well as the calcium-regulated phosphatase calcineurin [10–12]. Post-translational modifications induced by these calcium-regulated signaling proteins subsequently modulate gene transcription by altering the function, localization, or expression of transcriptional regulators in the nucleus [1, 8].

© 2011 Elsevier Ltd. All rights reserved.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

A powerful first step toward identifying the transcription factors that mediate activity-regulated gene expression has been to map the calcium-response elements (CaREs) in activity-regulated promoters. By placing reporter genes such as choline acetyltransferase or luciferase under control of activity-regulated promoter fragments, it is possible to conduct scanning mutagenesis to identify promoter regions required for activity-regulated induction of reporter gene expression. The sequence of a particular CaRE may have homology to a known transcription factor binding site, suggesting candidate regulators, or it can serve as a template to screen for binding proteins. Ideally the functional significance of such CaREs and their binding proteins in regulation of the gene under study can then be confirmed through the generation of transgenic animals.

BDNF is a secreted protein of the neurotrophin family that has numerous functions in the nervous system including the promotion of neuronal survival and the modulation of synaptic function [13, 14]. Neuronal activity drives robust transcription from the *Bdnf* gene locus, providing a useful assay for the identification of activity-dependent transcriptional mechanisms [15, 16]. Unlike the ubiquitous inducibility of other well-studied activity-regulated genes such as *Fos* [17], inducible transcription of *Bdnf* is selective for calcium signaling pathways and restricted to neurons [18]. Thus understanding regulation of this gene has the potential to reveal neural selective mechanisms of transcription that may be particularly important for neuronal plasticity.

The mammalian *Bdnf* gene is comprised of nine exons with eight alternative promoters that are differentially used during development, across brain regions, and in different cell types [19, 20] (Fig. 1). Promoter IV (formerly referred to as “promoter III” in the five-exon nomenclature of the *Bdnf* gene, see [21]) is the most activity-responsive promoter in cultured embryonic neurons and thus has been the most extensively studied at the molecular level [22, 23]. The addition of 170bp of promoter IV is sufficient to confer calcium-dependent and neuronal selective induction upon a luciferase reporter plasmid, suggesting that key CaREs reside in this region proximal to the transcription start site (TSS). Concerted mutagenesis identified three distinct 10-12 base pair elements required for neuronal calcium-induced transcription from the *Bdnf* (pIV-170) reporter plasmid [23, 24] (Fig. 2). Mutation of any one of the three CaREs is sufficient to abrogate calcium-dependent transcription, raising the possibility that the factors binding these elements may work cooperatively to induce transcription from this *Bdnf* promoter.

The CaRE closest to the *Bdnf* promoter IV TSS (CaRE3) resembles a consensus calcium/cAMP response element (CRE), which is bound by transcription factors of the CREB family [22, 23]. Calcium-regulated neuronal signaling cascades induce CREB-dependent transcription via phosphorylation of CREB at Serine 133, which facilitates recruitment of the transcriptional co-activator and histone acetyltransferase CREB-binding protein (CBP) [25, 26]. The requirement for CaRE3 in activity-dependent regulation of *Bdnf* transcription was elegantly demonstrated by generation of a mouse strain bearing a mutation knocked into *Bdnf* promoter IV that changes the CaRE3 sequence (TCACGTCA) to a sequence that does not support CREB family protein binding (CAGCTGCA) [27]. Neurons from CaRE3 mutant mice have normal basal levels of BDNF but lack activity-inducible transcription from promoter IV, validating the requirement for this CaRE in activity-dependent *Bdnf* gene regulation *in vivo*. Interestingly, disruption of CaRE3 is associated with impaired *Bdnf* promoter IV recruitment of other transcriptional regulators including MEF2, which binds to a DNA sequence distinct from CaRE3. These data provide experimental support for the role of a multifactor transcriptional complex at *Bdnf* promoter IV, and suggest a function for CREB in nucleating assembly of this complex.

The CaRE2 site contains an E-box element (CANNTG), which is a consensus binding site for members of the basic helix-loop-helix (bHLH) family of transcription factors. Although many of the most well-known members of this family are involved in cell fate determination, other bHLH family proteins are more ubiquitously expressed [28]. A yeast one-hybrid screen using the CaRE2 sequence as bait identified two members of this latter class of bHLH proteins, the Upstream Stimulatory Factors USF1 and USF2, as CaRE2 binding proteins [24]. Genetic analysis of these factors in CNS development and function has been limited both by the fact that USF1 and USF2 are partially functionally redundant and because double *Usf1/Usf2* knockouts die during embryogenesis [29]. However the transcriptional activity of the USFs is subject to calcium-dependent regulation in neurons, and the USFs are found bound to a number of activity-regulated neuronal promoters, consistent with a role for these factors in regulation of activity-regulated programs of neuronal transcription [24]. In addition to the USFs, recent data have implicated an additional bHLH domain containing transcription factor, the bHLH-PAS domain factor Npas4, as a regulator of *Bdnf* promoter IV [30, 31]. However whether this protein binds *Bdnf* promoter IV via CaRE2 and whether the USFs and Npas4 play distinct or overlapping roles in the transcriptional regulation of *Bdnf* remain to be determined.

The CaRE1 site is the most distal to the *Bdnf* promoter IV TSS and has no obvious homology to known transcription factor binding sites. A yeast one-hybrid screen identified a CaRE1-binding protein encoded by a novel cDNA that we named Calcium-Response Factor (CaRF) to reflect its activity in this assay [18]. CaRF is a nuclear protein that is widely expressed in many tissues and cell types. Within the CNS, CaRF is preferentially expressed in neurons compared with glial cells, and expression levels peak during early postnatal development before falling back in adulthood [32]. The domain of CaRF that is required for DNA binding is highly similar between CaRF orthologs from Cnidarians to mammals, suggesting an evolutionarily conserved function for CaRF as a DNA binding protein [33]. Surprisingly, unlike most transcription factors, which belong to large families defined by similarity of their DNA binding domains [7], the DNA binding domain of CaRF is unique and shares no homology with any other protein. Yet CaRF binds DNA directly and it acts as a CaRE1-dependent transcriptional activator in heterologous cells [18, 33]. Importantly, CaRF's ability to bind variant CaRE1 sequences *in vitro* strongly correlates with the ability of those sequences to promote activity-dependent transcription of the *Bdnf* (pIV-170) reporter plasmid [18]. Based on these data we proposed CaRF as a strong candidate for a transcription factor that regulates *Bdnf* and presumably other neural genes.

Stimulus- and brain region-selective CaRF-dependent regulation of *Bdnf*

Just because a transcription factor binds a sequence from a gene promoter does not mean that the factor is required for the functions of that regulatory element *in vivo*. For example, although both USF1 and USF2 are bound to *Bdnf* promoter IV in neurons, knockout of either one of factors alone has no impact on activity-dependent *Bdnf* exon IV transcription, suggesting they have redundant or at least overlapping functions in CaRE2-dependent transcriptional regulation of *Bdnf* [24].

Evidence that CaRF is required for transcriptional regulation of *Bdnf* promoter IV in neurons *in vivo* comes from analysis of *Bdnf* expression in the brains of *Carf* knockout (KO) mice. These mice lack exon VIII of the *Carf* gene and are functional nulls [32]. *Carf* KO mice are viable and fertile, and they show no overt behavioral or neurological deficits. Although *Bdnf* mRNA and protein levels are unchanged in the hippocampus and striatum of adult *Carf* KO mice compared with their wildtype (WT) littermates, there is a significant reduction in the expression of both *Bdnf* mRNA and BDNF protein in the frontal cortex of the KO mice. This reduction is specific for exon IV-containing *Bdnf* transcripts, whereas expression of

transcripts generated by the three other most transcriptionally active *Bdnf* promoters in the adult brain (exon I, II, and VI-containing isoforms) are unchanged. Although these data do not prove that CaRF regulates *Bdnf* transcription directly via CaRE1 binding, the restriction of the deficit to exon IV-containing forms of *Bdnf* is consistent with a role for CaRF at this promoter.

What is surprising about these data is the regional selectivity of the requirement for CaRF, especially given the widespread and largely similar expression of CaRF across most brain regions [32]. Historically there exists some precedence for regional differences in the regulation of *Bdnf* transcription, though the mechanisms have not been fully understood [34–36]. In addition, since CaRE1 is required for activity-dependent transcription of the *Bdnf* (pIV-170) reporter plasmid, it might have been anticipated that *Carf* KO mice would show impaired activity-inducible *Bdnf* exon IV expression. By contrast, although cultured cortical *Carf* KO neurons show reduced *Bdnf* exon IV expression prior to membrane depolarization, the activation of L-type voltage gated calcium channels robustly induces *Bdnf* exon IV expression in *Carf* KO neurons in a manner that is not discernibly different from that seen in WT neurons [32]. The fact that the loss of CaRF fails to phenocopy loss of CaRE1 in this assay indicates that there must exist additional CaRE1 binding proteins in addition to CaRF that contribute to the actions of this element. The identity of these factors remains unknown, however the evidence of their likely existence further adds to the rich and complex mechanisms that confer tight temporal and spatial regulation on the transcription of *Bdnf* in the CNS.

Learning and memory in *Carf* KO mice

BDNF is a secreted protein that plays numerous important roles in the brain by binding to and activating signaling through its transmembrane receptors, p75 and TrkB [13, 37]. At the cellular level, BDNF signaling has been shown to contribute to neuronal survival and death, dendrite outgrowth, synapse formation, and synaptic plasticity. Constitutive null mutations in *Bdnf* significantly impair postnatal survival [38, 39], however mouse models bearing hypomorphic mutations or conditional deletions of *Bdnf* have been used to study the role of this signaling pathway in adult brain plasticity and behavior [40, 41]. These studies have suggested important roles for BDNF in the regulation of feeding behavior [42], reward processing [43], social behaviors [44], and learning and memory [45]. *Carf* KO mice are cortical BDNF hypomorphs, suggesting that they too might provide a useful model system for studying behavioral functions of BDNF.

There is a particularly rich set of behavioral tasks that can be used to assay learning and memory in rodents. Tests of declarative memory rely on the recollection of facts as well as remembering relationships among the elements being learned. The Morris water maze tests for memory of spatial locations [46], while in the novel object task the spontaneous preference for novelty provides an index of memory for the recognition of a familiar object [47]. By contrast contextual fear conditioning is an associative learning task that tests emotional memory [48]. Each of these tasks requires intact hippocampal function, and for each BDNF has been implicated in the learning and/or memory process [49, 50].

During the acquisition and early retention phases of all three of these learning and memory tasks, *Carf* KO mice perform no differently from their WT littermates [32]. However at later time points of remote memory testing in the novel object task, and in relearning tasks that include platform reversal learning in the Morris water maze and extinction of condition fear, the *Carf* mice show significant abnormalities. One commonality amongst these tasks is the involvement of frontal cortical brain regions. Performance in the remote memory phase of the novel object recognition task, which takes place 10 days after initial exposure to the

objects, is not affected by hippocampal lesions and is thought instead to depend on transfer of the memory trace to the cortex [51]. Similarly, the relearning of the context associations in fear extinction and the cognitive flexibility that permits reversal learning are thought to involve aspects of frontal cortex function [48, 52]. Furthermore these tasks are affected by expression of BDNF. For example, extinction of conditioned fear is associated with induced expression of *Bdnf* exons I and IV in the prefrontal cortex [53] and both mice and humans bearing a mutation in the *Bdnf* gene (Val66Met) that reduces levels of secreted mature BDNF protein have defects in the extinction of fear conditioning [45].

These behavioral data demonstrate that CaRF is important for the organization and/or function of neural circuits that underlie essential aspect of learning and memory. However by what mechanisms CaRF regulates memory in these tasks remains unknown. It is possible that stimulus-dependent regulation of CaRF contributes to the expression of genes including *Bdnf* that mediate the plasticity underlying learning and memory. Alternatively because the *Carf* KO mice are constitutive mutants, it is also possible that abnormal performance in these tasks could arise as a secondary consequence of altered brain development. Insight into these alternative possibilities may be derived from understanding how CaRF works at the cellular level.

Transcriptional targets and cellular functions of CaRF

The potential set of target genes for any transcription factor depends on its DNA binding specificity, which determines where in the genome the factor can bind. However not all binding sites are occupied by any given transcription factor at any given time [54, 55]. It is thought that the differential usage of binding sites arises in part because secondary structural constraints of chromatin differentially regulate the availability of DNA elements for transcription factor binding [56]. In addition, the apparent affinity of transcription factors for specific binding sequences in the genome may be substantially enhanced by local protein-protein interactions *in vivo* [27]. For these reasons although clues to transcriptional targets may be derived from knowledge of a protein's DNA binding specificity, the most important recent insights into transcription factor biology have come from the application of technologies that experimentally capture the actual sites of transcription factor binding across the genome *in vivo* [57]. These methods rely upon chromatin immunoprecipitation (ChIP), in which formaldehyde crosslinking is used to stabilize endogenous transcription factor-DNA interactions [58]. After cell lysis, genomic DNA is sheared into small fragments by sonication and an antibody against the transcription factor is used to immunoprecipitate protein-DNA complexes. The coimmunoprecipitated DNA is then hybridized to promoter microarrays or analyzed by high-throughput sequencing to map sites of transcription factor binding across the genome [59].

ChIP-Seq for CaRF identified 176 binding sites that were significantly enriched in anti-CaRF immunoprecipitates from *Carf* WT neurons compared with *Carf* KO neurons [33]. Most of the CaRF binding sites contain a common 10bp element with significant similarity to the optimal CaRF-binding sequence, consistent with the possibility that CaRF binds these sites directly. However a fraction of sequences lack an obvious CaRF binding site and may represent sites to which CaRF is recruited through protein-protein interactions with other DNA binding proteins, or these may be regions of DNA that make long-distance interactions with CaRF binding sites through the secondary structure of chromatin [57]. Proximal promoter regions are highly over-represented among CaRF binding sites, suggesting that CaRF is likely to be among the class of transcription factors (e.g. E2F and CREB) that regulate gene expression largely through their association with sequences near the transcriptional start site of genes [60, 61]. However a subset of CaRF binding sites are also found outside proximal promoters raising the possibility that CaRF could also be acting at

other kinds of DNA regulatory elements such as long-distance enhancers or promoters for non-coding transcripts [31, 60].

Because genes that neighbor the sites of CaRF binding represent a likely set of targets for CaRF-dependent transcriptional regulation, categorical analysis of the functions of these target genes can offer insight into the potential cellular functions of CaRF. One way to analyze function in a set of target genes is to test for statistical over-representation of specific gene ontology (GO) categories. In the set of CaRF targets this analysis points toward the importance of gene products that play a role in phosphorous metabolism and regulation of post-translational protein modifications [33]. 19 of the CaRF binding sites neighbor genes whose products function in important intracellular signaling pathways, including the cAMP-dependent protein kinase regulatory subunit *Prkar1a*, the mitogen-activated protein kinase pathway members *Map3k3*, and *Map4k4*, and the E3-ubiquitin ligase *Park2*. Although the requirement for CaRF in regulation of all of these gene products has not yet been tested, *Carf* knockout mice show altered expression of at least a subset of the potential target genes tested so far consistent with the possibility that CaRF may affect the activity of these signaling cascades by regulating expressing of specific members of these pathways. Although CaRF was first characterized as a transcriptional activator, *Carf* KO neurons show upregulated expression of some of the putative target genes, while showing downregulation of others. These data suggest that CaRF could function as both an activator and a repressor, although the detailed mechanisms of how CaRF modulates transcription remain to be fully elucidated.

Interestingly, an additional six gene products among the CaRF targets are notable for their involvement in neuronal calcium signaling pathways. These include calcium-dependent regulators of cytoskeleton (*Camsap111*) [62], transcription (*Camta1*) [63], kinase pathways (*Camk2n1*) [64], synaptic vesicle release (*Syt1*) [65], and postsynaptic neurotransmitter receptor trafficking (*Caly* and *Cacng2*) [66, 67]. This finding is particularly interesting in light of the essential importance of calcium signaling pathways for the regulation of both neuronal transcription and synaptic plasticity [1, 68]. Whether there are changes in synaptic function or synaptic plasticity in *Carf* KOs has not been investigated, however these mice do show altered composition of striatal GABAergic synapses [32]. These synaptic changes include increased expression of the GABAA receptor $\gamma 2$ and $\beta 2/3$ subunits as well as increased synaptic localization of the GABA synthesizing enzyme Gad65. These changes could arise as a primary effect of CaRF on GABAergic synapse development or they could reflect compensatory changes at GABAergic synapses for altered synaptic input or cell excitability. In either case, these observations indicate that CaRF is required at some level for the regulation of synapses, and they further suggest that GABAergic synapse development may be a useful cellular assay for analyzing potential functions of the CaRF regulon.

Is CaRF a target of activity-dependent regulation?

The line of investigation that led to the discovery of CaRF began with the goal of understanding how neuronal activity leads to changes in gene transcription. Since the CaREs mediate calcium-regulated transcription of *Bdnf* promoter IV, it was presumed that the transcription factors bound to the CaREs could be targets of calcium-regulated signaling pathways. Neuronal activity can impinge on the regulation of transcription factors by modulating their nuclear localization, their protein-protein or protein-DNA interactions, or their expression [8]. CaRF is a constitutively nuclear protein, and *in vitro* DNA binding assays revealed no evidence for activity-dependent changes in CaRF's affinity for CaRE1 binding [18].

Stimulus-dependent regulation of transcription factor activity is commonly studied by tethering the activation domain of the transcription factor of interest down onto a reporter gene as a fusion protein with the DNA binding domain of the yeast transcription factor Gal4, which binds with high affinity and selectivity to the upstream activating sequence (UAS). When the transcriptional activation domain of CaRF is tethered to a UAS-Luciferase reporter in neurons, membrane depolarization drives a significant increase in CaRF-dependent luciferase expression that is blocked by the L-type voltage gated calcium channel inhibitor nimodipine, suggesting that activation of calcium-dependent signaling pathways is required for this increase in CaRF-dependent transcription [18]. Whether CaRF activity can also be enhanced by calcium influx through other channels such as NMDA-type glutamate receptors has not been tested. Intriguingly, like the activation of *Bdnf* transcription, the enhancement of CaRF's transcriptional activity in this assay is both calcium- and neuronal-selective, although the mechanism of this selectivity remains unknown. For many transcriptional regulators, calcium-dependent signaling pathways change factor activity by inducing post-translational modifications that promote protein-protein interactions and ultimately RNA polymerase II recruitment [1]. When FLAG-tagged human CaRF was overexpressed in 293T cells, and tryptic peptides of purified CaRF were subjected to tandem mass spectrometry, we detected phosphorylation at 3 serine residues (Ser140, SPRD; Ser 541, SPEP; and Ser 589, SPST) and acetylation at one lysine residue (Lys 453, ELFK) that are conserved between mouse and human (A.E. West and S. P. Gygi, unpublished observations) (Fig. 3A). Whether these sites are functionally important for CaRF-dependent transcription or regulated by calcium-signaling pathways remains to be determined.

Although the studies described above indicate that CaRF-dependent transcription can be enhanced by L-type voltage gated calcium channel activated signaling cascades, demonstration of the importance of this regulatory mechanism for CaRF-dependent gene transcription *in vivo* has proved elusive. As described in the *Bdnf* section above, *Carf* KO neurons show normal membrane depolarization-induced expression of *Bdnf* exon IV. In addition, very few activity-induced genes are found among the set of CaRF targets identified by ChIP-Seq. However this does not rule out the possibility that CaRF may be subject to other forms of stimulus-dependent regulation. For example, a recent study reported that repeated serotonin transporter blockade for 7 or 21 days *in vivo* with the drug escitalopram enhanced expression of CaRF in the prefrontal cortex of rats and depressed expression in the hippocampus [34]. Furthermore, the magnitude, regional distribution, and time course of these changes parallel changes in *Bdnf* expression. Unlike some other activity-regulated transcription factors such as CREB, which have little ability to promote transcription until phosphorylated by stimulus-induced signaling cascades, CaRF shows significant basal transcriptional activity in unstimulated neurons [18]. Thus changing expression levels of this transcription factor may be sufficient to have a significant impact on expression of its target genes. How escitalopram regulates CaRF expression is not known, however in a recent genome-wide RNA-Seq study, *Carf* was among a set of genes whose RNA levels were significantly *reduced* in cultured hippocampal neurons 6 hours following membrane depolarization [31]. This finding raises the interesting possibility that calcium signaling pathways could upregulate the activity but downregulate the expression of CaRF on different time scales, allowing for differential temporal regulation of the transcription of CaRF target genes (Fig. 3B).

Conclusions

The identification of CaRF as a regulator of *Bdnf* transcription raised the possibility that this transcription factor might contribute to neuronal development and/or plasticity. The studies reviewed here have shown memory defects in *Carf* knockout mice and identified the enrichment of signaling pathways including calcium signaling proteins among the set of

CaRF target genes, strongly supporting this hypothesis. Future studies that address the role of CaRF in regulating the structure and function of synapses, as well as further elucidation of mechanisms by which neuronal activity regulates CaRF-dependent transcription will substantially advance understanding of this unique transcription factor.

References

1. Greer PL, Greenberg ME. From synapse to nucleus: calcium-dependent gene transcription in the control of synapse development and function. *Neuron*. 2008; 59:846–60. [PubMed: 18817726]
2. Polleux F, Ince-Dunn G, Ghosh A. Transcriptional regulation of vertebrate axon guidance and synapse formation. *Nat Rev Neurosci*. 2007; 8:331–40. [PubMed: 17453014]
3. Kandel ER. The molecular biology of memory storage: a dialogue between genes and synapses. *Science*. 2001; 294:1030–8. [PubMed: 11691980]
4. Nestler EJ. Molecular basis of long-term plasticity underlying addiction. *Nat Rev Neuro*. 2001; 2:119–128.
5. Zhang SJ, Steijaert MN, Lau D, Schutz G, Delucinge-Vivier C, Descombes P, Bading H. Decoding NMDA receptor signaling: identification of genomic programs specifying neuronal survival and death. *Neuron*. 2007; 53:549–62. [PubMed: 17296556]
6. Nedivi E, Hevroni D, Naot D, Israeli D, Citri Y. Numerous candidate plasticity-related genes revealed by differential cDNA cloning. *Nature*. 1993; 363:718–722. [PubMed: 8515813]
7. Vaquerizas JM, Kummerfeld SK, Teichmann SA, Luscombe NM. A census of human transcription factors: function, expression and evolution. *Nat Rev Genet*. 2009; 10:252–63. [PubMed: 19274049]
8. West AE, Griffith EC, Greenberg ME. Regulation of transcription factors by neuronal activity. *Nat Rev Neuro*. 2002; 3:921–931.
9. Sabatini BL, Maravall M, Svoboda K. Ca(2+) signaling in dendritic spines. *Curr Opin Neurobiol*. 2001; 11:349–56. [PubMed: 11399434]
10. Deisseroth K, Mermelstein PG, Xia H, Tsien RW. Signaling from synapse to nucleus: the logic behind the mechanisms. *Curr Opin Neurobiol*. 2003; 13:354–65. [PubMed: 12850221]
11. Wayman GA, Lee YS, Tokumitsu H, Silva A, Soderling TR. Calmodulin-kinases: modulators of neuronal development and plasticity. *Neuron*. 2008; 59:914–31. [PubMed: 18817731]
12. Burgoyne RD. Neuronal calcium sensor proteins: generating diversity in neuronal Ca²⁺ signalling. *Nat Rev Neurosci*. 2007; 8:182–93. [PubMed: 17311005]
13. Poo, M-m. Neurotrophins as synaptic modulators. *Nat Rev Neuro*. 2001; 2:24–32.
14. Lewin GR, Barde YA. Physiology of the neurotrophins. *Annu Rev Neurosci*. 1996; 19:289–317. [PubMed: 8833445]
15. West AE, Chen WG, Dalva MB, Dolmetsch RE, Kornhauser JM, Shaywitz AJ, Takasu MA, Tao X, Greenberg ME. Calcium regulation of neuronal gene expression. *Proc Natl Acad Sci, USA*. 2001; 98:11024–11031. [PubMed: 11572963]
16. Zafra F, Hengerer B, Leibrock J, Thoenen H, Lindholm D. Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. *EMBO J*. 1990; 9:3545–3550. [PubMed: 2170117]
17. Sheng M, Dougan ST, McFadden G, Greenberg ME. Calcium and growth factor pathways of c-fos transcriptional activation require distinct upstream regulatory sequences. *Mol Cell Biol*. 1988; 8:2787–96. [PubMed: 3136322]
18. Tao X, West AE, Chen WG, Corfas G, Greenberg ME. A calcium-responsive transcription factor, CaRF, that regulates neuronal activity-dependent expression of BDNF. *Neuron*. 2002; 33:383–395. [PubMed: 11832226]
19. Liu QR, Lu L, Zhu XG, Gong JP, Shaham Y, Uhl GR. Rodent BDNF genes, novel promoters, novel splice variants, and regulation by cocaine. *Brain Res*. 2006; 1067:1–12. [PubMed: 16376315]
20. Aid T, Kazantseva A, Piirsoo M, Palm K, Timmusk T. Mouse and rat BDNF gene structure and expression revisited. *J Neurosci Res*. 2007; 85:525–35. [PubMed: 17149751]

21. Timmusk T, Palm K, Metsis M, Reintam T, Paalme V, Saarma M, Persson H. Multiple promoters direct tissue-specific expression of the rat BDNF gene. *Neuron*. 1993; 10:475–489. [PubMed: 8461137]
22. Shieh PB, Hu S-C, Bobb K, Timmusk T, Ghosh A. Identification of a signaling pathway involved in calcium regulation of BDNF expression. *Neuron*. 1998; 20:727–740. [PubMed: 9581764]
23. Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME. Ca^{2+} influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. *Neuron*. 1998; 20:709–726. [PubMed: 9581763]
24. Chen WG, West AE, Tao X, Corfas G, Szentirmay M, Sawadogo M, Vinson C, Greenberg ME. Upstream stimulatory factors are mediators of calcium-responsive transcription in neurons. *J Neurosci*. 2003; 23:2572–2581. [PubMed: 12684442]
25. Sheng M, Thompson MA, Greenberg ME. CREB: A Ca^{2+} -regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science*. 1991; 252:1427–1430. [PubMed: 1646483]
26. Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR, Goodman RH. Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature*. 1993; 365:855–859. [PubMed: 8413673]
27. Hong EJ, McCord AE, Greenberg ME. A biological function for the neuronal activity-dependent component of *Bdnf* transcription in the development of cortical inhibition. *Neuron*. 2008; 60:610–624. [PubMed: 19038219]
28. Ross SE, Greenberg ME, Stiles CD. Basic helix-loop-helix factors in cortical development. *Neuron*. 2003; 39:13–25. [PubMed: 12848929]
29. Sirito M, Lin Q, Deng JM, Behringer RR, Sawadogo M. Overlapping roles and asymmetrical cross-regulation of the USF proteins in mice. *Proc Natl Acad Sci U S A*. 1998; 95:3758–63. [PubMed: 9520440]
30. Lin Y, Bloodgood BL, Hauser JL, Lapan AD, Koon AC, Kim TK, Hu LS, Malik AN, Greenberg ME. Activity-dependent regulation of inhibitory synapse development by Npas4. *Nature*. 2008; 455:1198–1204. [PubMed: 18815592]
31. Kim TK, Hemberg M, Gray JM, Costa AM, Bear DM, Wu J, Harmin DA, Laptewicz M, Barbara-Haley K, Kuersten S, Markenscoff-Papadimitriou E, Kuhl D, Bito H, Worley PF, Kreiman G, Greenberg ME. Widespread transcription at neuronal activity-regulated enhancers. *Nature*. 2010; 465:182–187. [PubMed: 20393465]
32. McDowell KA, Hutchinson AN, Wong-Goodrich SJE, Presby MM, Su D, Rodriguiz RM, Law KC, Williams CL, Wetsel WC, West AE. Reduced cortical BDNF expression and aberrant memory in *Carf* knockout mice. *J Neurosci*. 2010; 30:7453–7465. [PubMed: 20519520]
33. Pfenning AR, Kim TK, Spotts JM, Hemberg M, Su D, West AE. Genome-wide identification of calcium-response factor (CaRF) binding sites predicts a role in regulation of neuronal signaling pathways. *PLoS ONE*. 2010; 5:e10870. [PubMed: 20523734]
34. Alboni S, Benatti C, Capone G, Corsini D, Caggia F, Tascetta F, Mendlewicz J, Brunello N. Time-dependent effects of escitalopram on brain derived neurotrophic factor (BDNF) and neuroplasticity related targets in the central nervous system of rats. *Eur J Pharmacol*. 2010; 643:180–187. [PubMed: 20599917]
35. Dennis KE, Levitt P. Regional expression of brain derived neurotrophic factor (BDNF) is correlated with dynamic patterns of promoter methylation in the developing mouse forebrain. *Brain Res Mol Brain Res*. 2005; 140:1–9. [PubMed: 16054725]
36. Poulsen FR, Lauterborn J, Zimmer J, Gall CM. Differential expression of brain-derived neurotrophic factor transcripts after pilocarpine-induced seizure-like activity is related to mode of Ca^{2+} entry. *Neuroscience*. 2004; 126:665–76. [PubMed: 15183516]
37. Chao MV. Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nat Rev Neurosci*. 2003; 4:299–309. [PubMed: 12671646]
38. Jones KR, Farinas I, Backus C, Reichardt LF. Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. *Cell*. 1994; 76:989–999. [PubMed: 8137432]

39. Ernfors P, Lee K-F, Jaenisch R. Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature*. 1994; 368:147–150. [PubMed: 8139657]
40. Sakata K, Jin L, Jha S. Lack of promoter IV-driven BDNF transcription results in depression-like behavior. *Genes Brain Behav*. 2010 May 26. ePub.
41. Rios M, Fan G, Fekete C, Kelly J, Bates B, Kuehn R, Lechan RM, Jaenisch R. Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity. *Mol Endocrinol*. 2001; 15:1748–57. [PubMed: 11579207]
42. Cordeira JW, Frank L, Sena-Esteves M, Pothos EN, Rios M. Brain-derived neurotrophic factor regulates hedonic feeding by acting on the mesolimbic dopamine system. *J Neurosci*. 2010; 30:2533–41. [PubMed: 20164338]
43. Graham DL, Edwards S, Bachtell RK, DiLeone RJ, Rios M, Self DW. Dynamic BDNF activity in nucleus accumbens with cocaine use increases self-administration and relapse. *Nat Neurosci*. 2007; 10:1029–37. [PubMed: 17618281]
44. Berton O, McClung CA, Dileone RJ, Krishnan V, Renthal W, Russo SJ, Graham D, Tsankova NM, Bolanos CA, Rios M, Monteggia LM, Self DW, Nestler EJ. Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. *Science*. 2006; 311:864–8. [PubMed: 16469931]
45. Soliman F, Glatt CE, Bath KG, Levita L, Jones RM, Pattwell SS, Jing D, Tottenham N, Amso D, Somerville L, Voss HU, Glover G, Ballon DJ, Liston C, Teslovich T, Van Kempen T, Lee FS, Casey BJ. A Genetic Variant BDNF Polymorphism Alters Extinction Learning in Both Mouse and Human. *Science*. 2010; 327:863–6. [PubMed: 20075215]
46. Vorhees CV, Williams MT. Morris water maze: procedures for assessing spatial and related forms of learning and memory. *Nat Protoc*. 2006; 1:848–58. [PubMed: 17406317]
47. Clark RE, Martin SJ. Interrogating rodents regarding their object and spatial memory. *Curr Opin Neurobiol*. 2005; 15:593–8. [PubMed: 16150589]
48. Myers KM, Davis M. Mechanisms of fear extinction. *Mol Psychiatry*. 2007; 12:120–50. [PubMed: 17160066]
49. Rattiner LM, Davis M, French CT, Ressler KJ. Brain-derived neurotrophic factor and tyrosine kinase receptor B involvement in amygdala-dependent fear conditioning. *J Neurosci*. 2004; 24:4796–806. [PubMed: 15152040]
50. Heldt SA, Stanek L, Chhatwal JP, Ressler KJ. Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. *Mol Psychiatry*. 2007; 12:656–70. [PubMed: 17264839]
51. Frankland PW, Bontempi B. The organization of recent and remote memories. *Nat Rev Neurosci*. 2005; 6:119–30. [PubMed: 15685217]
52. Arnsten AF, Li BM. Neurobiology of executive functions: catecholamine influences on prefrontal cortical functions. *Biological Psychiatry*. 2005; 57:1377–84. [PubMed: 15950011]
53. Bredy TW, Wu H, Crego C, Zellhoefer J, Sun YE, Barad M. Histone modifications around individual BDNF gene promoters in prefrontal cortex are associated with extinction of conditioned fear. *Learn Mem*. 2007; 14:268–76. [PubMed: 17522015]
54. Cooper SJ, Trinklein ND, Nguyen L, Myers RM. Serum response factor binding sites differ in three human cell types. *Genome Research*. 2007; 17:136–44. [PubMed: 17200232]
55. Cha-Molstad H, Keller DM, Yochum GS, Impey S, Goodman RH. Cell-type-specific binding of the transcription factor CREB to the cAMP-response element. *Proc Natl Acad Sci U S A*. 2004; 101:13572–7. [PubMed: 15342915]
56. Boyle AP, Davis S, Shulha HP, Meltzer P, Margulies EH, Weng Z, Furey TS, Crawford GE. High-resolution mapping and characterization of open chromatin across the genome. *Cell*. 2008; 132:311–22. [PubMed: 18243105]
57. Farnham PJ. Insights from genomic profiling of transcription factors. *Nat Rev Genet*. 2009; 10:605–616. [PubMed: 19668247]
58. Wells J, Farnham PJ. Characterizing transcription factor binding sites using formaldehyde crosslinking and immunoprecipitation. *Methods*. 2002; 26:48–56. [PubMed: 12054904]
59. Park PJ. ChIP-seq: advantages and challenges of a maturing technology. *Nat Rev Genet*. 2009; 10:669–80. [PubMed: 19736561]

60. Impey S, McCorkle SR, Cha-Molstad H, Dwyer JM, Yochum GS, Boss JM, McWeeney S, Dunn JJ, Mandel G, Goodman RH. Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions. *Cell*. 2004; 119:1041–54. [PubMed: 15620361]
61. Bieda M, Xu X, Singer MA, Green R, Farnham PJ. Unbiased location analysis of E2F1-binding sites suggests a widespread role for E2F1 in the human genome. *Genome Res*. 2006; 16:595–605. [PubMed: 16606705]
62. Baines AJ, Bignone PA, King MD, Maggs AM, Bennett PM, Pinder JC, Phillips GW. The CKK domain (DUF1781) binds microtubules and defines the CAMSAP/ssp4 family of animal proteins. *Mol Biol Evolution*. 2009; 26:2005–14.
63. Bouche N, Scharlat A, Snedden W, Bouchez D, Fromm H. A novel family of calmodulin-binding transcription activators in multicellular organisms. *J Biol Chem*. 2002; 277:21851–61. [PubMed: 11925432]
64. Lepicard EM, Mizuno K, Antunes-Martins A, von Hertzen LS, Giese KP. An endogenous inhibitor of calcium/calmodulin-dependent kinase II is up-regulated during consolidation of fear memory. *Eur J Neurosci*. 2006; 23:3063–70. [PubMed: 16819996]
65. Brose N, Petrenko AG, Sudhof TC, Jahn R. Synaptotagmin: a calcium sensor on the synaptic vesicle surface. *Science*. 1992; 256:1021–5. [PubMed: 1589771]
66. Chen L, Chetkovich DM, Petralia RS, Sweeney NT, Kawasaki Y, Wenthold RJ, Brecht DS, Nicoll RA. Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature*. 2000; 408:936–43. [PubMed: 11140673]
67. Davidson HT, Xiao J, Dai R, Bergson C. Calcyon is necessary for activity-dependent AMPA receptor internalization and LTD in CA1 neurons of hippocampus. *Eur J Neurosci*. 2009; 29:42–54. [PubMed: 19120439]
68. Lisman J, Schulman H, Cline H. The molecular basis of CaMKII function in synaptic and behavioural memory. *Nat Rev Neurosci*. 2002; 3:175–90. [PubMed: 11994750]

Figure 1. Organization of the *Bdnf* gene

Genbank mRNAs encoding *Bdnf* mapped onto chromosome 2 from *Mus musculus*. The numbers show genomic coordinates for the *Bdnf* gene from the July 2007 mm9 assembly of the mouse genome (<http://genome.ucsc.edu>). Black boxes represent exons and the thicker black region of exon IX indicates the coding sequence. The gray lines show the splice variants of *Bdnf* arising from alternate promoter usage.

Figure 2. Transcription factors associated with the CaREs of *Bdnf* promoter IV

Three CaREs are found in the 170bp upstream of the TSS of *Bdnf* exon IV. The CaREs are found within a nucleosome-free region of the proximal promoter flanked by two well-positioned nucleosomes (B. Li and A.E. West, unpublished observations). The histones (H3 and H4) in these nucleosomes are modified by acetylation (Ac) and methylation (me1 or me3) at specific lysine (K) residues [31]. Although both MEF2 and Npas4 have been localized to *Bdnf* promoter IV by ChIP, their binding elements have not yet been reported. The question mark in the circle on CaRE1 represents the likely presence of additional CaRE1 binding proteins that mediate the function of this element in the absence of CaRF. The numbers represent base position with respect to the TSS. PolII, RNA polymerase II; CBP, the histone acetyltransferase CREB binding protein; P, phosphorylation of CREB at serine 133, which recruits CBP to *Bdnf* promoter IV.

Figure 3. Mechanisms of activity-dependent regulation of CaRF

A) Post-translational modifications of CaRF. The numbers represent amino acid positions from human CaRF. P, phosphorylation; Ac, acetylation; S, serine; K, lysine; DBD, DNA binding domain; TAD, transcriptional activation domain. B) Model for the bimodal calcium-dependent regulation of CaRF-mediated transcription. Membrane depolarization induced by the elevation of extracellular potassium leads to the opening of voltage-gated calcium channels. This stimulus is associated with an increase in the transcriptional activity of the CaRF activation domain tethered via UAS-Gal4 to a luciferase reporter plasmid, whereas the same stimulus results in a decrease in *Carf* mRNA expression 6 hours after membrane depolarization.