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Mechanisms of specificity in neuronal activity-regulated gene transcription

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Abstract

The brain is a highly adaptable organ that is capable of converting sensory information into changes in neuronal function. This plasticity allows behavior to be accommodated to the environment, providing an important evolutionary advantage. Neurons convert environmental stimuli into long-lasting changes in their physiology in part through the synaptic activity-regulated transcription of new gene products. Since the neurotransmitter-dependent regulation of *Fos* transcription was first discovered nearly 25 years ago, a wealth of studies have enriched our understanding of the molecular pathways that mediate activity-regulated changes in gene transcription. These findings show that a broad range of signaling pathways and transcriptional regulators can be engaged by neuronal activity to sculpt complex programs of stimulus-regulated gene transcription. However, the shear scope of the transcriptional pathways engaged by neuronal activity raises the question of how specificity in the nature of the transcriptional response is achieved in order to encode physiologically relevant responses to divergent stimuli. Here we summarize the general paradigms by which neuronal activity regulates transcription while focusing on the molecular mechanisms that confer differential stimulus-, cell-type-, and developmental-specificity upon activity-regulated programs of neuronal gene transcription. In addition, we preview some of the new technologies that will advance our future understanding of the mechanisms and consequences of activity-regulated gene transcription in the brain.

Keywords

neuron; activity-regulated transcription; calcium signaling

1. Introduction

Stimulus-regulated transcription factors play an essential role in cell biology by coupling extracellular stimuli to coordinated intracellular responses. The basic principles of regulated transcription first emerged from the study of growth-factor dependent induction of *Fos* transcription in fibroblasts (Cochran *et al.*, 1984; Greenberg and Ziff, 1984; Müller *et al.*, 1984). Addition of growth factors promotes cell division, and some of the first growth factor-induced genes to be identified (i.e. *Fos* and *Myc*) were known proto-oncogenes, suggesting a role for this transcriptional program in mitogenesis (Stiles, 1985). Thus, it came as something of a surprise when *Fos* transcription was shown to be induced in

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neuroendocrine cells by stimuli including Nerve Growth Factor (NGF), which promotes differentiation, not division (Curran and Morgan, 1985; Greenberg and Ziff, 1984). The regulation of *Fos* transcription by neurotransmitters was quickly generalized to post-mitotic neurons of the central nervous system (CNS), where *Fos* transcription is robustly induced following seizure (Morgan *et al.*, 1987). These exciting results suggested the possibility that changes in neuronal activity levels might be capable of exerting long-lasting effects on neuronal gene expression, which could in turn influence various aspects of a given neuron's life and metabolism.

An important breakthrough in the understanding of neuronal activity-regulated transcription came from a series of studies that used differential cloning strategies to screen for activity-induced mRNAs (Hevroni *et al.*, 1998; Lanahan and Worley, 1998; Nedivi *et al.*, 1993; Qian *et al.*, 1993). These findings revealed that many of the genes induced by activity were selectively expressed in neurons and furthermore that some had known functions in neuronal or synaptic physiology (see review by Leslie and Nedivi, this issue). More recently, increasingly comprehensive screens have described complex patterns of gene transcription induced and/or repressed following different kinds of stimuli that act in concert to effect adaptations upon neuronal and synaptic physiology (Flavell *et al.*, 2008; Guan *et al.*, 2005; Kim *et al.*, 2010; Xiang *et al.*, 2007; Zhang *et al.*, 2007). A key theme to emerge from these studies is that of specificity, meaning that different kinds of stimuli up- and down-regulate distinct sets of genes (Bading *et al.*, 1993; Bartel *et al.*, 1989; Hardingham *et al.*, 2002). A major focus of the research effort directed at activity-regulated gene transcription has been to identify the molecular mechanisms that underlie this specificity. These findings have enhanced understanding of how neurons use information from the environment to properly and constantly adapt their development and plasticity to the world outside.

The goal of this review is to summarize major molecular advances that have been made in the understanding of neuronal activity-regulated gene transcription to date, and to describe where the field is heading in the future. We will 1) review the classes of genes and transcription factors that are regulated by neuronal activity, 2) describe how calcium signaling pathways differentially modulate transcription factors to confer specificity upon cellular responses to stimuli, 3) discuss the importance of activity-regulated transcriptional pathways in synaptic homeostasis, and finally 4) describe some of the technological developments that will advance future studies in this field. Because we focus on the idea of signaling specificity at many stages along the synapse to nucleus pathway, we do not cover every step in equal detail. Alternative reviews that offer additional detail about key steps in these pathways will be cited in the course of the discussion as they arise.

2. Transcriptional control of neuronal activity-regulated genes

Many studies have characterized the programs of gene transcription that are induced in response to altered activity states (Guan *et al.*, 2005; Hevroni *et al.*, 1998; Lanahan and Worley, 1998; Nedivi *et al.*, 1993; Qian *et al.*, 1993; Zhang *et al.*, 2007). Although these gene expression programs vary by brain region and stimulus type, there are some important commonalities. Specifically, these studies have shown that there are two particularly important classes of genes that are regulated by neuronal activity: 1) classic immediate-early gene transcription factors, which are general response factors in a broad range of cell types and 2) neuronally-enriched gene products with specific functions at synapses. The evidence that neural activity can change the expression of synaptic gene products suggests that study of this second class in particular may reveal neural-selective mechanisms of transcription that are of particular importance for synaptic plasticity. Here we review the transcriptional mechanisms that regulate key genes in these two classes to exemplify the molecular pathways that differentially control stimulus-regulated gene transcription in neurons.

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2.1. *Fos*: the archetype

In fibroblasts, *Fos* is only one of a set of genes whose transcription is rapidly induced by growth factors. Since induction of this transcriptional program proceeds without the need for prior protein synthesis, these genes were termed "immediate early genes" (IEGs) in analogy to the protein synthesis-independent gene expression program that underlies viral oncogenesis (Lau and Nathans, 1987). IEGs include members of the Fos, Jun, early growth response (Egr), and nuclear receptor (Nr) families of transcription factors. In addition to *Fos*, many of the classic growth factor regulated IEGs are part of the neural activity-dependent transcriptional program including *Fosb*, *Fosl1*, *Fosl2*, *Jun*, *Junb*, *Egr1* (also termed *zif/268; Ngfi-a; Krox-24*), *Egr3*, and *Nr4a1* (also termed *nur77*, *Ngfi-b*) (Herdegen and Leah, 1998). These genes encode transcription factors that are induced in many cells types in response to a broad range of stimuli. Thus, rather than having a specific cellular function, the IEG program appears to play a more general role in coupling extracellular stimuli with intracellular adaptations. The different functional consequences of IEG induction arise through cell type- and stimulus-specific recruitment of complexes of these transcription factors to distinct sets of late response genes promoters (Hill and Treisman, 1999). In the CNS, IEG transcription factors have been shown to contribute to diverse processes, including neurite outgrowth, neurotransmitter fate, and synapse plasticity (Dragunow *et al.*, 2000; Li *et al.*, 2007; Marek *et al.*, 2010; Maze *et al.*, 2010).

Because *Fos* was the first of the activity-regulated genes to be identified, and because it is so widely and strongly induced, considerable effort has been devoted to understanding the molecular mechanisms that regulate its transcription. The details of *Fos* transcription exemplify many of the general principles of activity-dependent transcription, and thus will be described in detail here. Specifically, *Fos* induction has been shown to be dependent upon 1) the association of sequence-specific DNA binding transcription factors with stimulus-response elements in the proximal promoter, 2) the stimulus-dependent recruitment to these transcription factors of transcriptional co-activators and co-repressors that post-translationally modify promoter-associated histones, 3) the modulation of promoter function by distant enhancer elements, and 4) the regulation of transcriptional elongation (FIGURE 1).

Mutagenesis of the *Fos* promoter revealed two elements that are differentially required for promoter activity in response to a variety of stimuli (Sheng *et al.*, 1990). One element about 300bp upstream of the *Fos* transcription start site (TSS) is required for serum- and growth factor-dependent induction of *Fos* and was therefore named the Serum Response Element (SRE) (Sheng *et al.*, 1988; Treisman, 1986). A second element, about 60bp upstream of the TSS is required for calcium- and cAMP-dependent regulation of *Fos* and was named the Calcium/cAMP-Response Element (CRE) (Hyman *et al.*, 1988; Sheng *et al.*, 1988). Finally, a distinct element just 5′ to the CRE is required for regulation of *Fos* transcription by the retinoblastoma tumor suppressor (Rb) and was named the Retinoblastoma Control Element (RCE) (Udvadia *et al.*, 1992). DNA elements control transcription because they are binding sites for transcription factors, thus once identified the elements are powerful tools for purifying or cloning the binding proteins. In the case of the SRE, the binding protein was called the Serum Response Factor (SRF) (Norman *et al.*, 1988). Subsequent studies showed that SRF binds the SRE in cooperation with additional proteins called the Ternary Complex Factors (TCF), which are a subfamily of E-twenty six (Ets) domain-containing transcription factors exemplified by Ets like gene 1 (Elk-1) (Buchwalter *et al.*, 2004; Dalton and Treisman, 1992). CRE binding proteins were named the Calcium-Response Element Binding protein (CREB) family (Montminy and Bilezikjian, 1987). The RCE is bound by the zinc-finger transcription factor Sp1 (Udvadia *et al.*, 1993).

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Even prior to neural activity, all of these transcription factors as well as the preinitiation form of the RNA polymerase II complex are bound to the *Fos* promoter (Kim *et al.*, 2010; Sheng *et al.*, 1988). The primed state of this promoter is likely to be important for the very rapid induction of *Fos* transcription by stimuli. However, this state also implies that active repression of the promoter may be required in the absence of a stimulus to prevent *Fos* from being constitutively transcribed.

Genomic DNA is wound into a complex secondary and tertiary structure called chromatin via its interactions with structural proteins. The basic repeating unit of chromatin in the nucleosome, which consists of 147bp of DNA wound around an octamer of histone proteins: 2 each of histones H2A, H2B, H3, and H4 (Horn and Peterson, 2002). These histones are subject to extensive post-translational modifications (e.g. acetylation, methylation, phosphorylation, ubiquitination) at specific amino acid residues in their flexible N-terminal tails. The observed correlation between specific modifications and transcriptional activity has given rise to the hypothesis of a "histone code" that determines the transcriptional state of a gene (Strahl and Allis, 2000). Although the details of this relationship between histone modifications and transcription remains an active area of investigation (Lee *et al.*, 2010), substantial evidence suggests that stimulus-dependent regulation of histone H3 and H4 acetylation is a major mechanism of promoter activation and repression (Bernstein *et al.*, 2007; Clayton *et al.*, 2006; Roh *et al.*, 2005). In the case of *Fos*, one mechanism of promoter repression is via the RCE, which recruits a protein complex consisting of Sp1, the transcriptional co-repressor Rb, and the scaffolding protein Calcium-Responsive Transactivator (CREST). In the absence of synaptic activity, Rb is bound to histone deacetylases (HDACs), which enzymatically remove acetyl groups from the histones surrounding the *Fos* TSS, repressing transcription (Qiu and Ghosh, 2008). HDACs can also be recruited to the SRF-binding protein Elk-1, although the functional requirement for this interaction at the *Fos* gene is not known (Yang *et al.*, 2002). Following neuronal activity, calcium-dependent signaling events drive the dissociation of the HDACs from both Rb and Elk, and also induce the active export of class II HDACs (HDAC4 and HDAC5) from the nucleus (Chawla *et al.*, 2003; Qiu and Ghosh, 2008; Yang and Sharrocks, 2006). Simultaneously, signaling cascades lead to the recruitment of the histone acetyltransferase (HAT) CREB-binding protein (CBP) to both CREB and CREST, acetylating promoter histones and promoting transcription (Chrivia *et al.*, 1993; Impey *et al.*, 2002; Qiu and Ghosh, 2008).

In addition to these local events at the *Fos* promoter, membrane depolarization of neurons is associated with widespread, activity-induced recruitment of both CBP and RNA polymerase II to enhancer elements that neighbor *Fos* and other activity-regulated genes (Kim *et al.*, 2010). Biochemically, enhancers have been defined across the genome as regions that show a characteristic chromatin signature that is enriched for histone H3 lysine 4 monomethylation and that shows hypersensitivity to cleavage with enzyme DNAseI (Heintzman *et al.*, 2007). Functionally, enhancers are defined as any DNA element that promotes transcription of a given gene regardless of its location in the genome or its orientation with respect to the regulated gene. Enhancers can be found at great distances from the TSS, as is well documented for regulation of the β-globin locus and the *Hoxd* gene cluster (Hérault *et al.*, 1999; Tuan *et al.*, 1985). How distant enhancers regulate promoter activity remains an active area of investigation, however these mechanisms may involve looping of chromatin to bring the enhancers in close proximity to the gene promoter (Li *et al.*, 2006a), and/or large scale chromosome reorganization that brings multiple actively transcribed regions of chromatin to pre-formed transcriptional "factories" near the nuclear periphery (Sutherland and Bickmore, 2009). Similar to promoter regulatory elements, many enhancer elements are pre-bound by activity-regulated transcription factors including SRF, CREB, and Myocyte Enhancer Factor 2 (MEF2), which may act to recruit CBP (Flavell *et*

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al., 2008; Kim *et al.*, 2010). Interestingly, activity-dependent RNA polymerase II recruitment to enhancers is associated with the induced expression of short, non-coding enhancer RNA transcripts (eRNAs) that initiate at these non-coding elements (Kim *et al.*, 2010). Although the functions of eRNAs are not known, they may play a role in recruiting chromatin modifying enzymes to maintain the chromatin landscape in a transcriptionally permissive state.

Finally, in addition to the regulation of transcriptional initiation, evidence suggests that *Fos* transcription may also be regulated at the level of transcriptional elongation. Productive transcriptional elongation is an active process that requires dynamic CDK9/pTEFb-dependent phosphorylation of amino acids in the C-terminal domain of the large subunit of RNA polymerase II (Buratowski, 2009). For at least a subset of genes, regulation of RNA polymerase II phosphorylation causes the polymerase complex to undergo promoter proximal stalling following initiation until signal-dependent elongation is cued (Nechaev *et al.*, 2010). Consistent with the possibility that *Fos* transcription may be subject to regulation of elongation, nuclear run-on assays of *Fos* transcription in quiescent fibroblasts show that these cells have constitutive transcription of short 5′ transcripts that fail to extend past a *Fos* intragenic regulatory element (FIRE) near the end of exon I prior to serum stimulation (Lamb *et al.*, 1990). Genome-level chromatin immunoprecipitation (ChIP) studies have begun to address the localization and phosphorylation state of the polymerase subunits at large sets of activity-regulated genes (Kim *et al.*, 2010), and further studies of this kind are likely to enhance understanding of the relative importance of the regulation of transcriptional elongation for neuronal activity-dependent changes in mRNA expression.

2.2. Brain-Derived Neurotrophic Factor (Bdnf): promoter complexity

BDNF is a secreted protein of the neurotrophin family that has numerous functions in nervous system development and plasticity (Lewin, 1996; Poo, 2001). Exposure to a wide range of environmental stimuli leads to induced expression of *Bdnf* mRNA in corresponding activated brain regions, suggesting that the induction of *Bdnf* transcription may be a common mechanism for environmental modulation of neural function (Lu, 2003). Neural activity also regulates both the local secretion of BDNF protein and the trafficking of the BDNF receptor TrkB to the plasma membrane, allowing BDNF-TrkB signaling to be tightly linked to activity (Balkowiec and Katz, 2000; Meyer- Franke *et al.*, 1998). Mutations that partially reduce either overall levels of BDNF or BDNF secretion have substantial effects on brain development and plasticity (Chen *et al.*, 2006; Egan *et al.*, 2003; Genoud *et al.*, 2004). Taken together, these data suggest that precise temporal and spatial control of BDNF expression is essential to its function.

The mammalian *Bdnf* gene is comprised of nine exons with at least eight alternative promoters that are differentially used during development, across brain regions, and in different cell types (Aid *et al.*, 2007; Liu *et al.*, 2006). All of the *Bdnf* promoters are active to at least some degree in the CNS, and transcription from each of these promoters can be induced by neuronal activity (Aid *et al.*, 2007). Promoter IV (formerly referred to as “promoter III” in the five-exon nomenclature of the *Bdnf* gene, see (Timmusk *et al.*, 1993)) is strongly activity-responsive in cultured embryonic neurons and has been the most extensively studied at the molecular level (Shieh *et al.*, 1998; Tao *et al.*, 1998; West *et al.*, 2001; Pruunsild *et al.*, 2011).

One of the key insights to be derived from studies of *Bdnf* promoter IV is that the tight temporal, spatial, and stimulus-specific regulation of this single promoter is achieved by a complex interplay between multiple activity-regulated transcriptional pathways (FIGURE 2). Three calcium-response elements (CaREs) within the proximal 170bp of the major embryonic promoter IV TSS cooperatively regulate calcium-induced transcription of *Bdnf*

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exon IV (Chen *et al.*, 2003b; Shieh *et al.*, 1998; Tao *et al.*, 1998). Starting at the element most distal to the TSS, these CaREs are selectively bound and regulated by the unique transcription factor Calcium-Response Factor (CaRF) (Tao *et al.*, 2002), the upstream stimulatory factors USF1/2 (Chen *et al.*, 2003b), and members of the CREB family (Shieh *et al.*, 1998; Tao *et al.*, 1998). In addition, the activity-inducible transcription factor Neuronal Per-Arnt-Sim (PAS) Domain Protein 4 (Npas4) has been shown to interact with another activity-response element 5′ to CaRE1 in human *Bdnf* promoter IV (Pruunsild *et al.*, 2011). An alternative TSS about 100bp downstream of the 5′-most end of *Bdnf* exon IV is regulated by the association of the Nuclear Factor κB (NF-κB) (Lipsky *et al.*, 2001) and the basic helix-loop-helix (bHLH) protein bHLHB2 (Jiang *et al.*, 2008) with elements that fall between the two TSSs. The Nuclear Factor of Activated T Cells (NFAT) is reported to regulate *Bdnf* exon IV expression in response to N-methyl-D-aspartate (NMDA) receptor (NMDAR) activation via its association with an intragenic element located 140–156bp 3′ to the alternative TSS (Vashishta *et al.*, 2009). Finally, ChIP studies have shown that *Bdnf* promoter IV is bound by MEF2 (Hong *et al.*, 2008), however the specific binding site for this factor within *Bdnf* promoter IV remains to be identified.

Distinct functions of these transcription factors in the regulation of *Bdnf* promoter IV have been revealed through the generation of transgenic mice that either lack expression of one of these transcription factors or that block the ability of specific factors to regulate *Bdnf*. For example, studies in mice lacking the CaRE1-binding protein CaRF show that this factor appears to play a brain region-specific role in regulation of *Bdnf* transcription (McDowell *et al.*, 2010). *Carf* knockout mice show reduced levels of *Bdnf* exon IV-containing mRNA transcripts and reduced BDNF protein in the frontal cortex compared with their wildtype littermates, however *Bdnf* expression is unchanged in the hippocampus and striatum of the knockout mice (McDowell *et al.*, 2010). Furthermore, although CaRE1 is required for activity-dependent transcription of *Bdnf* exon IV, CaRF is selectively required for the activity-*in*dependent regulation of *Bdnf* promoter IV activity (McDowell *et al.*, 2010). The failure of the *Carf* knockout to fully phenocopy the loss of the CaRE1 site implies that there likely exist additional CaRE1 binding proteins that are yet to be identified. By contrast with CaRF, the binding of CREB to CaRE3 is selectively required for the activity-dependent regulation of *Bdnf* exon IV transcription. The functional importance of this interaction was elegantly demonstrated by generation of a mouse strain bearing a mutation knocked into *Bdnf* promoter IV that selectively mutates the CRE/CaRE3 site (Hong *et al.*, 2008). 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 the assembly of this complex.

2.3. Activity-Regulated Cytoskeletal Protein (Arc): signal pathway integration

Arc (also known as Arg3.1) was first identified in 1995 as a novel IEG that is rapidly induced in neurons following increased activity (Link *et al.*, 1995; Lyford *et al.*, 1995). Stimulus-inducible transcription of *Arc* depends on elements within the proximal promoter as well as distal enhancer elements (FIGURE 3). The proximal promoter is not sufficient to confer acute cAMP- or calcium-inducible regulation on a reporter plasmid (Waltereit *et al.*, 2001). However, this region is bound *in vivo* by the activity-induced transcription factors Egr1 and Egr3 and overexpression of Egr1 or Egr3 is sufficient to drive endogenous *Arc* expression (Li *et al.*, 2005). Analysis of *Arc* mRNA expression in *Egr1* and *Egr3* knockout mice suggests that these transcription factors may function to prolong *Arc* transcription

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following its acute induction. These studies showed that although acute stimulus-induced expression of *Arc* mRNA does not require new protein synthesis, the persistent expression of *Arc* mRNA 4 hours after kainate-induced seizure is blocked by administration of protein synthesis inhibitors, and this late phase of *Arc* expression is significantly diminished in *Egr3* knockout mice (Li *et al.*, 2005). Intriguingly, the acute induction of *Arc* transcription by synaptic activity requires distal enhancer elements located several thousand base pairs upstream of the *Arc* TSS (Kawashima *et al.*, 2009; Pintchovski *et al.*, 2009). The Synaptic Activity Response Element (SARE) located 6.9kB upstream of the TSS is highly conserved across mammalian species, and is comprised of a cluster of binding sites for the activity-regulated transcription factors CREB, MEF2, and SRF (Kawashima *et al.*, 2009). The importance of this regulatory region for the activity-dependent regulation of Arc was elegantly demonstrated in cerebellar Purkinje cells, in an experiment in which the expression of Arc in *Arc* knockout neurons was rescued by transgenic expression from a BAC construct containing the intact upstream enhancer region, but not by expression from a BAC construct in which the SRE in the SARE had been mutated to a sequence that does not support SRF binding (Smith-Hicks *et al.*, 2010).

Transcription of *Arc* is regulated through the integration of multiple synaptic signaling cascades. As a result of the conversion of these signaling pathways, both the levels and distribution of Arc are particularly sensitive to changes in synaptic activity. An important early finding was the observation that although forskolin-induced elevation of intracellular cAMP can drive induction of *Arc* in the neuroendocrine PC12 cell line in a manner that depends on the protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) signaling pathways, this stimulus is not sufficient to induce *Arc* in the neuroblastoma line Neuro2a or in NIH3T3 fibroblasts (Waltereit *et al.*, 2001). These data raised the possibility that cAMP signaling might interact with additional neural-selective signaling pathways to regulate *Arc* transcription. Subsequent studies have suggested that calcium influx through NMDARs can provide the second signal. For example, treatment of neuron cultures with the growth factor BDNF drives robust *Arc* induction, however this induction is dependent upon synaptic activity and inhibited by antagonists of NMDARs (Rao *et al.*, 2006). Interestingly, BDNF-dependent *Arc* induction is antagonized by α-amino-3-hydroxyl-5-methyl-4-isoxazole-proprionate (AMPA) receptor (AMPAR) activation, through a mechanism that involves pertussis toxin–sensitive $G_{i/o}$ proteins (Rao *et al.*, 2006). Further support for coactivation of NMDAR and cAMP pathways in the control of *Arc* transcription comes from the observation that the cAMP-dependent activation of *Arc* induced by agonists of G_s-coupled dopamine and beta-adrenergic receptors is blocked in the presence of NMDAR antagonists (Bloomer *et al.*, 2008). However, the specific point at which NMDARs and G-protein coupled signaling pathways converge to regulate *Arc* transcription remains to be characterized.

2.4. *Homer1a:* alternative polyadenylation

The Homer family of synaptic scaffolding proteins is comprised of three genes (*Homer1-3*), each of which has multiple splice isoforms (Bottai *et al.*, 2002). Only the *Homer1* gene shows neuronal activity-induced transcription (Brakeman *et al.*, 1997). This gene is comprised of 10 exons spanning over 100kB in the mouse with alternative exon usage giving rise to two full length splice variants called *Homer1b* and *Homer1c* (Bottai *et al.*, 2002). Neuronal activity sharply increases the rate of transcriptional initiation at the *Homer1* promoter, however the activity-induced transcripts from the *Homer1* gene (*Homer1a* and *Ania-3*) contain only exons 1–5 and part of intron 5. The short forms of *Homer1* lack a C-terminal oligomerization domain and are thought to act in a dominant negative fashion to disrupt the formation or function of synaptic signaling complexes (Kammermeier and Worley, 2007; Sala *et al.*, 2003). These short splice variants are generated through the use of

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alternative polyadenylation sites within intron 5 of the *Homer1* gene (Bottai *et al.*, 2002; Niibori *et al.*, 2007). Interestingly, an mRNA profiling study that analyzed changes in exon usage following neuronal membrane depolarization identified a large number of additional genes that show similar activity-dependent alternative polyadenylation site usage (Flavell *et al.*, 2008). In many of these cases, recognition of the intragenic polyadenylation site leads to the expression of a truncated gene product that lacks functional C-terminal domains. These data raise the possibility that alternative polyadenylation site usage may be a common mechanism to modulate the function of proteins in a neural activity-dependent manner.

3. Transcription factors regulated by neuronal activity

Activity-regulated signaling pathways modulate gene transcription by altering the function, localization, or expression of transcriptional factors in the nucleus (West *et al.*, 2002). A growing number of transcriptional regulators have been shown to be targets of activity-dependent signaling cascades in neurons (FIGURE 4). Here we describe the most well-studied activity-regulated transcription factors in neurons, and review the mechanisms of their regulation.

3.1. Activation of prebound transcription factors

One of the most striking features of neuronal activity-regulated transcription is its very rapid initiation. Newly synthesized nuclear RNA transcripts can be detected within one minute of stimuli that induce calcium influx suggesting that some gene promoters are primed for rapid response (Greenberg *et al.*, 1986). At these promoters, such as *Fos*, transcriptional activators are prebound before stimulation arrives (Kim *et al.*, 2010). Activity-dependent signaling pathways are required to post-translationally modify these factors and/or their associated proteins to change their activity state.

3.1.1. cAMP-Response element binding protein (CREB)—The CREB family of transcription factors is comprised of *Creb*, cAMP Response Element Modulatory Protein (*Crem*), and activating transcription factor 1 (*Atf1*). In the CNS, CREB family members have been shown to be essential for neuronal survival and are thought to modulate both synaptic and intrinsic plasticity in response to neuronal activity (Benito and Barco, 2010; Lonze and Ginty, 2002). Loss-of-function genetic studies show that CREB and CREM have overlapping functions in the developing and mature CNS, whereas ATF1 plays particularly important roles early in development (Bleckmann *et al.*, 2002; Mantamadiotis *et al.*, 2002). All stimuli that activate neuronal CREB-dependent transcription (e.g., receptor tyrosine kinases, calcium signaling pathways, cAMP) do so by inducing phosphorylation of CREB at serine 133 (Ser133) (Shaywitz and Greenberg, 1999). Distinct Ser133 kinases including PKA, Ca^{2+}/calmodulin (CaM)-dependent protein kinases (CaMKs), MAPK, and Akt (also known as protein kinase B, PKB) mediate phosphorylation of CREB in response to different stimuli in different cell types (Gonzalez and Montminy, 1989; Lonze and Ginty, 2002; Mayr and Montminy, 2001; Sheng *et al.*, 1991). In neurons, CaMKs mediate rapid but transient phosphorylation of CREB following membrane depolarization, whereas MAPKs make a major contribution to later, sustained CREB phosphorylation (Dolmetsch *et al.*, 2001; Wu *et al.*, 2001a). Phosphorylation of CREB at Ser133 activates transcription by inducing the association of CREB with the transcriptional coactivators CBP and p300, which promote transcription by acetylating promoter histones (Chrivia *et al.*, 1993; Goodman and Smolik, 2000). CREB can also bind directly to elements of the RNA polymerase complex in a phosphorylation-independent manner, which may allow it to recruit the pre-initiation form of RNA polymerase II onto the promoters of primed IEGs such as *Fos* (Felinski *et al.*, 2001; Hong *et al.*, 2008).

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In addition to phosphorylation of Ser133, activation of neuronal calcium signaling pathways induces phosphorylation of CREB at Ser142 and Ser143 (Kornhauser *et al.*, 2002). These phosphorylation events occur concurrently with activation of CREB-dependent transcription, and mutation of these serines to non-phosphorylatable alanine residues selectively inhibits calcium-regulated CREB activity in a reporter gene assay, while leaving cAMP-dependent transcription unaffected (Kornhauser *et al.*, 2002). Consistent with a role for these phosphorylation sites in regulation of CREB-dependent transcription *in vivo*, phosphorylation of CREB Ser142 is induced in neurons of the suprachiasmatic nucleus by light exposure, and mice bearing a mutation that changes Ser142 to alanine knocked into the *Creb1* gene show altered circadian rhythms (Gau *et al.*, 2002). Interestingly, phosphorylation of CREB at Ser142/143 inhibits the ability of Ser133 phosphorylated CREB to bind to the KID-interacting domain (KIX) domain of CBP (Parker *et al.*, 1998). This raises the possibility that activation of calcium signaling pathways may selectively promote the association of CREB with other transcriptional coactivators than just CBP/p300. Alternate coactivators include the CREB-regulated transcription coactivators (Crtc1-3, previously known as TORCs). The Crtcs are CREB coactivators that bind the C-terminal leucine zipper domain of CREB, a domain distinct from the Ser133-containing kinase-inducible domain (KID) domain bound by CBP/p300 (Conkright *et al.*, 2003). Under basal conditions, Crtc1 and Crtc2 are sequestered in the cytoplasm through a phosphorylation-dependent interaction with the chaperone protein 14-3-3 (Screaton *et al.*, 2004). Calcium-dependent activation of the phosphatase calcineurin combined with cAMP-dependent inhibition of the AMP-activated protein kinase (AMPK) family of Ser/Thr kinases salt-inducible kinase 2 (SIK2), leads to dephosphorylation and nuclear import of Crtc1 and 2, where they co-activate CREB-dependent transcription (Kovács *et al.*, 2007; Sasaki *et al.*, 2011; Screaton *et al.*, 2004). Consistent with a role for these CREB co-activators in neuronal biology, absence of Crtc1 in neurons impairs the late phase of long-term potentiation (LTP), BDNF-dependent dendrite outgrowth, and neuronal survival after ischemic injury (Finsterwald *et al.*, 2010; Kovács *et al.*, 2007; Sasaki *et al.*, 2011). Future studies that dissect the relative roles of CBP/p300 and Crtc-dependent regulation of CREB in different contexts may facilitate new understanding into the distinct roles of these regulatory mechanisms.

3.1.2. Serum-Response Factor (SRF)—SRF is a versatile transcription factor expressed in many cell types that plays an important role in coupling actin signaling to changes in gene expression that control cell motility (Arsenian *et al.*, 1998; Knöll *et al.*, 2006; Olson and Nordheim, 2010). In the CNS, disruption of SRF function is associated with impaired axon pathfinding during development and impaired synaptic plasticity in the adult hippocampus (Etkin *et al.*, 2006; Knöll *et al.*, 2006; Ramanan *et al.*, 2005). Furthermore the inducibility of many of the immediate-early gene transcription factors is abolished in the absence of SRF expression, implying an essential role for this factor in activating the general cellular response to changes in extracellular stimuli (Parkitna *et al.*, 2010; Ramanan *et al.*, 2005).

Unlike the CREB family of transcription factors, SRF acts as a homodimer and is encoded by a single gene (Norman *et al.*, 1988). Although activity-regulated signaling pathways can induce phosphorylation of SRF at Ser103, the functional significance of this phosphorylation event for SRF-dependent transcription remains largely unknown (Rivera *et al.*, 1993). However, SRF interacts with a diverse set of transcriptional co-regulators, many of which are highly modulated by activity-regulated signaling cascades (Knöll and Nordheim, 2009). Evidence suggests that it is the differential regulation and use of these cofactors that confers functional versatility upon SRF-dependent programs of gene transcription.

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SRF-dependent transcription is activated by two sets of cofactors: 1) the TCFs (Buchwalter *et al.*, 2004), and 2) the myocardin family of transcriptional co-factors, which includes myocardin, myocardin-related transcription factor A (MKL1, also known as MAL or MRTF-A) and MKL2 (also known as MRTF-B) (Pipes *et al.*, 2006). The TCFs Elk-1, Net, and SRF accessory protein 1 (Sap-1) comprise a subfamily of the Ets domain transcription factors, and were first shown to complex with SRF on the SRE of the *Fos* gene (Shaw *et al.*, 1989). In addition to being recruited to SRF target genes via their interactions with SRF, the TCFs also contain a well-conserved DNA binding domain that interacts with a DNA sequence element neighboring a subset of SRF binding sites (Treisman, 1992). The N-terminal domain of the TCF factors recruits co-repressors to keep target genes off in the absence of stimuli (Yang *et al.*, 2001). Following neuronal activity- and calcium-dependent activation of MAPK signaling pathways, the TCFs are extensively phosphorylated (Marais *et al.*, 1993; Miranti *et al.*, 1995). Phosphorylation of TCFs regulates their activity by inducing a conformational change that both enhances DNA binding and also activates the TCF-bound HAT CBP (Gille *et al.*, 1992).

In contrast to the dominant role of the MAPKs in regulation of the TCFs, the myocardin family of SRF coactivators is regulated by actin signaling (Miralles *et al.*, 2003; Sotiropoulos *et al.*, 1999). In resting cells, myocardin family members such as MKL1 are bound to monomeric G-actin, which keeps them sequestered in the cytoplasm. Extracellular signals that activate intracellular Rho-GTPases stimulate the assembly of filamentous F-actin, freeing MKL1 to translocate into the nucleus where it can bind to SRF and activate SRF-dependent transcription (Vartiainen *et al.*, 2007). Although the relative importance of these two sets of cofactors in neurons for SRF-dependent transcription is not fully, known, evidence suggests that the myocardins may be particularly important during neuronal development. For example, whereas *Elk1* knockout mice show grossly normal brain development (Cesari *et al.*, 2004), mice lacking both *Mkl1 and Mkl2* in the brain have abnormal neuronal migration, neurite outgrowth, and SRF-dependent gene expression (Mokalled *et al.*, 2010), phenocopying many of the early neurodevelopmental defects observed in *Srf* knockout mice (Knöll *et al.*, 2006).

3.1.3. Myocyte Enhancer Factor 2 (MEF2)—The MEF2 family was first identified for its role in muscle differentiation (Molkentin *et al.*, 1995), however the four members of the MEF2 family (MEF2A-D) are also found in distinct but overlapping expression patterns in neurons throughout the developing and adult CNS (Leifer *et al.*, 1993; Lyons *et al.*, 1995). Various members of the MEF2 family have been implicated in the regulation of neuronal survival during development (Mao *et al.*, 1999), in the activity-dependent elimination of excitatory synapses (Flavell *et al.*, 2006), and in behavioral adaptations to drugs of abuse (Pulipparacharuvil *et al.*, 2008).

The transcriptional activity of MEF2 is highly sensitive to regulation by a complex array of stimulus-dependent post-translational modifications that modulate MEF2’s interactions with multiple transcriptional cofactors (McKinsey *et al.*, 2002; Shalizi *et al.*, 2006). The intricate details of these regulatory mechanisms suggest that post-translational modification of MEF2 by various signaling enzymes may provide a way for distinct stimuli to differentially regulate MEF2-dependent transcriptional programs. MEF2 can be either an activator or a repressor of transcription, and stimulus-induced signaling pathways switch it between these states. In its repressor state, class IIa HDACs (HDAC 4,5, 7 and 9) bind to the N-terminal domain of the MEF2s (Lemercier *et al.*, 2000). This interaction contributes to repression of MEF2-dependent transcription both by reducing the acetylation of histones at MEF target gene promoters and by deacetylating a key residue in MEF2, Lys403 (note, all amino acid numbers presented in this section refer to sequence positions in human MEF2A) (Shalizi *et al.*, 2006). Deacetylation of Lys403 is correlated with modification of this residue by the

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small ubiquitin-like modifier (SUMO) moiety, which appears to stabilize MEF2 in the repressor state (Shalizi *et al.*, 2006; Zhao *et al.*, 2005).

Neuronal activity-induced calcium signaling pathways activate MEF2-dependent transcription by at least three mechanisms. First, CaMK-dependent phosphorylation of the HDACs leads to their release from MEF2 and their export from the nucleus (Chawla *et al.*, 2003; Lu *et al.*, 2000). Since the HDACs compete for binding to the same region on MEF2 as the HATs p300 and CBP, HDAC unbinding promotes the ability of MEF2 to recruit co-activators (McKinsey *et al.*, 2001). Second, calcium-dependent activation of the p38-MAPK leads to phosphorylation of MEF2s at multiple sites (Han *et al.*, 1997; Yang *et al.*, 1999; Zhao *et al.*, 1999). At least three sites of p38-induced phosphorylation contribute to the transcriptional activity of the MEF2s (Thr312, Thr319, and Ser453). These sites are conserved in MEF2A, C, and D, and these isoforms of MEF2 are targets of p38-MAPK regulation (Yang *et al.*, 1999). In addition, the extracellular signal-regulated kinase 5 (Erk5) MAPK is capable of phosphorylating MEF2A, C, and D and has been implicated in neuronal MEF2 regulation downstream of BDNF signaling (Kato *et al.*, 1997; Shalizi *et al.*, 2003). Third, calcium-dependent activation of the phosphatase calcineurin leads to dephosphorylation of Ser and Thr residues on MEF2 (Mao and Wiedmann, 1999; Wu *et al.*, 2001b). Dephosphorylation at Ser408 is particularly important for the ability to switch MEF2 from repressor to activator and is required for activity to induce the switch at Lys403 from SUMOylation to acetylation (Grégoire *et al.*, 2006; Shalizi *et al.*, 2006). Interestingly, these sites of regulation are conserved in MEF2A and D, however they are contained within an alternatively spliced region of MEF2C (the γ domain) that is only present in a fraction of the MEF2C protein expressed in brain (Zhu *et al.*, 2005). Finally, whereas calcium and cAMP cooperate to activate the transcription factor CREB, cAMP can either prevent or inhibit MEF2 activation by blocking HDAC export from the nucleus and by inhibiting import of the MEF2 co-activator NFATc3/c4 (Belfield *et al.*, 2006). Taken together, these data raise the possibility that differential activation of signaling cascades, combined with differential expression of MEF2 isoforms, is a mechanism of transcriptional specificity for MEF2-dependent gene expression.

3.2. Transcription factors that undergo regulated nucleocytoplasmic shuttling

An alternative mechanism by which activity can induce transcription factor activity is via the regulation of nuclear import. Cytoplasmic sequestration of transcription factors provides a robust means to keep transcription off in the absence of stimuli, and local tethering of transcription factors near channels may also allow them to be directly regulated by signaling events that occur near the cell membrane. In the nervous system it has been suggested that transcription factors may be capable of being locally activated at dendritic synapses or in the distal growing axon and then translocating to the nucleus (Cox *et al.*, 2008; Meffert *et al.*, 2003). In addition, for transcription factors that are retained in the nucleus, key transcriptional coactivator and corepressor proteins can be shuttled in and out of the nucleus to regulate transcriptional activity (Parkitna *et al.*, 2010; Soriano *et al.*, 2011). Compared with transcription induced by prebound transcription factors, the transcriptional response to shuttling factors is slower and constitutes a late wave of activity-regulated transcription. Termination of transcription through these pathways is primarily mediated by signaling pathways that induce nuclear export.

3.2.1. Activity-dependent nuclear translocation—The two most widely studied transcription factors that translocate into the nucleus following stimulation are NF-κB and NFAT. The calcium-dependent regulation of these factors was first studied in immune cells, where these pathways play crucial roles is gene expression activated by T and B cell receptors. Although the sources of calcium and the biological context of the signaling

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pathways are different in neurons, these factors appear to play an important role in regulating intracellular neuronal responses to stimuli that include synaptic activity.

NF-κB refers to the activity of a set of five mammalian Rel-domain DNA binding subunits: NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and c-Rel (Liou and Baltimore, 1993). In some cell types, most notably activated immune cells, NF-κB activity is constitutive. However, in many cells, NF-κB is held in the cytosol in an inactive form through its association with one of the Inhibitor of NF-κB (IκB) proteins (Meffert and Baltimore, 2005). The ankyrin repeat domains of IκB mask the nuclear localization signal (NLS) of NF-κB, and thus keep this transcription factor in an inactive form in the cytoplasm. Nuclear translocation of NF-κB is induced by stimuli that lead to phosphorylation and subsequent degradation of IκB (Mercurio *et al.*, 1997). Phosphorylation of IκB is mediated primarily by the IκB kinase (IKK), which is composed of the IKKα and IKKβ subunits along with the regulatory protein NF-κB essential modulator (NEMO).

NF-κB is best known for its role in inflammation and immune responses. However, this transcription factor is also expressed in neurons, where the most prominent species are the canonical p50-p65 heterodimer, which is a regulated transcriptional activator, and the inactive p50-p50 homodimer (Meffert and Baltimore, 2005). Mice lacking expression of p65 show defects in a spatial learning task, suggesting that NF-κB-dependent transcription could contribute to activity-induced synaptic plasticity (Meffert and Baltimore, 2005). Some of the same stimuli that activate NF-κB in the immune system also regulate this transcription factor in the CNS. These stimuli include cytokines such as TNFα, viral infections, and oxidative stress. However, in the CNS NF-κB activity can also be induced by glutamate activation of synaptic NMDARs or the opening of L-VGCCs (Guerrini *et al.*, 1995; Meffert *et al.*, 2003). Both Ca^{2+}/CaM-dependent protein kinase II (CaMKII) and MAPKs have been implicated in CNS regulation of NF-κB activity (Lilienbaum and Israël, 2003; Meffert *et al.*, 2003; Takeuchi and Fukunaga, 2004). However, some controversy exists over whether glutamate induces NF-κB activity in neurons as opposed to other CNS cell types such as glia or microglia (Massa *et al.*, 2006). The need to identify the cell types in which transcription is activated is not unique to NF-κB, but rather is a concern for any of the transcription factors that are expressed in both neurons and glia (this concern applies to CREB, for example). However in the case of NF-κB, resolving whether this transcription factor can be inducible in neurons is particularly important because apparently constitutive NF-κB transcriptional activity has been detected throughout the CNS during development in a transgenic mouse strain that expresses β-galactosidase under the control of an NF-κB-responsive promoter (Bhakar *et al.*, 2002). Future development of cell type-specific genetic tools for manipulating NF-κB activity in the CNS will be important for addressing the different biological functions of this transcription factor in neurons, glia, and microglia.

Although evolutionarily related to the Rel/NF-κB family, the NFAT transcription factors (NFAT1-4) are distinguished by their sensitivity to intracellular Ca^{2+} and their regulation by the Ca^{2+}-dependent serine/threonine phosphatase calcineurin (Hogan *et al.*, 2003). A fifth NFAT family member (NFAT5/TonEBP) shares homology to the NFAT DNA binding domain but lacks calcium sensitivity and instead plays important roles in cellular responses to changes in extracellular tonicity (Miyakawa *et al.*, 1999). NFAT family members have been most highly studied for their role in regulation of cytokine gene expression in T cells, however these proteins are widely expressed in numerous tissues including the nervous system (Vihma *et al.*, 2008). Genetic studies have revealed that NFAT is required for neurotrophin- and netrin-dependent axon outgrowth during embryonic development (Graef *et al.*, 2003) and for serum- and activity-dependent survival of cerebellar granule neurons (Benedito *et al.*, 2005). However, the substantial redundancy in function among the four calcium-regulated members of this family and the requirement for these factors in

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development of other organ systems have limited genetic analysis of adult brain functions in constitutive knockout mice.

In response to stimuli that elevate intracellular calcium and/or induce the release of calcium from intracellular stores, the phosphatase calcineurin dephosphorylates multiple Ser and Thr residues on NFAT, which facilitates nuclear translocation (Okamura *et al.*, 2000). Although NFAT can bind DNA as a dimer at regulatory elements that resemble NF-kB binding sites, NFAT also binds and cooperates with a number of other nuclear transcription factors including Fos/Jun family members and MEF2 (collectively known as "NFATn" for the NFAT "nuclear" component) to synergistically promote gene transcription (Hogan *et al.*, 2003). NFAT-dependent transcription is terminated by nuclear export following rephosphorylation of the protein by a number of constitutively active kinases including dual specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A) and glycogen synthase kinase 3 (GSK3) (Graef *et al.*, 1999; Gwack *et al.*, 2006).

Experimental observations combined with mathematical modeling of this molecular regulatory loop have led to intriguing ideas about the ways in which temporal information encoded in neuronal firing patterns may modulate the activation of NFAT-dependent transcription (Arron *et al.*, 2006; Cai *et al.*, 2008; Crabtree and Graef, 2008). Activation of NFAT nuclear translocation and transcriptional activity in both lymphocytes and developing muscles is known to be sensitive to the frequency of calcium oscillations (Dolmetsch *et al.*, 1997; Dolmetsch *et al.*, 1998; McCullagh *et al.*, 2004). Although the specific mechanisms that confer frequency-specific activation of NFAT target genes are not fully understood, they may involve changes in the intensity of the calcium signal such that a threshold for activation of target genes is passed (Fiering *et al.*, 1990). Another possibility is that frequency modulation of the nuclear transport of NFAT could induce "waves" of NFAT translocation to the nucleus, such that bursts of nuclear protein are able to cooperatively activate sets of target genes (Cai *et al.*, 2008).

3.2.2. Synaptic activity-dependent nuclear export—In addition to driving transcription factors into the nucleus, increased activity can also lead to nuclear export. The transcription factor forkhead box O3 (FOXO3) is best known for its regulation of cell death and stress responses (Brunet *et al.*, 1999; Brunet *et al.*, 2004; Tran *et al.*, 2002). FOXO3 is phosphorylated at three sites by the growth factor-regulated Akt signaling pathway, promoting 14-3-3 association and nuclear export (Brunet *et al.*, 1999). By contrast, growth factor withdrawal and oxidative stress are associated with FOXO3 dephosphorylation and nuclear entry, and the activation of FOXO3 target genes that promote cell death. In neurons the NMDAR-dependent activation of calcium signaling pathways can also lead to regulated shuttling of both FOXO3 and FOXO1 (Al-Mubarak *et al.*, 2009; Dick and Bading, 2010; Papadia *et al.*, 2008; Soriano *et al.*, 2006). For example, stimulation of extrasynaptic NMDARs, which can induce cell death, drives nuclear translocation of FOXO3 (Dick and Bading, 2010). By contrast, prolonged synaptic activation prior to growth factor withdrawal reduces nuclear translocation of FOXO3 and protects against cell death through a signaling pathway that requires the activation of synaptic NMDARs, the elevation of nuclear calcium, and Ca^{2+}/CaM-dependent protein kinase IV (CaMKIV). This bidirectional regulation of the FOXO3 and FOXO1 pathways demonstrates one of the many downstream pathways through which synaptic and extrasynaptic NMDARs can have opposing effects on neuronal biology. Importantly, this example highlights the fact that calcium signaling pathways can drive the nuclear export as well as the nuclear import of transcription factors.

A relative newcomer to the field of stimulus-regulated neuronal transcription factors is the calcium channel associated transcription regulator (CCAT). Experiments in both neurons and cardiac myocytes had suggested that the C-terminal domain of the L- VGCC α subunit

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Ca_V1.2 could be cleaved from the membrane-associated channel (De Jongh *et al.*, 1994; Gerhardstein *et al.*, 2000). When Gomez-Ospina and colleagues raised an antibody against this C-terminal domain of the protein, they noticed that it strongly localized to the nucleus, especially in glutamic acid decarboxylase 65 (GAD65)-expressing γ-aminobutyric acid (GABA)-ergic interneurons (Gomez-Ospina *et al.*, 2006). In the nucleus, CCAT binds to the transcriptional regulator p54(nrb)/NonO, and acts as a transcriptional activator of a number of genes including that encoding the connexin Cx31.1 (*Gjb5)*. Most interestingly, CCAT localization is regulated by intracellular calcium, and it accumulates in the nucleus under conditions of *low* intracellular calcium. These data raise the intriguing possibility that CCAT could contribute to transcriptional regulation induced under periods of *low* rather than high synaptic activity, providing an important counterbalance to the programs of transcription driven by activity-induced factors such as CREB, SRF, and MEF2.

3.2.3. Translocation from dendrites and axons—Transcription factors have been demonstrated to translocate between the nucleus and the cytoplasm in many cell types. Although small molecules can diffuse freely into the nucleus through the nuclear pore complex, larger molecules including most transcription factors are actively transported through the pore by associating with a family of nuclear transport proteins called the importins (Otis *et al.*, 2006). Classically, importin α subunits bind directly to nuclear localization sequences on their target proteins whereas importin β subunits mediate the transport of the complex through the nuclear pore. Given the central importance of this mechanism in cellular physiology, it is not surprising the function of the core components of the importin pathway are conserved in neurons. However, neurons are unique with respect to the shape and the size of their cytoplasmic compartment. Unlike most other kinds of cells, in which no part of the plasma membrane is more than 100 μm from the nucleus, neurons have large and complex shapes. Furthermore, the most important functional parts of the cell, the pre- and post-synaptic regions of the axons and dendrites, can be found at very great distances from the nucleus. This unusual morphology has raised the intriguing possibility that transcription factors might sample environmental information at the neuronal periphery and then, via the traditional transport pathways described above, translocate to the nucleus to effect changes in gene expression.

Consistent with this possibility, several studies have shown that key elements of the nuclear translocation machinery are localized to distal parts of the neuron. For example, in dorsal root ganglion neurons not only are the importins found in the axon at significant distances from the cell body, but importin β protein levels are also rapidly increased in the axon after the nerve is lesioned via a mechanism that involves local translation of axonal importin β mRNA (Hanz *et al.*, 2003). In dendrites, α-importin has been shown to bind to the cytoplasmic tail of the NMDAR subunit NR1-1a at postsynaptic sites in synapses (Jeffrey *et al.*, 2009). This α-importin-NR1 interaction is disrupted by activation of NMDARs in a protein kinase C (PKC)-dependent manner in cultured neurons, allowing α-importin to translocate to the nucleus. Transcription factors that depend on importin translocation are also found at pre- and post-synaptic sites. Activating transcription factor 4 (ATF4 or CREB2) is localized to distal dendrites under resting conditions and translocates to the nucleus in an α-importin-dependent manner following stimuli that induce long-term depression but not long-term facilitation of *Aplysia* sensory neurons (Lai *et al.*, 2008). mRNA encoding CREB1 has been found in axons of developing neurons, where CREB is both inducibly translated and retrogradely trafficked in response to NGF application (Cox *et al.*, 2008). Finally, several studies have suggested that NF-κB may translocate from neurites to the nucleus in an activity-dependent manner (Meffert *et al.*, 2003; Wellmann *et al.*, 2001).

These data demonstrate that signal-dependent translocation of transcription factors can occur in neurites, but substantial questions about the mechanisms and consequences of this process

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remain to be solved. Is it possible for a single transcription factor molecule activated at a synapse or axon tip to translocate all the way to the nucleus? Is there something special about the way in which transcription factors become activated in the distal periphery of neurons that alters their transcriptional potential, or would somatic activation of the same transcription factor provide an equivalent signal? These questions as well as other ideas about synapse to nucleus signaling have been discussed in detail elsewhere (Jordan and Kreutz, 2009) and represent intriguing potential areas for the discovery of new mechanisms that could contribute specificity to the control of activity-regulated transcription.

3.3. Transcription factors that show regulated binding

The specificity of transcription factor function is determined in large part by the factor's sites of DNA binding, which in turn determine which genes that transcription factor can regulate. For this reason, inducible binding of a transcription factor to different sites in the genome would have an obvious impact on its function. Perhaps the most clear example of calcium-dependent regulation of transcription factor binding has been that described for the Downstream Repressor Element Antagonist Modulator (DREAM) (Carrión *et al.*, 1999). DREAM (also known as KChIP3 and calsenillin (An *et al.*, 2000; Buxbaum *et al.*, 1998)) belongs to the Neuronal Calcium Sensor (NCS) family of EF-hand proteins (Burgoyne, 2007). This protein family is related to the ubiquitous calcium effector protein CaM, but NCS family members also have domains distinct from those found in CaM. The 14 members of the NCS family have functions ranging from vesicle trafficking to ion channel regulation to transcriptional control (Burgoyne, 2007). DREAM was first characterized as a transcription factor when it was shown to bind to a repressor element in the first intron of the gene encoding prodynorphin (*Pdyn)* (Carrión *et al.*, 1999). In the absence of calcium signaling, DREAM acts as a repressor of *Pdyn* by sterically hindering progression of the polymerase. Upon a rise in nuclear calcium, DREAM binds calcium through its EF-hands and its affinity for DNA is subsequently reduced (Lusin *et al.*, 2008; Osawa *et al.*, 2005; Osawa *et al.*, 2001). Mice lacking DREAM (*Kcnip3*$^{-/-}$ mice) have elevated *Pdyn* mRNA in the spinal cord and reduced behavioral responses to painful stimuli, indicating a physiological requirement for DREAM in this pathway (Cheng *et al.*, 2002).

Although a few other factors including some bHLH proteins (Corneliussen *et al.*, 1994) and members of the MEF2 family (Mao and Wiedmann, 1999) have been shown to exhibit regulated DNA binding *in vitro*, direct regulation of DNA binding capacity has not emerged as a major mechanism of transcriptional regulation for most activity-regulated factors. Instead, it seems likely that inducible binding may be more likely to occur as a consequence of multiple coordinated cellular signaling events that either shift the subcellular localization of transcription factors and cofactors or that change the structure of genomic DNA. A good example of how these kinds of processes may result in regulated DNA binding has recently been described for CREB. Following the initial identification of CREB as the "cAMP response element binding protein," substantial effort was devoted to understanding the mechanisms by which phosphorylation of CREB at Ser133 led to transcriptional activation. Most *in vitro* studies concluded that CREB was constitutively bound to the CRE, and that Ser133 phosphorylation did not change CREB's DNA binding affinity (Montminy and Bilezikjian, 1987; Yamamoto *et al.*, 1988). However, a set of *in vivo* studies showed evidence for inducible protein binding to at least some CREs (Boshart *et al.*, 1991; Weih *et al.*, 1990; Wölfl *et al.*, 1999). Consistent with these early studies, a recent genome-wide study mapped sites of CREB binding before and after membrane depolarization of neurons and provided support for both of these scenarios (Kim *et al.*, 2010). Although CREB was found prebound at most sites identified across the genome, a subset of binding sites showed enhanced CREB association after membrane depolarization. A potential mechanistic explanation for this observation is that changes in chromatin conformation may underlie the

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apparent induction of CREB binding by altering the availability of the CREs at some CREB target genes (Riccio *et al.*, 2006). Upon exposure of neurons to BDNF, CREB becomes rapidly bound to some sites of DNA coincident with phosphorylation at its transcriptional regulatory site, Ser133. However, this inducible CREB-DNA binding is independent of CREB Ser133 phosphorylation and is not affected by inhibition of the Erk or phosphatidylinositol 3-kinase (PI3K) signaling pathways. Instead, BDNF appears to regulate CREB binding by initiating a nitric oxide-dependent signaling pathway that leads to S-nitrosylation of nuclear proteins including the histone deactylase HDAC2 (Nott *et al.*, 2008). S-nitrosylation of HDAC2 induces its release from chromatin and is associated with increased histone acetylation and transcriptional induction of neurotrophin-regulated and CREB-dependent target genes. Traditionally most transcription factor binding assays have been conducted in cell-free systems, where this sort of chromatin regulatory mechanism of inducible binding would be missed. However, with the growing ease of performing genome-wide transcription factor binding assays *in vivo*, it will be of great interest in the future to determine how commonly these kinds of regulated binding site mechanisms are used to modulate transcription factor function following changes in neuronal activity.

3.4. Neuronal activity-regulated transcription factor expression

As initially described for Fos and the other IEGs, an important class of transcription factors are regulated by neuronal activity at the level of their expression. The promoters of these genes are targets of the transcription factors in the categories above. Robust induction of the IEG transcription factors is a cardinal feature of the transcriptional response to a wide variety of stimuli in many cell types (Herdegen and Leah, 1998; Morgan and Curran, 1991). Notably these transcription factors are also subject to additional forms of activity-dependent regulation. For example, after synthesis, Fos and Jun family transcription factors are subject to phosphorylation, which can change their nuclear localization or protein-protein interactions (Chen *et al.*, 1993). Furthermore, the specific complexes of Fos/Jun proteins bound to activator protein 1 (AP-1) elements are subject to change under different conditions (Herdegen and Leah, 1998). For example, through an alternative splicing event that does not appear to be sensitive to neuronal activity, the *Fosb* locus gives rise to two transcripts that encode either full length FosB or a C-terminally truncated protein termed ΔFosB (Mumberg *et al.*, 1991; Nakabeppu and Nathans, 1991). The short ΔFosB protein lacks a protein domain that promotes FosB degradation and thus has a much longer half-life than full length FosB. Thus, in response to repeated activation of *Fosb* transcription, over time ΔFosB protein accumulates and becomes the dominant FosB protein isoform in cells (Hope *et al.*, 1994). Although the functional differences between ΔFosB and full length FosB are incompletely understood, these two isoforms show differences in their ability to repress target genes suggesting they may have distinct roles in regulation of genes that contain AP-1 regulatory elements (Mumberg *et al.*, 1991; Nakabeppu and Nathans, 1991).

A more recent addition to the set of activity-induced transcription factors is the bHLH-PAS domain family member Npas4 (Lin *et al.*, 2008). Like other IEGs, Npas4 is very rapidly and robustly induced by membrane depolarization of cultured neurons (Lin *et al.*, 2008). However, unlike the Fos and Jun families of transcription factors, expression of Npas4 is largely restricted to neurons and its transcription is selectively induced by stimuli that activate intracellular calcium signaling pathways (Coba *et al.*, 2008; Lin *et al.*, 2008; Ooe *et al.*, 2004; Zhang *et al.*, 2009). Interestingly, acute RNAi-mediated knockdown of *Npas4* during synaptogenesis in cultured hippocampal neurons reduces GABAergic synapse numbers while overexpression of Npas4 selectively increases GABAergic synapses (Lin *et al.*, 2008). By contrast these manipulations have no effect on the number of glutamatergic synapses, indicating a selective role for Npas4 in regulating GABAergic synapse development. Npas4 forms functional heterodimers with other members of the bHLH-PAS

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domain protein family including Arnt1 and Arnt 2 (Ooe *et al.*, 2009). Genome-wide ChIP-sequencing (ChIP-Seq) for Npas4 shows that this protein is widely found not only at promoters but also at activity-regulated distant enhancers (Kim *et al.*, 2010). The function of Npas4 recruitment to enhancers remains to be determined, however one speculative idea is that late recruitment of Npas4 may be required to prolong the transcription of activity-regulated genes such that they reach critical expression levels for regulation of downstream processes (Greer and Greenberg, 2008). RNA interference (RNAi)-mediated knockdown of *Npas4* affects the expression of a large number of gene products, many of which are activity-regulated, consistent with the possibility that this transcription factor contributes to stimulus-dependent regulation of gene expression (Lin *et al.*, 2008).

Finally, the CREB family provides a particularly intriguing example of how the interaction between different regulatory mechanisms can confer specificity upon the control of gene transcription. The inducible cAMP early repressor (ICER) is a stimulus-inducible transcriptional repressor of the CREB family (Foulkes *et al.*, 1996). ICER is inducibly transcribed from an intragenic promoter of the *Crem* gene (Borlikova and Endo, 2009; Stehle *et al.*, 1993). This promoter contains four CRE elements that are bound by phosphorylated CREB. Once synthesized, the ICER protein can dimerize with other members of the CREB family including both CREB and CREM, changing the nature of the CRE-binding complex. ICER contains a DNA binding domain but it lacks a transcriptional activation domain and thus acts in a dominant negative manner to suppress CREB family-dependent transcription (Stehle *et al.*, 1993). In this way ICER may function as a feedback mechanism to help turn off activity-stimulated transcription of CREB target genes. After its expression is induced by activity, ICER competes with CREB for binding to the CREs in its own promoter, shutting off its own expression and thereby defining the temporal window during which this mechanism is active. Furthermore, after expression, the stability of ICER is subject to regulation by the ubiquitin-proteasome system (Folco and Koren, 1997). Taken together, the mechanisms that regulate ICER provide a means for fine-tuning the temporal regulation of CRE-dependent transcription during physiologically relevant stimuli.

3.5. Regulation of chromatin

In order to fit into the nucleus, long strands of genomic DNA are twisted into complex secondary and tertiary structures. Although all cells contain the same genomic DNA, the architecture of chromatin structure differs between cell types (Xi *et al.*, 2007). These changes in chromatin structure are established and maintained by epigenetic mechanisms that biochemically alter chromatin, and are thought to underlie cell type specific differences in gene expression by altering the availability of transcription factor binding sites. The major epigenetic mechanisms of transcriptional regulation involve both post-translational modifications of the histone proteins that comprise nucleosomes, which are the basic repeating unit of chromatin structure, along with covalent modifications of genomic DNA itself (Jaenisch and Bird, 2003) (FIGURE 5). Intriguingly, an accumulating body of evidence has shown that these epigenetic modifications are not static components of genomic DNA; instead both histone and DNA modifications are subject to regulation by stimuli that converge to regulate the function of histone- and DNA-modifying enzymes (Dulac, 2010; Riccio, 2010). Furthermore, genetic and pharmacological manipulation of the enzymes that mediate epigenetic regulation of chromatin have been associated with changes in learning and memory as well as alterations in affective- and addictive-like behaviors (Dulac, 2010; Roth and Sweatt, 2009; Tsankova *et al.*, 2007). Our understanding of the relationship between the dynamic regulation of the epigenome and transcriptional potential is still in its infancy. However, here we review evidence for the major activity-regulated processes that have an impact on epigenetic mechanisms of chromatin regulation.

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3.5.1. Regulation of enzymes that modify histones—Histones can be post-translationally modified at specific amino acid residues in their N-terminal tails by several different moieties, including phosphorylation, acetylation and methylation (Strahl and Allis, 2000). Multiple studies have shown that both phosphorylation and acetylation of histones are subject to rapid activity-dependent regulation in neurons (Crosio *et al.*, 2000). It is less clear whether histone methylation can be acutely regulated by changes in neuronal activity (Chen *et al.*, 2003a; Kim *et al.*, 2010).

The ability of activity-regulated signaling pathways to modulate histone phosphorylation and acetylation has been well described. Histone H3 can be phosphorylated at a number of sites including Ser3, Ser10, Thr11, and Ser28. Histone H3 Ser10 phosphorylation was first linked with chromosome condensation during mitosis – a condition under which every Ser10 of every histone H3 is phosphorylated (Gurley *et al.*, 1978; Wei *et al.*, 1999). However, growth factors and neuronal activity can also lead to the rapid, transient, and gene-specific phosphorylation of Ser10 in non-dividing cells, where this histone modification has been associated with transcriptional activation of IEGs including *Fos* and *Jun* (Bilang-Bleuel *et al.*, 2005; Crosio *et al.*, 2000). Thus, the context in which phosphorylation of histone H3 Ser10 is induced may determine the functional consequence of this modification for cell physiology. Study of histone H3 Ser 10 phosphorylation by epidermal growth factor (EGF) and the PKC agonist TPA implicated activation of the Erk/MAP kinase pathway (Cheung *et al.*, 2000; Sassone-Corsi *et al.*, 1999). The relevant Ser10 kinase activated by these stimuli was suggested to be the ribosomal S6 kinase 2 (Rsk2), which is mutated in Coffin-Lowry Syndrome, a rare syndromic form of X-linked mental retardation (Sassone-Corsi *et al.*, 1999). MAPK signaling has also been implicated in neuronal activity-induced phosphorylation of histone H3 Ser10 since inhibitors of the MAPK pathway block the inducible Ser10 phosphorylation found in the hippocampus following contextual fear conditioning (Chwang *et al.*, 2006). However, many kinases have been shown to be capable of inducing histone H3 Ser10 phosphorylation in different cell types following distinct stimuli, including a number that are activated by neural activity such as PKA, PKC, the p38 MAPK, Akt, and the mitogen- and stress-induced kinases 1 (MSK1) and 2 (MSK2), among others (Bode and Dong, 2005). The functional consequences of differential kinase regulation of histone H3 Ser10 phosphorylation remain largely unknown.

Histones H3 and H4 are subject to rapid and transient stimulus-dependent changes in acetylation at a number of residues, all of which have been associated with transcriptional activation (Renthal *et al.*, 2009; Riccio, 2010; Wang *et al.*, 2008). The acetylation of histones is regulated by the balance between HATs and HDACs, which are either locally recruited or activated at genetic regulatory elements. As described in the section on transcriptional regulation of *Fos* above, both HATs and HDACs are targets of activity-regulated signaling cascades. The HAT CBP is recruited to gene promoters and enhancers following membrane depolarization in a manner that depends on its association with transcription factors such as CREB that undergo calcium-regulated phosphorylation (Impey *et al.*, 2002; Kim *et al.*, 2010). In addition, CBP itself is subject to phosphorylation by stimulus-dependent signaling cascades including the calcium-activated kinase CaMKIV, though the functional consequences of these modifications for recruitment or the HAT activity of CBP remain unclear (Chawla *et al.*, 1998; Hardingham *et al.*, 1999; Hu *et al.*, 1999; Impey *et al.*, 2002; Riccio, 2010).

HDACs are a large family of proteins with different regulation and function. The eleven “classical” HDAC proteins remove acetyl groups via hydrolysis and are characterized into three families (class I, class IIa/b, and class IV) based on their structure, enzymatic function, and pattern of expression (Butler and Bates, 2006). HDACs 1, 2, 3, and 8 comprise the class I family and are ubiquitously expressed and predominantly localized to the nucleus. As

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described in the section on inducible transcription factor binding above, HDAC2 is a target of BDNF signaling which reduces HDAC2 association with its target genes (Nott *et al.*, 2008). HDACs 4, 5, 7, and 9 belong to the class IIa family, while HDACs 6 and 10 form class IIb. Class II HDACs can associate with transcription factors, but unlike class I, they shuttle between the nucleus and the cytoplasm in a calcium-dependent fashion, regulating the function of their transcription factor partners (Chawla *et al.*, 2003). Finally, HDAC11 is the only member of the class IV family and is localized to the nucleus though little is known about its function. Despite their nomenclature, members of the HAT and HDAC family can also regulate acetylation of other proteins in addition to histones, and the non-nuclear HDACs have several biological functions that are unrelated to transcriptional regulation (Pandey *et al.*, 2007). Interestingly, the different HDACs may control distinct groups of genes. By ChIP, HDAC2 but not HDAC1 is found to be bound to the promoters of a number of genes (including *Bdnf, Egr1,* and *Fos)* involved in activity-regulated brain plasticity (Guan *et al.*, 2009). Consistent with a functional role for HDAC2 in regulation of these genes, neuron-specific overexpression of HDAC2 but not HDAC1 decreases synapse number, synaptic plasticity, and memory formation. These data raise the possibility that differential regulation of gene subsets by members of the large family of HDACs may provide a mechanism for specificity in the activity-regulated transcription of gene expression programs.

3.5.2. Regulation of DNA methylation—DNA methylation is known to be a key chromatin regulatory mechanism underlying many very long-lasting changes in gene expression (Reik, 2007). For example, DNA methylation is involved in the imprinting of genes, which permits only the maternal or paternal allele of a specific gene to be expressed (Reik and Walter, 1998). DNA methylation is also required for X-chromosome inactivation and it controls the activity of many cell-type specific gene promoters. For these reasons, for many years DNA methylation was largely perceived as a static phenomenon unlikely to be important for transcriptional plasticity. However, a growing body of data has provided increasingly compelling evidence that DNA methylation is subject to stimulus-dependent regulation even in post-mitotic cells. The first evidence for rapid DNA demethylation was shown in sperm cells at the moment of fertilization. Erasure of DNA methylation in the sperm and egg is essential for resetting gene expression potential in the pluripotent cells of the early embryo (Reik *et al.*, 2001). Maternal methylation is lost slowly during early cell divisions of the fertilized egg by failing to transfer methyl marks to the newly synthesized DNA at each cell division. By contrast, paternal methylation is lost rapidly over a period of hours beginning at the moment the sperm invades the egg (Oswald *et al.*, 2000). Rapid demethylation of DNA has also been reported in a stimulus-dependent manner in other cell types. For example, following T-cell receptor activation, multiple sites in the *Il2* gene promoter are demethylated within 20 minutes – far too fast to be mediated by passive loss during cell division (Bruniquel and Schwartz, 2003). In the hippocampus, seizure has been shown to drive a significant decrease in DNA methylation within regulatory regions of the fibroblast growth factor (FGF) gene *Fgf1* as well as the *Bdnf* gene, coincident with their activity-dependent transcriptional induction (Ma *et al.*, 2009). Interestingly, genetic and pharmacological manipulations of the enzymes that regulate DNA methylation affect brain plasticity (Feng *et al.*, 2010; Miller and Sweatt, 2007). Thus, these data raise the possibility that stimulus-dependent changes in DNA methylation may contribute to activity-regulated neuronal transcription.

Given the evidence that DNA methylation may be regulated in the nervous system, these data raise the question of the mechanisms that modulate this chromatin modification. DNA is methylated at CpG dinucleotides across the genome by a small family of DNA methyltransferases. These enzymes include the maintenance DNA methyltransferase Dnmt1, which mediates transfer of DNA methylation patterns to the newly synthesized strand of

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DNA during cell division, and the *de novo* DNA methyltransferases Dnmt3a and Dnmt3b (Jaenisch and Bird, 2003). There is some evidence suggesting that the expression of Dnmt3a in particular may be regulated by the activation of neuronal signaling cascades (LaPlant *et al.*, 2010; Miller and Sweatt, 2007), however whether these changes in expression are causative for to local activity-dependent differences in DNA methylation has not been determined. Loss of DNA methylation is also presumed to have an enzymatic mediator. The nature of the enzymes that demethylate DNA remains a subject of some controversy but emerging data point toward a role for base excision repair mechanisms in this process (Fritz and Papavasiliou, 2010; Gehring *et al.*, 2009; Wu and Zhang, 2010). Notably, it has recently been discovered that genomic DNA can also be modified by hydroxymethylation (Ito *et al.*, 2010; Kriaucionis and Heintz, 2009; Tahiliani *et al.*, 2009). This modification is induced by the enzymes Tet1-3, which hydryoxylate 5-methyl-cytosine in genomic DNA. Intriguingly, Tet1-dependent cytosine hydroxymethylation promotes DNA demethylation via a mechanism that requires the AID (activation-induced deaminase)/APOBEC (apolipoprotein B mRNA-editing enzyme complex) family of cytidine deaminases (Guo *et al.*, 2011). Viral short hairpin RNA (shRNA)-mediated knockdown of *Tet1* or *Apobec1* in the dentate gyrus of the hippocampus inhibits electroconvulsive stimulation-induced demethylation of the *Fgf1* and *Bdnf* genes suggesting that this mechanism could be engaged by activity-dependent signaling pathways to demethylate neuronal DNA (Guo *et al.*, 2011). Future study of the mechanisms by which neural activity regulates these enzymes will be an essential step toward understanding the importance of this regulatory process for brain plasticity.

DNA methylation can have an impact on transcription through a number of different mechanisms, most commonly either by sterically hindering the association of transcription factors with their binding sites, or by recruiting the binding of proteins with methyl-DNA binding domains (Klose and Bird, 2006). Biochemical purification of methyl-DNA binding proteins has led to the identification of a small family of methyl-DNA binding domain (MBD) proteins comprised of MBD1, MBD2, MBD4, and the founding member methyl CpG binding protein 2 (MeCP2). A fifth methyl-DNA binding protein named Kaiso lacks an MBD, and instead recognizes methylated DNA through a zinc- nger domain. These proteins act as effectors of the DNA methyl mark by recruiting complexes of chromatin regulatory proteins to methylated regions of the genome.

MeCP2 is of particular interest in the nervous system because loss-of-function mutations in human *MECP2* cause the neurodevelopmental disorder Rett Syndrome (Chahrour and Zoghbi, 2007). Rett Syndrome is characterized by motor dysfunction, lack of language development, and severe mental retardation. Many children with Rett Syndrome show grossly normal neurological function at birth but begin to lose developmental milestones at 6–18 months of age. This is the peak period of synapse formation in the brain, which suggested the hypothesis that Rett Syndrome might be a disorder of synapse development (Zoghbi, 2003). Consistent with this possibility, mice bearing loss-of-function mutations in *Mecp2* show multiple abnormalities of synapse development, including decreased strength of excitatory glutamatergic synapses and alterations in the number of GABAergic synapses (Dani and Nelson, 2009; Deng *et al.*, 2010; Moretti *et al.*, 2006; Wood *et al.*, 2009; Zhang *et al.*, 2010b). Furthermore, loss of MeCP2 appears to trap cortical synapses in an immature state of development (Tropea *et al.*, 2009). Specifically, it has been shown in mice lacking MeCP2, that monocular deprivation induces an ocular dominance shift in the visual cortex long after the critical period for cortical plasticity has closed in their wildtype littermates.

MeCP2 is subject to phosphorylation by calcium-activated signaling cascades, raising a potential mechanism to link function of this protein with activity-dependent plasticity. Mass spectrometry has identified calcium-regulated sites of MeCP2 phosphorylation at Ser80 and

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Ser421 (Tao *et al.*, 2009; Zhou *et al.*, 2006). MeCP2 has also been shown to be modified by O-GlcNAc glycosylation, and the fraction of glycosylated MeCP2 is more likely to be phosphorylated at Ser80, suggesting a potential interaction between these two modifications (Rexach *et al.*, 2010). MeCP2 Ser80 is rapidly dephosphorylated following membrane depolarization of cultured neurons (Tao *et al.*, 2009), whereas MeCP2 Ser421 is inducibly phosphorylated by CaMKs following L-VGCC or NMDAR activation (Zhou *et al.*, 2006). Overexpression of a nonphosphorylatable Ser421Ala mutant MeCP2 blocks activity-dependent *Bdnf* transcription, suggesting that phosphorylation of this site is required for functional regulation of a least a subset of MeCP2-sensitive genes. However, the mechanisms by which Ser421 phosphorylation may alter MeCP2 function remain largely unknown. Phosphorylation at this site is correlated with the dissociation of MeCP2 from *Bdnf* promoter IV (Chen *et al.*, 2003a), raising the possibility that phosphorylation could be associated with a change in the genomic distribution of MeCP2. However, MeCP2 is very highly expressed in neurons and it is bound widely across the genome essentially wherever DNA is methylated (Skene *et al.*, 2010). Whether Ser421 phosphorylation of MeCP2 occurs at specific genomic loci and/or whether this modification has a selective impact on the transcription of a subset of gene products remains unknown. Future studies that address the functional relevance of activity-dependent regulation of MeCP2 will be expected to increase understanding of this neurologically important epigenetic transcriptional regulator.

4. Calcium channel-dependent regulation of transcription

Transcription occurs in the cell nucleus, and up to this point, we have been focused on the nuclear proteins that regulate transcription in response to neuronal activity. However, the signals that set these nuclear events in motion begin at the synaptic plasma membrane, where neurotransmission occurs. In this section we turn our attention toward the synaptic and cytoplasmic mechanisms by which calcium signaling pathways encode specificity toward the transcriptional pathways they activate.

Presynaptic action potentials release glutamate into the synaptic cleft, activating glutamate receptors of NMDA-, AMPA-, and kainate-types. This postsynaptic glutamate receptor activation then sets into motion a series of intracellular signaling events that culminate in the regulation of the transcription factors and stimulus-regulated genes described above. One of the most salient second messengers in cell biology is the calcium ion (Clapham, 2007). The importance of calcium signaling pathways for activity-regulated gene transcription was first demonstrated by experiments that showed removing calcium from the extracellular solution ablates neurotransmitter-induced gene induction in cultured cells (Greenberg *et al.*, 1986; Morgan and Curran, 1986). Over the course of the following decade, it became apparent that this single signal – the calcium ion – is capable of exerting diverse effects on neuronal physiology and metabolism depending upon the mode of calcium entry and the cellular context in which the calcium signal acts (Ghosh and Greenberg, 1995). The two most physiologically relevant modes of calcium entry into neurons in terms of regulating changes in gene transcription are entry through L-VGCCs and NMDARs (Bading *et al.*, 1993; Ghosh *et al.*, 1994b; Ghosh and Greenberg, 1995; Lerea *et al.*, 1992). Additional sources of calcium include other voltage- and ligand-gated plasma membrane calcium channels as well as the release of calcium from intracellular stores.

Calcium can activate a number of different signaling cascades, and the differential activation of these pathways by distinct pools of intracellular calcium contributes to the specificity of transcriptional responses. Calcium acts on cell physiology by virtue of its ability to bind to proteins, which then changes their conformations and functions. One of the most important calcium adaptors in the cell is the protein CaM, which can coordinate up to four calcium ions with its four EF hand domains (Clapham, 2007). The Ca^{2+}/CaM complex is then

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capable of activating several different types of effector molecules, including the family of CaMKs and the Ca^{2+}/CaM-dependent protein phosphatase calcineurin (Wayman *et al.*, 2008b). Other Ca^{2+}/CaM binding proteins including Ras-specific guanine nucleotide-releasing factor (Ras-GRF), along with CaMKIV, lead to activation of MAPK signaling pathways. Additionally, Ca^{2+}/CaM-dependent adenylate cyclases elevate cellular cAMP levels to activate PKA. Activation of these kinase cascades drives the changes in transcription factor activation discussed above that regulate gene transcription. Factors such as calcium channel location, channel subunit composition, calcium microdomain dynamics, and protein-protein interactions can all play a role in determining which signaling cascades – and therefore which genes – are differentially regulated following neuronal stimulation (Bading *et al.*, 1993; Ginty, 1997). In this section, we will discuss the different sources of calcium that, upon activation, can lead to alterations in gene transcription in neurons, and we will discuss the mechanisms by which these various routes of calcium influx convey specificity to the nucleus. We note that much has been written about the potential mechanisms that underlie long-distance synapse to nucleus signaling in neurons, and we refer the reader to other, more comprehensive reviews for the details of this discussion (Adams and Dudek, 2005; Carrasco and Hidalgo, 2006).

4.1. NMDA Receptors (NMDARs)

NMDARs are glutamate-gated non-selective cation channels that play a key role in the activation of intracellular calcium signaling cascades following synaptic activity (Dingledine *et al.*, 1999; McBain and Mayer, 1994). Because NMDARs require both presynaptic depolarization (for neurotransmitter release into the synaptic cleft) and postsynaptic depolarization (for the removal of the Mg^{2+} ion that usually blocks the channel pore) for activation, they play an essential role as coincidence detectors in LTP, a Hebbian form of synaptic plasticity that is a cellular mechanism for learning and memory (Collingridge and Singer, 1990; McBain and Mayer, 1994). The ability of NMDARs to allow calcium to enter the cell has been shown to be important for many neurobiological processes including neuronal survival, migration, neurite outgrowth, synapse formation, and other forms of plasticity such as homeostatic plasticity and metaplasticity (Choi, 1988a, b). Importantly, it has been demonstrated by a myriad of studies that one critical way in which NMDARs are capable of exerting their effects on a neuron's life and metabolism is via regulation of gene transcription (Hardingham and Bading, 2003; Wayman *et al.*, 2006; West *et al.*, 2002).

4.1.1. NMDARs regulate transcription in vitro and in vivo—There is a substantial body of evidence in support of the idea that calcium influx through NMDARs contributes to induction of gene transcription. The first evidence for NMDARs being critical for the induction of IEGs following electrical stimulation came from a study published by Cole and colleagues (Cole *et al.*, 1989). Briefly, the authors showed that high frequency stimulation of the perforant path-granule cell synapse *in vivo* leads to robust induction of *Egr1* mRNA in an NMDAR-dependent manner. A later study showed that in cultured hippocampal neurons, glutamate-induced increases in mRNA transcript levels of the IEGs *Fos*, *FosB*, *Jun*, *JunB*, *Egr1* and *Nr4a1* were almost entirely due to NMDAR activation (Bading *et al.*, 1995). Interestingly, while high frequency stimulation – which is known to induce LTP – is capable of triggering *Egr1* mRNA induction, low frequency stimulation – which does not induce LTP – is not sufficient to induce *Egr1* transcription (Cole *et al.*, 1989). These correlational findings were also among the first to suggest that IEG transcription might play a role in synaptic plasticity.

NMDAR-regulated gene transcription has been implicated in numerous cellular processes. NMDARs are the critical source of calcium influx for the initiation of LTP (Collingridge *et al.*, 1983a, b; Kauer *et al.*, 1988; Malenka *et al.*, 1988; Muller *et al.*, 1988), and subsequent

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work has suggested that the late phase of LTP relies on NMDAR-induced, transcription-dependent cellular events (Frey and Morris, 1997; Nguyen *et al.*, 1994). NMDARs also contribute to transcription that promotes neural development. For example, NMDAR activation regulates *Wnt-2* transcription in a manner dependent upon CaMK kinase (CaMKK), the Ca^{2+}/CaM-dependent protein kinase I (CaMKI) α and γ isoforms, MAPK, and CREB activation (Wayman *et al.*, 2006). This increase in *Wnt2* transcription following synaptic NMDAR stimulation results in increased Wnt-2 protein secretion and a subsequent increase in dendrite outgrowth and branching in hippocampal neurons. Finally, NMDAR activation has been shown to be essential for the regulation of neuronal survival during development (Hardingham and Bading, 2002, 2003; Hardingham *et al.*, 2002; Sala *et al.*, 2000). Interestingly, cell survival can be bidirectionally regulated by NMDARs, and this process appears to rely largely on which specific NMDARs are activated on a given neuron. We will discuss this phenomenon in greater detail below.

4.1.2. Molecular biology and patterns of expression of the NMDARs—NMDAR channels are heterotetramers that can be made up of different subunit compositions. There are seven known NMDAR subunits: NR1, which can be alternatively spliced to yield eight different variants; NR2A-D, which are encoded by four different genes; and NR3A-B, which are also encoded by distinct genes (Ciabarra *et al.*, 1995; Dingledine *et al.*, 1999; Matsuda *et al.*, 2002; McBain and Mayer, 1994; Nishi *et al.*, 2001; Sucher *et al.*, 1995). Functional glutamate-sensing NMDARs require at least one NR1 and one NR2 subunit (Cull-Candy *et al.*, 2001; Köhr, 2006; Rosenmund *et al.*, 1998), however the exact subunit composition of individual receptors varies. The NR1 and NR3A/B subunits contain glycine-binding sites, while the four NR2 subunits all contain glutamate-binding sites. In order to achieve full opening of the ion channel pore, co-activation by both glutamate and glycine is required (Clements and Westbrook, 1991; McGurk *et al.*, 1990). Most CNS NMDARs are thought to contain two NR1 subunits and two NR2 subunits (Dingledine *et al.*, 1999). NMDARs that contain NR3 subunits probably contain NR1, NR2, and NR3 (Sasaki *et al.*, 2002).

NR1 is expressed throughout the nervous system and is an obligate NMDAR subunit; without it, functional NMDARs cannot form (Ishii *et al.*, 1993; Kutsuwada *et al.*, 1992; Meguro *et al.*, 1992; Monyer *et al.*, 1992). Several important, functional differences have been identified that distinguish the four NR2 subunits (NR2A-D) from each other. NR2A and NR2B are most highly expressed in brain structures in the forebrain, while NR2C and NR2D are more highly expressed in the hindbrain and the cerebellum (Monyer *et al.*, 1994; Monyer *et al.*, 1992). The subunits NR2B and NR2D are both expressed in embryonic CNS structures, while there is no notable expression of either NR2A or NR2C until some time after birth (Monyer *et al.*, 1994; Monyer *et al.*, 1992). After birth, expression of NR2A and NR2C slowly increases until their levels plateau shortly before adulthood, while NR2B and NR2D levels either remain unchanged or decrease. The developmental "switch" that occurs between NR2B and NR2A over the course of development is thought to be critical for a number of neurological developmental milestones (Shi *et al.*, 1997). Furthermore, there is evidence that this switch is not only regulated by developmental progression, but also by neuronal activity, as visual deprivation retards this switch in the visual cortex (Nase *et al.*, 1999; Quinlan *et al.*, 1999; Roberts and Ramoa, 1999). Functionally, this switch is important because NR2B-containing NMDARs exhibit longer-lasting currents and carry more calcium per unit of current (Monyer *et al.*, 1994; Sobczyk *et al.*, 2005). This means that for each episode of presynaptic glutamate release, NR2B-containing NMDARs allow more calcium to enter the cell than do NR2A-containing NMDARs. Finally, like NR2A and NR2B, NR3A is most highly expressed in regions of the forebrain, while NR3B is more highly expressing in the midbrain, hindbrain, and in peripheral motor neurons (Al-Hallaq *et al.*, 2002; Matsuda *et al.*, 2002; Nishi *et al.*, 2001; Sasaki *et al.*, 2002; Wong *et al.*, 2002). Both NR3 subunits

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are highly developmentally regulated, with expression increasing in the early postnatal period and peaking ~P8–10 in the rodent brain before decreasing down to low levels that persist through adulthood.

4.1.3. Different pools of NMDARs differentially regulate transcription—The subunit composition of NMDARs has a strong effect on their function (Hardingham and Bading, 2010). While all NMDARs must contain an NR1 subunit, one key distinction between different receptors is which NR2 subunit they contain, as well as whether they contain an NR3 subunit. The combinations of NMDAR subunit assemblages allows for NMDARs with distinct pharmacological, physiological, and signaling properties (Monyer *et al.*, 1994; Monyer *et al.*, 1992). Subunit composition can significantly affect subcellular localization as well as which signaling cascades are activated downstream of NMDAR stimulation, and subsequently which transcriptional programs are induced in response to that stimulation (Cheng *et al.*, 1999; Hardingham and Bading, 2002, 2003; Hardingham *et al.*, 2002; Martel *et al.*, 2009; Yashiro and Philpot, 2008).

One transcription factor that is particularly important for NMDAR-regulated transcription is CREB (Hardingham and Bading, 2002, 2003; Hardingham *et al.*, 2002; Sala *et al.*, 2000). Interestingly, CREB is not only activated by NMDAR signaling, its transcriptional activity can also be turned off by NMDARs (Hardingham *et al.*, 2002). Specifically, synaptic NMDAR stimulation induces CREB Ser133 phosphorylation, which is required for transcriptional *activation*. By contrast, extrasynaptic NMDAR stimulation results in CREB Ser133 dephosphorylation, which leads to transcriptional *deactivation* (Hardingham and Bading, 2002; Hardingham *et al.*, 2002). One possible mechanism by which extrasynaptic NMDARs might induce CREB dephosphorylation is via the nuclear translocation of the recently-identified protein Jacob (Dieterich *et al.*, 2008). Jacob has been shown to be alternately bound by either the NCS Caldendrin – during periods then dendritic calcium levels are very high, such as would occur during synaptic NMDAR stimulation – or by α-importin, which following extrasynaptic NMDAR stimulation localizes Jacob to the nucleus (Dieterich *et al.*, 2008; Thompson *et al.*, 2004). Nuclear localization of Jacob has been shown to be both necessary and sufficient for extrasynaptic NMDAR-dependent CREB dephosphorylation and subsequent cell death pathway initiation (Dieterich *et al.*, 2008).

Importantly, even though they inactivate CREB under some conditions, extrasynaptic NMDARs are capable of inducing other programs of gene transcription (Hardingham and Bading, 2002; Hardingham *et al.*, 2002; Wahl *et al.*, 2009). However, unlike synaptic NMDAR activity, which promotes cell survival, transcription regulated by extrasynaptic glutamate receptors is able to trigger activation of cell death pathways in neurons. For example, as described in section 3.2.2, stimulation of extrasynaptic NMDARs has been shown to drive nuclear translocation of the FOXO family of transcription factors, which functions as a trigger for apoptosis through upregulation of cell-death promoting genes (Brunet *et al.*, 1999; Dick and Bading, 2010), whereas synaptic NMDAR activation induces FOXO export (Papadia *et al.*, 2008; Soriano *et al.*, 2006). Among the extrasynaptically-regulated genes that might damage neurons are *Clca1*, which encodes a putative calcium-activated chloride channel that has been implicated in other forms of cellular injury (Wahl *et al.*, 2009), and *Txnip*, a thioredoxin inhibitor that leaves cells more vulnerable to oxidative stress (Papadia *et al.*, 2008).

An important point of uncertainty lies in the identity of the subunits that comprise synaptic versus extrasynaptic NMDARs, and furthermore to what degree the subunit composition of these different pools of receptors matters for downstream transcriptional regulation. In the studies done on synaptic versus extrasynaptic regulation of CREB phosphorylation and CREB-dependent gene transcription, it has been demonstrated that blockade of NR2B-

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containing NMDARs by ifenprodil greatly impairs the ability of extrasynaptic NMDARs to effect CREB Ser133 dephosphorylation (Hardingham *et al.*, 2002). This raises the intriguing possibility that NMDAR NR2 subunit composition might dictate the specificity of signaling cascades activated downstream of NMDAR stimulation (Cheng *et al.*, 1999; Hardingham *et al.*, 2002; Martel *et al.*, 2009), and specifically suggests that NR2B is critical in mediating extrasynaptic NMDAR-dependent gene transcription programs. Some studies have suggested that synaptic NMDARs might be predominantly NR2A-containing, while extrasynaptic NMDARs are predominantly NR2B-containing (Tovar and Westbrook, 1999). This is interesting given that at birth, NMDARs are almost exclusively NR1/NR2B heteromers, and NR2A subunit expression in the rodent CNS doesn't begin until 6–10 days after birth, a particularly dynamic period in synapse development (Flint *et al.*, 1997; Kew *et al.*, 1998; Kirson and Yaari, 1996; Li *et al.*, 1998; Monyer *et al.*, 1994; Sheng *et al.*, 1994; Tovar and Westbrook, 1999). However, more recent evidence suggests that this model may be overly simplistic. In addition, at least one study has suggested that there are roughly equal proportions of NR2A and NR2B localized both synaptically and extrasynaptically (Thomas *et al.*, 2006). In dissociated hippocampal neuron cultures NMDARs have been found to be mobile and to traffic in and out of synapses via lateral diffusion (Groc *et al.*, 2006; Tovar and Westbrook, 2002), although a similar study in acutely dissected hippocampal slices reported contrary results (Harris and Pettit, 2007). One possible explanation for this apparent discrepancy could be that the extracellular matrix plays a role in NMDAR stabilization at the plasma membrane, which would only be left intact in whole tissue preps. More work is required to determine where NR2A and NR2B are localized, how their localization is regulated developmentally, and to what degree subunit composition versus localization is the deciding factor in what kind of signal is transmitted from NMDARs to the nucleus.

4.1.4. Protein interactions that underlie NMDAR signaling specificity—The large intracellular C-termini of the NMDAR subunits are variable and have been shown to confer differential protein-protein interactions with NMDARs of distinct subunit composition (FIGURE 6). These interactions have important functional implications for NMDAR localization as well as interaction with signaling molecules and may be responsible for the specificity upon receptor activation of downstream events including transcription. Given the differential effects of NR2A- and NR2B-containing receptors on many biological processes, including the regulation of CREB Ser133 phosphorylation, considerable effort has been devoted to determining whether these two NR2 subunits may differentially interact with signaling proteins.

Some signaling proteins bind to both NR2A and NR2B, however they may show differential regulation at these subunits. NR2A and NR2B both have PDZ-binding domains at the C-terminal tails, allowing them to interact with the membrane-associated guanylate kinase (MAGUK) family of synaptic scaffolding proteins (Kennedy, 2000). These scaffolds in turn associate with important synaptic signaling molecules and tether NMDARs to intracellular signaling pathways. The mammalian MAGUK family is comprised of four members: postsynaptic density-95 (PSD-95), synapse-associated protein-97 (SAP-97), postsynaptic density-93 (PSD-93), and synapse-associated protein-102 (SAP-102) (Sheng and Sala, 2001). Although some studies suggested that NR2A-containing NMDARs might be more likely to bind PSD-95, while NR2B-containing NMDARs could preferentially bind SAP102 (Sans *et al.*, 2000; Townsend *et al.*, 2003), others found that PSD-95 and SAP102 interact with diheteromeric NR1/NR2A and NR1/NR2B receptors in the adult brain *in vivo* at roughly comparable levels (Al-Hallaq *et al.*, 2007). However, NR2A-containing receptors co-immunoprecipitate with a greater fraction of the synaptic proteins neuronal nitric oxide synthase, Homer, and β-catenin (Al-Hallaq *et al.*, 2007). None of these proteins bind directly to NMDARs; instead they are thought to interact indirectly via their association with the MAGUKs or other scaffolding molecules such as the Shank family (Sheng, 2001). These

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data are consistent with the possibility that the MAGUKs may be key for differential formation of signaling complexes at distinct NMDARs, although the mechanism of specificity remains to be determined. Additionally, whether these differential interactions with scaffolding molecules influences specificity of signals transmitted to the nucleus remains an unanswered question.

Another signaling molecule that binds to both NR2A and NR2B but exhibits distinct properties at these subunits is CaMKII. NR2A and NR2B have both been shown to bind CaMKIIα and both subunits can be substrates for CaMKII phosphorylation (Bayer *et al.*, 2001; Gardoni *et al.*, 1999; Strack and Colbran, 1998; Strack *et al.*, 2000). However, the affinity of CaMKIIα binding to NR2B is greatly enhanced upon CaMKIIα autophosphorylation, suggesting a mechanism that may explain the stimulus-dependent trafficking of CaMKII to synapses (Bayer and Schulman, 2001; Shen and Meyer, 1999). Furthermore, the sites of CaMKIIα binding to the subunits differ – in NR2A the CaMKII binding site is near the C-terminus and competes with the PDZ domain interactions of the C-terminal tail (Gardoni *et al.*, 2001), whereas in NR2B the binding site is farther away from the C-terminus (Bayer *et al.*, 2001), potentially allowing NR2B to simultaneously interact with both CaMKII and PSD-95. Thus, while NR2A and NR2B can both bind to CaMKII, their specific modes of interaction with this important signaling molecule are fundamentally different, and this may differentially affect CaMKII-dependent gene transcription.

The ability of NMDARs to regulate CREB is known to require activation of the MAPK signaling pathway; thus, several studies have examined the differential recruitment to NR2 subunits of the upstream regulators of MAPK activation. The classic calcium-regulated MAPK cascade in neurons begins with signaling events that change the ratio of GTP:GDP-bound Ras (Rosen *et al.*, 1994). Ras then activates the MAP kinase kinase Raf, which in turn activates the MAP kinase MEK, which then activates the MAP kinases Erk1 and Erk2. Erk1/2 activate various kinases including Rsk2, which then translocate to the nucleus to phosphorylate CREB at Ser133. The ratio of GTP:GDP-bound Ras is determined by the balance in the activation of the GTPase activating proteins (GAPs) that enhance the ability of Ras to hydrolyze bound GTP, and the GTP exchange factors (GEFs) that catalyze the release of GDP from Ras in exchange for binding GTP.

Two of the most important calcium-regulated Ras modulators in neurons are the synaptic Ras GTPase activating protein (SynGAP) and the GEF Ras-GRF1/2 (Cullen and Lockyer, 2002). SynGAP is highly concentrated in the PSD of excitatory glutamatergic synapses in the CNS and the GAP activity of this protein is enhanced upon phosphorylation by CaMKII (Chen *et al.*, 1998; Oh *et al.*, 2004). Ras-GRF1 contains a CaM-binding IQ domain that is required for the regulation of its ability to active MAPK signaling, suggesting that calcium-dependent regulation is conferred on Ras-GRF1 indirectly through calcium binding to CaM (Farnsworth *et al.*, 1995). Ras-GRF2 also contains an IQ domain, but has a somewhat more complex mechanism of activation by calcium that involves additional protein-protein and protein-lipid interactions (Cullen and Lockyer, 2002). SynGAP is selectively found in synaptic complexes with NR2B-containing NMDARs where it may contribute to the ability of these receptors to suppress activation of MAPK signaling (Kim *et al.*, 2005). Intriguingly, Ras-GRF1 is also enriched at NR2B-containing receptors (Krapivinsky *et al.*, 2003), whereas Ras-GRF2 has been shown to selectively mediate signaling to MAPKs downstream of NR2A-containing receptors (Li *et al.*, 2006b). How the effects of Ras-GRF1 and SynGAP signaling toward MAPK pathways are integrated at NR2B-containing receptors is not entirely clear, but combinatorial signaling through these Ras regulators appears to largely have a net effect of inhibiting Erk1/2 activation (Kim *et al.*, 2005). By contrast, the Ras-GRF2 is required for NMDAR-dependent LTP (Li *et al.*, 2006b), a cellular phenomenon largely ascribed as a downstream action of NR2A-containing NMDARs (Liu *et al.*, 2004).

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Finally, there are a few classes of proteins that do appear to be specifically bound to one or the other of the two NR2 subunits NR2A and NR2B. One protein that may be involved in the pro-death-associated signaling capabilities of extrasynaptic NMDARs (as apposed to the pro-survival signaling of synaptic NMDARs) is death-associated protein kinase 1 (DAPK1). DAPK1 has been shown to directly bind to the NMDAR subunit NR2B and to phosphorylate NR2B at Ser1303, thereby enhancing NR2B-containing NMDAR channel conductance (Tu *et al.*, 2010). Importantly, genetic deletion of DAPK1 appears to uncouple NR2B-containing, extrasynaptic NMDARs from cell death-associated signaling pathways, while leaving NMDAR-mediated synaptic transmission unaffected. In mice deficient for DAPK1, activation of extrasynaptic NMDARs does not activate cell death, thus protecting them from ischemic insults (Tu *et al.*, 2010).

In addition to proteins that associate with the different NR2 subunits, it is also possible to generate diversity amongst NMDAR-associated signaling complexes via proteins that differentially associate with alternatively spliced regions of the C-terminus of the NR1 subunit. The *Grin1* gene encoding NR1 contains three alternatively spliced exons: exon 5 in the N terminus (also called the N1 cassette) and exons 21 and 22 in the C terminus (also known as the C1 and C2 cassettes) (Dingledine *et al.*, 1999). There is evidence that the different C-terminal NR1 splice variants may couple differentially to downstream signaling cascades and subsequent changes in gene transcription. In conventional nomenclature, NR1-1 is the full-length NR1 variant containing both C-terminal exons, NR1-2 lacks exon 21 (the C1 cassette), NR1-3 lacks exon 22 (the C2 cassette), and NR1-4 lacks both (Dingledine *et al.*, 1999; Hollmann *et al.*, 1993). Exclusion of exon 22 by splicing removes the stop codon present in exon 22 and introduces a new coding region that encodes an unrelated domain termed C2′ (Zukin and Bennett, 1995). Bradley and colleagues have found that NMDARs lacking the C1 cassette (aka exon 21; NR1-2 and NR1-4 splice variants) are much less efficacious in their ability to activate gene transcription than are NMDARs that contain the C1 cassette (NR1-1 and NR1-3) (Bradley *et al.*, 2006). Additionally, it has been shown that the splicing of the C2 cassette is activity-dependent, with increased activity leading to more C2-containing NR1 subunits and activity blockade yielding more C2′-containing NR1 subunits (Mu *et al.*, 2003). This activity-dependent splicing could provide a mechanism of homeostatic control, as the C2′ domain is much more efficiently trafficked out of the ER and to the cell surface than the C2 domain.

A yeast two-hybrid screen for proteins that bind to NR1 identified Yotiao as a protein that interacts with the alternatively spliced C-terminal C1 exon cassette of the ion channel (Lin *et al.*, 1998). Yotiao binds to both the cAMP-dependent protein kinase PKA and the protein phosphatase 1 (PP1) and is thought to be important for anchoring these proteins to NR1-1 and 1-3 containing NMDARs (Westphal *et al.*, 1999). It has been suggested that activation of PP1, which can dephosphorylate CREB, is important for limiting the duration of NMDAR-mediated transcription (Bito *et al.*, 1996). However, what effect the interaction of Yotiao with alternatively spliced NR1 subunits might have on NMDAR dependent transcription remains to be determined. Finally, two other NR1-interacting proteins that may contribute to the regulation of transcriptional pathways are the EF-hand-containing transcriptional repressor DREAM and the Eph family receptor tyrosine kinase EphB2. DREAM has been shown to bind to the membrane proximal region of the NR1 C-terminal domain (the "C0" domain) (Zhang *et al.*, 2010a). The interaction of DREAM with NR1 significantly impairs NMDAR surface expression in neurons and is neuroprotective against NMDA-induced excitotoxic neuronal injury, however the functional consequence of this interaction for NMDAR-mediated transcription, and the regulation of DREAM-mediated transcription, is not known. Unlike the other interactions discussed so far, which are associated with the intracellular domains of the NMDA-R, EphB2 binds to the extracellular domain of NR1 (Dalva *et al.*, 2000). EphrinB2-mediated activation of EphB2 receptors in

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primary cortical neurons potentiates NMDAR-dependent influx of calcium and subsequent CREB-dependent gene transcription (Takasu *et al.*, 2002). The effects of EphB2 on NMDAR function are mediated by phosphorylation of the NR2B subunit at Tyr1252, Tyr1336, and Tyr1472 through the activity Src tyrosine kinase family members. The cooperative regulation of transcription by Eph receptors and NMDARs may be a mechanism to couple activity-independent and activity-dependent signaling pathways in early neuronal development (Takasu *et al.*, 2002).

Finally, NR3A has been shown to engage in numerous protein-protein interactions that could have important implications for the function of NMDARs that contain this subunit. The C-terminus of NR3A has been shown to interact with several intracellular proteins, including PACSIN1/syndapin-1 (Pérez-Otaño *et al.*, 2006), protein phosphatase 2A (PP2A) (Chan and Sucher, 2001), and the small GTPase Ras homologue enriched in brain (Rheb) (Sucher *et al.*, 2010). The interaction with PACSIN1/syndapin-1 has been the most extensively studied, and has been shown to mediate activity-dependent endocytosis of NR3A-containing NMDARs, which may be important for the downregulation of NR3A surface expression during development (Pérez-Otaño *et al.*, 2006). Association of PP2A with the C-terminal tail of NR3A increases the phosphatase activity of PP2A and leads to dephosphorylation of the NR1 subunit at the PKA-regulated site at Ser897 (Chan and Sucher, 2001). The association of NR3A and PP2A is inhibited by NMDAR stimulation, suggesting a mechanism by which control of this signaling event could be regulated by synaptic activity. The C-terminal tail of NR3B differs substantially from that of NR3A, suggesting that the protein-protein interactions of these two related subunits may be distinct. The role of either of the NR3 subunits in influencing NMDAR-dependent gene transcription remains unknown.

4.2. L-type Voltage-Gated Calcium Channels (L-VGCCs)

AMPARs comprise the other major class of glutamate receptors at synapses, and activation of these receptors by glutamate results in a large current influx that drives postsynaptic membrane depolarization. This depolarization then induces the opening of voltage-gated ion channels, including voltage-gated calcium channels (VGCCs). The VGCCs are commonly referred to by names that arose from aspects of the physiological properties of their currents – for example there are L-type (long-lasting activation), N-type (neurotransmitter release), T-type (transient activation), P/Q-type (found in Purkinje cells), and R-type (drug-resistant) channels. Molecular cloning of VGCC subunits has shown that a set of distinct α_1 subunits confers the specific physiological properties upon each of these calcium channel types. Among the classes of VGCCs expressed in neurons, L-VGCCs have been shown to be a particularly salient mode of calcium entry in terms of their ability to bring about alterations in gene transcription (Bading *et al.*, 1993).

4.2.1. Molecular biology of the L-VGCCs—All VGCCs are made up of a single, central α_1 subunit, which both forms the ion- conducting pore and defines the channel type, along with a variable number of auxiliary α_2-δ, β, and γ subunits (Dai *et al.*, 2009) L-VGCCs are categorized as being high-voltage-activated channels (calcium channels that require strong membrane depolarization for activation) and are pharmacologically defined by their sensitivity to dihydropyridine agonists or antagonists (Dai *et al.*, 2009; Dolphin, 2009; Lipscombe *et al.*, 2004). The best-characterized L-VGCC α_1 subunit in terms of ability to regulate gene transcription in neurons is $Ca_V1.2$ (Dolmetsch *et al.*, 2001; Weick *et al.*, 2003). Additionally, several biochemical and functional studies indicate that $Ca_V1.2$-containing channels account for at least 75–80% of all L-VGCCs in the brain (Hell *et al.*, 1993a; Hell *et al.*, 1993b; Koschak *et al.*, 2007). The $Ca_v1.3$ α_1 subunit is also highly expressed in the brain where it has been shown to contribute to activity-dependent induction

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of nuclear CREB Ser133 phosphorylation (Zhang *et al.*, 2006). The α_2-δ, β, and γ auxiliary subunits function to modulate channel surface expression, localization, biophysical properties, and intracellular protein-protein interactions (Chen *et al.*, 2004; Dai *et al.*, 2009; Lipscombe *et al.*, 2004; Opatowsky *et al.*, 2004; Van Petegem *et al.*, 2004).

4.2.2. Physiological relevance of L-VGCCs for transcription—L-VGCCs are present along the dendrites and the somata of neurons. They have been shown to couple membrane depolarization in neurons to numerous cellular processes, and are thought to exert many of these actions through their effects on activity- and calcium-regulated gene transcription. Significant data implicate L-VGCCs in the activity-dependent regulation of neuronal survival. For example, Ghosh and colleagues demonstrated that activation of L-VGCCs promotes cell survival in cultured cortical neurons in a manner that requires BDNF, which can be transcriptionally induced by L-VGCC signaling (Ghosh *et al.*, 1994a). L-VGCCs have also been shown to regulate dendritic growth in cultured cortical neurons in a calcium-, CREB-, and transcription-dependent manner (Redmond and Ghosh, 2005; Redmond *et al.*, 2002). Interestingly, evidence suggests that the unique biophysical properties of L-VGCCs–namely their ability to activate at relatively negative potentials, and their slow activation kinetics as compared with other VGCCs–may allow them to act as a kinetic filter to support spike–EPSP discrimination in the activation of CREB-dependent gene transcription (Mermelstein *et al.*, 2000).

Genetic evidence for a critical role of L-VGCCs in neuronal and other cellular physiology came when the $Ca_V1.2$ gene was shown to be mutated in Timothy Syndrome. This syndrome is characterized by multiorgan dysfunction including lethal arrhythmias, webbing of fingers and toes, congenital heart disease, immune deficiency, intermittent hypoglycemia, cognitive abnormalities, and autism (Splawski *et al.*, 2005; Splawski *et al.*, 2004). Patients with Timothy syndrome have a critical mutation in an alternatively spliced exon of the *Cacna1c* gene encoding $Ca_V1.2$, which results in the coding sequence mutation G406R. The G406R mutation produces maintained inward Ca^{2+} currents by causing nearly complete loss of voltage-dependent channel inactivation, a key property of normal L-VGCCs (Splawski *et al.*, 2004). This could hypothetically lead to calcium overload in many excitable cell types, including neurons. Intriguingly, while cardiac arrhythmia due to delayed cardiomyocyte repolarization is the primary cause of death in Timothy syndrome (Splawski *et al.*, 2005; Splawski *et al.*, 2004), it remains to be determined what effect the prolonged inward Ca^{2+} currents might have on calcium-dependent gene transcription – particularly in the nervous system – and what role that could then play in the manifestation of plasticity-related neurological deficits such as autism.

4.2.3. Signaling pathways and protein interactions that regulate L-VGCCs— Like NMDARs, L-VGCCs have long been known to play an important role in activity-regulated gene transcription (Greenberg *et al.*, 1986). However, several early studies demonstrated important differences in the ability of calcium influx through L-VGCCs versus NMDARs to activate specific transcriptional pathways (Bading *et al.*, 1993; Ghosh *et al.*, 1994b). For example, whereas the activation of transcription through the SRF-binding SRE element can be driven by calcium influx through NMDARs, the calcium rises that activate transcription through the CREB-binding CRE-element are more selectively coupled to L-VGCCs (Bading *et al.*, 1993; Hardingham *et al.*, 1997). Like other plasma membrane receptors, the specificity of L-VGCC signaling is thought to arise in part through the nature of the specific protein-protein interactions between these channels and components of intracellular signaling pathways. Strong evidence for this idea of local calcium signaling at the L-type channel came from an elegant study that examined the ability of calcium chelators with different calcium binding affinities and K_{ON} rates to block membrane depolarization induced Ser133 phosphorylation of CREB (Deisseroth *et al.*, 1996). These

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studies showed that only the very rapid chelation of calcium, which inhibits the elevation of calcium with the microdomain at the mouth of the channel, is sufficient to block CREB Ser133 phosphorylation following L-VGCC activation. These data suggested that a local submembranous calcium sensor bound at or near the channel was required for transmission of the calcium signal to CREB.

One way in which L-VGCCs may be capable of coupling to downstream transcriptional signaling pathways is via their direct interaction with the calcium sensor CaM. An isoleucine-glutamine ("IQ") motif in the C-terminus of the L-VGCC α_1 subunit $Ca_V1.2$ allows the channel to bind CaM and this interaction is critical for conveying the Ca^{2+} signal through the channel to the nucleus (Dolmetsch *et al.*, 2001). Specifically, cultured neurons transfected with a construct encoding a $Ca_V1.2$ in which the IQ domain has been mutated are deficient for activation of the Ras/MAPK pathway, which is normally activated following L-VGCC activation. Additionally, these neurons fail to exhibit CREB- or MEF2-dependent gene transcription following a stimulus that normally leads to L-VGCC activation (Dolmetsch *et al.*, 2001). How the local activation of CaM might selectively couple L-VGCCs to CREB-dependent transcription is not entirely clear. CaM is involved in gating the calcium-dependent inactivation of L-VGCCs (Peterson *et al.*, 1999), which could influence the amplitude or kinetics of downstream signaling pathway activation. In addition, some studies have suggested that CaM locally activated at calcium channels may translocate to the nucleus (Deisseroth *et al.*, 1998). Nuclear CaM levels are elevated within 15 seconds of calcium channel activation, and this elevation precedes the phosphorylation of CREB (Mermelstein *et al.*, 2001). However, in isolated preparations of hippocampal nuclei, elevation of calcium levels is sufficient to induce CREB phosphorylation in a CaMK-dependent manner indicating that CaM translocation from the synapse is not required to activate this signaling pathway (Hardingham *et al.*, 2001). Furthermore, nuclear expression of a CaM-binding peptide that inhibits nuclear CaM blocks 43% of all genes induced by action potential firing in cultured hippocampal neurons, indicating that elevation of nuclear calcium is essential for controlling the expression of a large subset of activity-regulated genes (Zhang *et al.*, 2009).

Protein-protein interactions that affect L-VGCC channel function may serve to regulate gene transcription programs by regulating the location or function of L-VGCCs themselves. For example, the tumor suppressor eukaryotic initiation factor 3 subunit E (eIF3E, also known as Int6) binds to the II-III loop of $Ca_V1.2$ in a calcium-dependent manner following electrical stimulation. This interaction facilitates the activity-dependent internalization of L-VGCCs into endosomes following neuronal activity (Green *et al.*, 2007), and thus has the potential to function in the homeostatic regulation of L-VGCCs and their downstream signaling effects on the neuron. Interestingly, the lipid kinase phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) has also been shown to bind to $Ca_V1.2$ in an activity-dependent manner (Tsuruta *et al.*, 2009). Following NMDAR- induced binding of PIKfyve to $Ca_V1.2$, $Ca_V1.2$ is endocytosed and targeted to lysosomes for degradation. Tsuruta and colleagues suggest that this phenomenon could be a novel mechanism by which neurons guard against excitotoxicity, as knockdown of PIKfyve prevents $Ca_V1.2$ degradation and increases neuronal susceptibility to excitotoxicity (Tsuruta *et al.*, 2009). It is important to note that the stimulus used in this study to activate NMDARs was the bath application of high concentrations of glutamate to cortical neurons in culture. It has been shown previously that this stimulus not only activates synaptic NMDARs (which induce pro-survival signaling pathways and gene transcription paradigms) but also activates extrasynaptic NMDARs, which have been shown to induce cell death in a manner that is dominant over the synaptic NMDAR-mediated signals (Hardingham and Bading, 2002, 2003; Hardingham *et al.*, 2002). It would be interesting to determine whether the binding of PIKfyve to $Ca_V1.2$ and the subsequent internalization and degradation of $Ca_V1.2$ is specifically induced by activation of

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extrasynaptic NMDARs (which would indicate a possible compensatory role for this interaction) or by synaptic NMDARs (which would indicate that it functions as part of a larger pro-survival signaling paradigm induced by synaptic activation).

Finally, the activity of L-VGCCs is also subject to modulation downstream of other signaling cascades in addition to changes in cellular calcium. Thus, modulation of L-VGCC function may be a pathway through which other signaling cascades can alter gene transcription. For example, it has long been known that activity of L-VGCCs in neurons was subject to modulation by cAMP signaling (Gray and Johnston, 1987). Phosphorylation of the L-VGCC α_1 subunit $Ca_V1.2$ by PKA enhances L-type Ca^{2+} currents by increasing the open probability (P_O) of the individual channels in cardiac myocytes (Bean *et al.*, 1984; Yue *et al.*, 1990), and PKA modulates L-VGCCs in the dendrites of hippocampal neurons as well (Hoogland and Saggau, 2004; Kavalali *et al.*, 1997). A substantial body of evidence indicates that the PKA-mediated enhancement of L-VGCC current specifically relies on the phosphorylation of Ser1928 in the C-terminal tail of the pore-forming $Ca_V1.2$ subunit (Catterall, 2000). The A kinase-anchoring protein 15 (AKAP15) is capable of directly interacting with the distal C terminus of the L-VGCC α_1 subunit $Ca_V1.2$ via a leucine zipper-like motif, and this interaction is important for the β-adrenergic regulation of CaV1.2 channels via the PKA pathway (Hulme *et al.*, 2003). However, while AKAP15 can direct PKA to its protein substrates, other AKAPs can function as signaling integrators by binding additional regulators other than PKA to target proteins. Recently, Oliveria and colleagues have shown that AKAP79/150 is capable of anchoring both PKA and calcineurin to $Ca_V1.2$ via a direct interaction between modified leucine zipper motifs in both proteins, and that this association is capable of modulating neuronal L-VGCC-mediated calcium currents (Oliveria *et al.*, 2007). Interestingly, these authors also showed that AKAP79/150 targeting of calcineurin is necessary to couple Ca^{2+} influx via L-VGCCs to activation of the transcription factor NFATc4, thus providing a novel mechanism by which L-VGCCs might be capable of signaling to the nucleus.

4.3. Other sources of calcium

Although NMDARs and L-VGCCs appear to play the major roles in the regulation of calcium-dependent transcription in neurons, other sources of calcium may be important under select conditions. For example, although N-type voltage-gated calcium channels (N-VGCCs), are generally inactivated during persistent neuronal depolarization, transient activation of these channels has been shown to contribute to the induction of tyrosine hydroxylase expression that occurs following 5 Hz electrical stimulation of primary sensory neurons (Brosenitsch and Katz, 2001).

In addition, subsets of AMPARs can provide a source of calcium entry at the synapse. AMPARs are tetramers comprised of various combinations of the GluA1-4 subunits (formerly called GluR1-4 (Collingridge *et al.*, 2009)). In the mature brain, the majority of AMPARs contain the GluA2 subunit and are impermeable to calcium due to the presence of a positively-charged arginine (R) residue in a key position in the GluA2 pore domain that blocks calcium influx. Notably, however, some GluA2-containing AMPARs are calcium permeable. This is because the calcium permeability of GluA2 is regulated post-transcriptionally by RNA editing. In the genome, the DNA sequence of the *Gria2* gene encodes a negatively charged glutamine (Q) residue at the key selectivity residue in the GluA2 pore, rather than the R found in edited *Gria2* mRNA (Lomeli *et al.*, 1994). The Q/R switch occurs by editing of the *Gria2* mRNA that is mediated by the enzyme RNA-dependent adenosine deaminase 2 (ADAR2) (Melcher *et al.*, 1996). In addition to AMPARs with unedited GluA2, some AMPARs pass calcium because they lack GluA2. For example, early in development a significant fraction of AMPARs lack GluA2 (Kumar *et al.*, 2002), and GluA2-lacking, calcium-permeable AMPARs are also expressed in interneurons of the

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adult cortex (Geiger *et al.*, 1995). Furthermore, following the induction of LTP, the selective insertion of GluA1-only AMPA receptors has been reported, leading to the transient appearance of GluA2-lacking, calcium permeable AMPARs at potentiated synapses (Liu and Cull-Candy, 2000; Plant *et al.*, 2006). This calcium could contribute to transcriptional regulation. It has been shown that pharmacological activation of AMPARs in striatal and cortical neurons leads to a MAPK- and calcium-dependent phosphorylation of the transcription factor CREB, raising the possibility that AMPARs could function in some cases to activate changes in gene transcription (Perkinton *et al.*, 1999; Tian and Feig, 2006). However, whether calcium influx through AMPARs contributes to the regulation of specific transcriptional programs has not been well established.

Finally, neuronal transcription can also be regulated by calcium release from intracellular calcium stores (Hardingham *et al.*, 2001). In order to utilize the highly abundant ion calcium as a second messenger, all cells tightly regulate intracellular calcium levels by binding or sequestering the vast majority of free calcium (Clapham, 2007). The endoplasmic reticulum (ER) serves as a particularly large and important reservoir of stored calcium. In response to extracellular signals that activate receptors coupled to G_q-type G proteins or phospholipase C (PLC), calcium is released from the ER into the cytoplasm through IP3- and ryanodine receptors (Berridge, 2002). In non-excitable cells such as lymphocytes, this depletion of intracellular calcium stores is coupled to the opening of calcium release-activated calcium (CRAC) channels in the plasma membrane. Lymphocytes lack the VGCCs that are so important for activity-dependent transcription in neurons, and in non-excitable cells it is the CRAC channels that serve as the major source of calcium for stimulus-dependent transcriptional regulation (Hogan *et al.*, 2010). Store-operated calcium entry depends on the proteins known as stromal interaction molecules 1 and 2 (Stim1 and Stim2), which sense the depletion of calcium in the ER. The Stim proteins then form a complex with the plasma membrane protein Orai, which forms the pore subunit of the CRAC channel.

Stim1 is expressed in the brain, where it has been shown to interact directly with the C-terminal tail of the L-VGCC subunit $Ca_v1.2$ (Park *et al.*, 2010). Interestingly, the association of Stim1 with $Ca_v1.2$ suppresses depolarization-induced opening of the L- VGCC channel and causes long-term depletion of L-type channels from the plasma membrane by promoting their internalization. These data suggest that the actions of Stim1 and L-VGCCs are reciprocally regulated in neurons, raising the possibility that they could signal the activation of distinct downstream events. One stimulus that may utilize this interplay between these calcium sources to differentially regulate transcription is the activation of nicotinic acetylcholine receptors (nAChRs). Activated nAChRs are capable of inducing CREB Ser133 phosphorylation and activating *Fos* expression (Chang and Berg, 2001; Hu *et al.*, 2002). This transcriptional response requires calcium influx across the plasma membrane as well as subsequent release of calcium from internal stores, and is thought to signal to the nucleus through CaMK and MAPK pathways. Interestingly, however, sustained CREB phosphorylation is only driven by nAChRs if L-VGCCs are silent (Chang and Berg, 2001). As described above, Stim1 is one potential mechanism that could couple intracellular calcium release and silencing of L-VGCCs (Park *et al.*, 2010). Future studies that address the requirements for Stim1/2 in stimulus-regulated transcription in the nervous system may reveal selective functions for intracellular calcium stores in response to different kinds of extracellular stimuli.

5. Roles for activity-regulated transcription in homeostatic synaptic plasticity

We have concentrated on describing the signaling pathways that confer specificity upon transcriptional regulatory processes induced by neuronal activity. Now we turn our attention

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to considering how this biochemical selectivity is translated into differential biological outcomes. As an example, we consider the transcription-dependent processes that regulate homeostatic synaptic plasticity in response to elevations versus reductions in synaptic activity.

Neuronal activity-regulated transcription has been shown to contribute to many biological processes in the brain including neurite outgrowth (Spitzer, 2006; Wayman *et al.*, 2006), cell fate determination (Borodinsky *et al.*, 2004), synapse development (Greer and Greenberg, 2008), critical period plasticity (Sugiyama *et al.*, 2008), long-lasting changes in synaptic strength (Alberini, 2009), and neural adaptations to drugs of abuse (Nestler, 2001). In some cases such as cortical development, the activity-dependent processes appear to be inductive. For example, in the visual cortex, the onset of patterned visual input from the two eyes and the subsequent activity-regulated, transcription-dependent maturation of inhibition induces the closure of the critical period for experience-dependent rewiring of inputs from the two eyes (Fagiolini *et al.*, 2004). However, more often the actions of activity-regulated transcription appear to be largely homeostatic. The term homeostasis refers to phenomena by which a single cell, an organ system, or an entire organism maintains a stable internal environment in the face of changing external stimuli (Cannon, 1932).

While some homeostatic mechanisms in the nervous system occur at the level of the neural network or circuit, there also exist cellular homeostatic plasticity mechanisms that are able to mediate the scaling of excitatory synaptic strength following changes in synaptic activity within a single cell (Burrone and Murthy, 2003; Turrigiano, 2007). It has been proposed that for a molecular mechanism to function as a homeostatic regulator of synaptic scaling that the integrator needs to be able to sample the overall activity of the cell’s synapses and feedback to modulate synaptic function globally (Turrigiano, 2007; Turrigiano and Nelson, 2004). Activity-regulated transcription factors are well-suited to play this role, as they serve as substrates for converging signaling pathways, they are centrally located in the cell nucleus, where they respond to signals integrated from all over the cell, and they function on a slow time course relative to synaptic signaling, allowing them to respond to accumulated changes in activity over time. Furthermore, many activity-regulated transcription factors control the expression of gene expression programs that function at synapses (West *et al.*, 2001).

However, one additional tenet of the integrator hypothesis is that a homeostatic mechanism is expected to be bidirectionally responsive – such that when activity is enhanced the integrator mechanism can scale the strength of synapses down, whereas when activity is reduced the mechanism can scale synapses up. Early gene expression studies primarily focused on the ability of neuronal transcription to be induced by increases in activity and even seemed to suggest that activity-dependent decreases in transcription were less common than increases in gene expression (Nedivi *et al.*, 1993). However, with improved sensitivity in the techniques for the detection of mRNA levels and an increasing range in the kinds of stimuli that can be used to alter neural activity, it has become clear that transcription can be bidirectionally regulated by changes in neural activity, expanding the range of biological stimuli for which this mechanism could be relevant (Kim *et al.*, 2010; Zhang *et al.*, 2007). Intriguingly, two recent complementary studies have demonstrated the importance of transcriptional mechanisms for both up- and downregulation of synaptic strength following changes in cell firing (Goold and Nicoll, 2010; Ibata *et al.*, 2008) (FIGURE 7). These studies highlight the idea that specificity in the regulation of transcriptional pathways can have highly specific, and in this case opposing, effects on cellular physiology.

In 2008, Ibata and colleagues published a landmark study demonstrating that transcription is required for the ability of cortical neurons to homeostatically adjust synaptic strengths in

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response to changes in their own firing rates (Ibata *et al.*, 2008). To monitor synaptic strength, cells were transfected with GluR2 tagged at the extracellular N-terminus with enhanced yellow fluorescent protein (eYFP). Since the fluorescence of eYFP is highly pH-sensitive, the signal is quenched when GluA2 is held in intracellular endosomes. Thus, plasma membrane insertion of GluA2 can be detected as an acute increase in eYFP fluorescence at synapses. Although synaptic scaling has previously been studied on longer time scales, using this live imaging method the authors discovered that bath application of the sodium channel blocker tetrodotoxin (TTX) for as little as 4 hours resulted in the accumulation of AMPARs at synapses. Local application of TTX to the soma of a single neuron in the culture was sufficient and necessary to induce this synaptic scaling effect, demonstrating that it is somatic action potential firing that underlies the change in synaptic AMPAR number. Inhibition of somatic calcium signaling by local application of either the broad spectrum calcium channel blocker $NiCl_2$ or the L-VGCC antagonist nifedipine was also sufficient to induce the increase in synaptic AMPARs, indicating that a decrease in somatic calcium signaling could underlie this effect. Phosphorylation of the calcium-regulated nuclear kinase CaMKIV was reduced when TTX was applied to the cells, and overexpression of a dominant-negative CaMKIV occluded the effects of TTX on AMPAR accumulation at synapses. Most strikingly, co-application of the transcriptional inhibitor actinomycin D (ActD) blocked the ability of either TTX or the CaMKK2-CaMKIV inhibitor Sto-609 to increase synaptic AMPARs. Taken together, these results demonstrate that these effects of *reducing* activity and calcium signaling on synaptic scaling depend on the activation of transcription.

In an aesthetically pleasing complement to this study, in 2010, Goold and Nicoll examined the cellular homeostatic mechanisms that are involved in synaptic scaling during periods of *increased* neuronal activity (Goold and Nicoll, 2010). To change the firing of single neurons within an organotypic hippocampal slice, the authors used optogenetic techniques, transfecting single cells with the light-gated ion channel channel rhodopsin 2 (ChR2). ChR2 is a non-selective cation channel that is opened upon exposure to blue light. Neurons expressing ChR2 can be made to fire following ChR2 activation at rates up to 50Hz (Boyden *et al.*, 2005). Using electrophysiological measures of synaptic function, the authors found that cell autonomous activation of neuronal firing resulted in a downregulation of both synaptic AMPARs and synaptic NMDARs. Pharmacologically, the scaling of these synaptic proteins relied on a CaMKK2- and CaMKIV-dependent signaling cascade. However, while the synaptic depression of AMPARs was blocked by inhibitors of transcription and translation, the synaptic depression of NMDARs was not, indicating a divergence in the cellular mechanisms responsible for the downregulation of these two different types of glutamate receptors following increased neuronal activity (Goold and Nicoll, 2010).

Taken together, these two studies point toward CaMKIV and GluA2 as key nodes in the signaling pathway that controls bi-directional transcription regulation of homeostatic synaptic scaling in single neurons. However, the transcription factors and gene targets that mediate this process remain unknown. CREB is a known target of regulation by CaMKIV, but most experimental manipulations of CREB appear to be positively coupled to synapse formation and thus seem unlikely to be homeostatic (Impey *et al.*, 2002; Wayman *et al.*, 2008a). MEF2 and SRF have been highly linked to homeostatic regulation of excitatory synapse number or strength (Flavell *et al.*, 2006; Smith-Hicks *et al.*, 2010), however the regulation of these factors by CaMKIV has not been reported. Furthermore, very little is known about transcriptional mechanisms that activate transcription during periods of decreased synaptic activity. As described in the transcription factor section above, nuclear translocation of the transcription factor CCAT has been observed following treatment of cells with TTX (Gomez-Ospina *et al.*, 2006). CCAT is derived from the C-terminal tail of the L-VGCC subunit Cav1.2 and it accumulates in the nucleus under conditions of low

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intracellular calcium (Gomez-Ospina *et al.*, 2006). It is highly likely that there are additional factors that respond to decreases in neuronal activity. Future studies that identify these factors and study their roles in synaptic scaling will expand our understanding of how transcriptional specificity is achieved in this paradigm.

6. Technological advances for studying neuronal activity-regulated transcription

Despite substantial progress in identifying the molecular components of neuronal activity-regulated transcription in neurons, important questions regarding the mechanisms and consequences of this biological process remain to be addressed. As indicated by the theme of this review, one of the most significant directions of recent research has been to elucidate mechanisms that can confer specificity upon transcriptional regulation. However, even though the activation of a transcription factor might be subject to differential phosphorylation downstream of calcium influx through L-VGCCs versus NMDARs, for many factors it remains to be resolved what the precise biological consequence of this specificity might be for a neuron *in vivo*. In addition, since the regulation and function of single transcriptional pathways are often studied in experimental isolation, much remains to be learned about how single transcription factors integrate into the highly interconnected signaling networks that comprise the native cellular context. Finally, because many of the methods used to study transcriptional regulatory pathways are biochemical techniques that average the effect of experimental manipulations over a population of cells, significant questions arise about whether there exist transcriptional variations between individual neurons and how these variations could influence brain function. These questions of biological significance, and mechanistic or cellular specificity, are likely to dominate the field of neuronal activity-regulated transcription in coming years. Here we review some of the technological innovations that are helping to advance each of these areas of inquiry.

6.1. Genetic techniques to study the function of transcription factors

Transcription factors are like any other gene product in that understanding the biology of these proteins has been greatly advanced by the developments in experimental molecular genetics that allow the generation of knockout mouse strains. However, activity-regulated transcription factors present three specific problems that limit the usefulness of conventional mouse knockouts. First, most transcription factors belong to large protein families with related DNA binding domains and overlapping functions. Thus, compensation by related family members can limit the detection of phenotypes in single transcription factor knockout strains, whereas embryonic lethality is a frequent complication of double or triple knockout combinations. Second, knockout technology has poor temporal resolution and thus is not well suited to isolating the activity-dependent component of transcription factor function independent of functions in brain development. Finally, since transcription factors regulate large sets of target genes, knockout of any given factor is likely to have effects on a large number of these genes, challenging the ability of this loss-of-function genetic approach to identify the biological significance of any given transcription factor-target gene interaction.

6.1.1. RNA interference (RNAi) – spatial, temporal, and cell-type specific gene disruption—The genetic analysis of protein function has been dramatically enhanced with the advent and widespread use of RNAi technology (Novina and Sharp, 2004; Zeringue and Constantine-Paton, 2004). A significant advantage of RNAi is that it is easier using this technology to disrupt several RNA species at the same time, compared with crossing multiple strains of knockout mice (Paradis *et al.*, 2007), and thus could be useful for the functional study of transcription factor families.

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One key application of RNAi technology for neuronal activity-dependent transcription studies is virally-mediated RNAi delivery into the brain of adult animals to study the function of specific transcription factors in stimulus-dependent behavioral plasticity. The regional knockdown of neuronal proteins using stereotactically injected viruses allows the functional contributions of these pathways to be isolated to specific parts of defined neuronal circuits. This spatial specificity has revealed that altering activity-dependent transcription in different brain regions can have substantially different effects on behavior. For example, genetic manipulations that reduce BDNF expression in subcortical regions (nucleus accumbens, caudate/putamen, ventral tegmental area) are associated with *impaired* susceptibility to addictive-like behaviors in rodents treated with psychostimulant drugs of abuse (Graham *et al.*, 2007; Im *et al.*, 2010; Pu *et al.*, 2006), whereas genetically reducing BDNF expression in the prefrontal cortex *enhances* addictive-like behaviors in these same paradigms (Sadri-Vakili *et al.*, 2010).

The temporally defined gene knockdown afforded by this methodology allows the function of proteins in the adult brain to be isolated from roles they may have during development. For example, both the activity-regulated transcriptional activator MEF2 and the methyl-DNA binding protein MeCP2 are known to play important roles in synapse development (Barbosa *et al.*, 2008; Dani and Nelson, 2009; Deng *et al.*, 2010; Wood *et al.*, 2009; Zhang *et al.*, 2010b). Yet these proteins are also expressed in the adult brain where both of these transcription factors were identified as targets of psychostimulant-induced signaling cascades, raising the possibility that they might contribute to the transcriptional programs that underlie psychostimulant-induced behavioral modifications (Deng *et al.*, 2010; Pulipparacharuvil *et al.*, 2008). Using virally-mediated RNAi in adult mice, knockdown of MEF2 in the nucleus accumbens was shown to impair behavioral sensitization to repeated cocaine treatment, while knockdown of MeCP2 enhanced the rewarding properties of amphetamine as measured by the expression of conditioned place preference (Deng *et al.*, 2010; Pulipparacharuvil *et al.*, 2008).

Other variants of viral vectors allow for cell-type specificity of the knockdown by combining overexpression or RNAi with cell-type specific Cre recombinase expression. In this method, the cDNA or RNAi target sequence is cloned into a viral vector either after a floxed stop codon or in reverse orientation between two sets of loxP sites (Atasoy *et al.*, 2008; Stern *et al.*, 2008). When injected into the brain, although the virus infects many cells, only in those cells expressing the Cre recombinase will the overexpression or RNA interference cassette be correctly positioned with respect to the promoter to drive expression. This is an important new addition to the toolkit for the study of specificity in activity-regulated transcription.

6.1.2. Strategies for isolating the activity-dependent functions of transcription factors—Perhaps the most significant genetic challenge for this field has been discovering ways to selectively disrupt the activity-dependent functions of transcription factors without altering their basal activities. There have been essentially two approaches to this problem. One is to alter the transcription factor or its cofactors in ways that selectively disrupt the activity-dependent component of their function. The other is to dissociate the actions of the transcription factor from its ability to confer activity-dependent regulation on a target gene.

Capitalizing on the fact that the stimulus-inducible activity of many transcription factors depends on the local recruitment of the HAT CBP, Korzus and colleagues devised a genetic strategy to inducibly block CBP function in the adult brain. This mouse strain overexpresses a tetracycline-inducible allele of full-length CBP that bears two amino acid substitutions that abolish the HAT activity of CBP (CBP[HAT−/−]). Animals in which CBP[HAT−/−] transgene expression is activated during adulthood exhibit long term, but not short term,

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memory deficits that can be fully rescued by subsequent suppression of transgene expression or administration of a HAT inhibitor (Korzus *et al.*, 2004). These data provide important support for the possibility that CBP-dependent stimulus-induced gene transcription is important for learning and memory.

Another strategy for disrupting stimulus-dependent functions of transcription factors that are targets of calcium signaling cascades is to generate knockin mice that bear germline mutations of activity-regulated transcription factor phosphorylation sites. For example, activation of calcium signaling cascades leads to phosphorylation of CREB on several serine residues (Shaywitz and Greenberg, 1999). Whereas phosphorylation of Ser133 is required for most CREB activity and is broadly induced by all stimuli that activate CREB-dependent transcription, induced phosphorylation of CREB at Ser142 and Ser143 is selective for calcium signaling pathways and may provide a modulatory influence on CREB function (Kornhauser *et al.*, 2002). *In vivo*, as described in section 3.1.1, CREB Ser142 phosphorylation is induced in neurons of the suprachiasmatic nucleus (SCN) by light exposure. Thus to address the potential functions of this modification in light-induced plasticity of SCN function, Gau and colleagues generated a transgenic mouse strain in which a mutation was knocked into the endogenous *Creb1* locus that changes Ser142 to an alanine residue, rendering CREB non-phosphorylatable at this site (Gau *et al.*, 2002). Light-induced phase shifts of locomotion and expression of c-Fos and Per1 in the SCN are significantly attenuated in the knockin mutants, demonstrating that Ser142 phosphorylation of CREB is involved in the entrainment of the mammalian clock.

The phosphorylation site knockin and RNAi concepts for studying activity-regulated transcription factors can be combined into viral replacement strategies. For example, Zhou and colleagues developed a bicistronic lentiviral vector that simultaneously expresses an shRNA to knockdown endogenous MeCP2 while also expressing an RNAi-resistant version of either wildtype or Ser421Ala mutant MeCP2 (Zhou *et al.*, 2006). Using this vector, the authors found that overexpression of wildtype MeCP2 in hippocampal neurons in organotypic slice culture leads to a decrease in dendritic spine density. By contrast, replacement of endogenous MeCP2 with overexpressed Ser421Ala mutant MeCP2 has no effect on spines. These data suggest that phosphorylation of MeCP2 is important for its ability to regulate dendritic spine density, supporting a role for the activity-dependent modulation of this protein in synapse development and plasticity.

Rather than targeting the transcription factor, knockin strategies can also be used to disconnect the activation of a transcriptional signaling pathway from its activity-regulated target gene. Transcription factors act on their target genes by binding to specific sequence elements in gene promoters or other genomic regulatory regions. Mutation of a genetic regulatory element to a sequence that does not permit transcription factor binding disrupts this r egulatory interaction without affecting other functions of the transcription factor and leaving intact alternative genomic regulatory elements through which the target gene can be regulated by other factors. For example, in the case of the *Bdnf* gene, to demonstrate the requirement for the CREB-regulated CaRE3 site in activity-dependent regulation of *Bdnf* exon IV transcription, Hong and colleagues generated 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) (Hong *et al.*, 2008). Neurons from CaRE3 mutant mice have normal basal levels of BDNF but selectively lack activity-inducible transcription from promoter IV. Interestingly, mice bearing this mutation have reduced numbers of GABAergic synapses. Although BDNF levels had been linked with GABAergic synapse development prior to this study (Genoud *et al.*, 2004; Huang *et al.*, 1999), the experiments in this mouse strain were the first to demonstrate that it

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is the activity-dependent component of BDNF expression that is selectively required for this synapse development process.

Finally, whereas transgenic mouse generation is a laborious process, the role of activity-regulated transcriptional elements can also be more rapidly assayed using the expression of proteins from recombineered bacterial artificial chromosomes (BACs). BACs are large DNA constructs that can be used to clone, propagate, and transfer genomic DNA regions that range from 150–300kbp. Thus, for any given gene, the BAC can contain the complete coding region as well as the large majority of the genetic regulatory elements required for proper regulated expression of the gene. Transfection of a BAC into a cell can be used to drive expression of a gene of interest within the genomic regions carried in the BAC, and mutation of genetic regulatory elements within the BAC can be used to test the function of these elements. For example, Smith-Hicks and colleagues recently used this strategy to study the role of SRF-dependent *Arc* transcription in cerebellar LTD. In this study, the authors showed that the impaired LTD found in cultured cerebellar neurons from *Arc* knockout mice can be rescued by re-expression of *Arc* from a transfected BAC (Smith-Hicks *et al.*, 2010). However, if the SRF binding site in the synaptic activity-regulated enhancer of the *Arc* gene was mutated to a sequence which does not support wildtype SRF binding, the BAC was no longer able to drive stimulus-dependent *Arc* expression or to rescue LTD. Most elegantly, when an SRF engineered to bind the mutated SRF binding site was co-transfected with the mutated BAC, *Arc* expression as well as LTD were once again restored. Additional future studies of this kind will undoubtedly expand our understanding of the functional consequences of activity-regulated gene transcription.

6.2. A systems biology approach to neuronal activity-regulated gene transcription

Advances in high-throughput sequencing technologies are revolutionizing the quantitative study of transcription. Large-scale sequencing efforts started evolving rapidly at the time of the publicly-funded International Human Genome Project (Watson, 1990). Although this project, and the parallel effort led by Celera Genomics, led to the development of many of the sequence alignment, sequence data visualization, and data storage tools still used today, the actual sequencing of the human genome was accomplished with incremental modifications of the Sanger sequencing methods first developed in the 1970s (Sanger *et al.*, 1977). However, in 2004, a new and far more rapid method of sequencing was introduced. Instead of sequencing a single strand of DNA at a time, the next generation sequencers (which include the Roche 454, the Solexa/Illumina Genome Analyzer, the SOLiD platform from Applied Biosystems, and the new single molecule platform from Helicos called the HeliScope) use massively parallel sequencing platforms that allow them to simultaneously sequence up to several hundred million DNA fragments in parallel, generating a very large number of short (50–500bp) sequence reads (McPherson, 2009).

There are three main advantages to next-generation sequencing. First, the large volume of sequence data from these techniques means that the content and dynamic range of the information obtained can outstrip that obtained with other technologies such as gene expression microarrays, for example. Second, large volumes of sequence information can be obtained very rapidly permitting analysis of large sets of experimental samples. Finally, the cost of next-generation sequencing has fallen to the point where it is within the reach of individual investigators and thus can be applied to a broad range of experimental questions. For these reasons, high-throughput sequencing represents one of the most rapidly expanding current experimental technologies. We will discuss three areas where sequencing has influenced the understanding of neuronal activity-regulated transcription – comparative genome sequencing, whole transcriptome sequencing (RNA-Seq), and genome-wide chromatin immunoprecipitation (ChIP-Seq).

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6.2.1. Comparative genomics for identification of genetic regulatory elements—One of the first applications of genomic technology for the study of transcription was the use of evolutionary comparisons between multiple species to identify conserved blocks of non-coding DNA sequences that contain gene regulatory elements (e.g., promoters, enhancers, insulators, silencers) (Pennacchio and Rubin, 2001). Like coding sequences, gene regulatory elements constitute a small fraction of the total genome. However, unlike coding sequences, which can be identified by aligning sequenced cDNA with genomic DNA, traditional methods for identifying regulatory elements have relied on labor-intensive functional evaluation in biochemical or cell biological laboratories. Once the genomes of multiple species became available (the first comparisons were of human and mouse, then human, mouse, and pufferfish), cross-species comparisons of sequence showed that there were significant regions of conservation not only within coding but also in non-coding regions of the genome. Analysis of these conserved regions has helped to localize certain kinds of gene regulatory elements, such as distal enhancers of transcription that control cell-type specific gene transcription (De Val *et al.*, 2008; Visel *et al.*, 2008). In addition, analysis of single-nucleotide polymorphisms within these conserved regions has increased understanding of the mechanisms by which variations within non-coding regions of the genome may confer individual risk for disease susceptibility (Visel *et al.*, 2009). An important limitation of this analysis is that sequence conservation does not necessarily imply conserved function, and many studies have shown that evolutionarily conserved sequences are utilized in a tissue-specific and/or species-specific manner (Heintzman *et al.*, 2009; Schmidt *et al.*, 2010).

6.2.2. Transcriptional complexity revealed by whole transcriptome sequencing—Quantitative analysis of RNA expression, whether performed by RNAse protection, quantitative polymerase chain reaction (PCR), or microarray hybridization, has always been an essential part of transcriptional studies. RNA-Seq is the latest addition to this arsenal (Wang *et al.*, 2009; Wilhelm and Landry, 2009). Like other RNA analysis techniques, this process begins with the synthesis of cDNA. However, in most of the methods described above, only specific target segments within the RNA are quantified. For RNAse protection, the 5′ end of the RNA is the target sequence; for quantitative PCR, whatever fragment lies between the gene-specific primers is measured; for microarray hybridization, it is usually a small region of homology to a short oligonucleotide that is quantified, though there are often several oligonucleotides on a microarray that recognize the RNA transcribed from any single gene. By contrast, in RNA-Seq the entire cDNA for each transcript is fragmented into small pieces and subjected to quantitative sequencing.

A major advantage of this technique is that it facilitates the identification and quantification of RNA variants, such as alternative transcriptional start sites, alternative exon usage, and different sites of polyadenylation (Cloonan *et al.*, 2008; Mortazavi *et al.*, 2008). Furthermore, because the sensitivity of the technique can be enhanced by increasing the number of sequence reads, it can potentially be sensitive enough for use with small amounts of starting material (Tang *et al.*, 2009). These are very early days for RNA-Seq, and to date only one landmark study has utilized the technique for the study of activity-regulated gene expression in neurons (Kim *et al.*, 2010). However, significant technological advances are likely to come quickly that will enhance the use of this technology. Specifically the large amount of information about RNA complexity that can be obtained from a single RNA-Seq experiment means that there remain significant bioinformatic hurdles to be crossed, such as the development of software tools for processing this information, before the technique can be widely applied. As software programs become available and the cost of sequencing continues to fall, it is likely that RNA-Seq will begin to replace microarray hybridization as the technique of choice for RNA profiling experiments, opening the potential to significantly expand our understanding of the complexity of transcriptional regulation.

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6.2.3. ChIP-Seq describes the chromatin landscape—Gene regulation by transcription factors depends on the binding of these proteins to specific sequences in genomic DNA. Where any given transcription factor binds largely determines its set of potential target genes, thus a comprehensive knowledge of the position of transcription factor binding sites across the genome is essential for understanding transcription factor biology. The major biochemical method used to study transcription factor-genomic DNA interactions *in vivo* is that of chromatin immunoprecipitation (ChIP) (Aparicio *et al.*, 2004) (FIGURE 8).

Traditionally, quantitative PCR has been used to measure the co-immunoprecipitation of specific target sequences with a transcription factor of interest. However, when combined with high-throughput sequencing techniques (ChIP-Seq) it is now possible to obtain an unbiased view of transcription factor binding across the genome (Schmidt *et al.*, 2009). In this variation of the technique, all of the genomic DNA fragments co-precipitated with the transcription factor are purified, size selected, and cloned with adaptors for sequencing on the SOLiD or Solexa/Illumina platforms to a depth of 20–40 million reads. Mapping these fragments to the reference genome gives a profile of the DNA regions that are enriched in the pulldown. An important challenge is to determine which of the enriched regions ("peaks") are statistically significant at a given threshold and which correspond to the genomic background.

Because ChIP-Seq makes no assumptions about where transcription factors are likely to be bound, the results of these studies have revealed new fundamental aspects of transcription factor biology (Farnham, 2009). For example, because traditional biochemical assays of transcription factor function rely on short regions of genomic regulatory sequences to drive the expression of reporter genes, the vast majority of studies in the literature have focused on the ability of transcription factors to bind sequences within a few hundred base pairs of the transcription start site for a candidate target gene. Genome-wide studies have shown that some transcription factors, such as the cell-cycle regulating factor E2F, CREB, and USF1/2 do in fact appear to preferentially bind to proximal gene promoters (Bieda *et al.*, 2006; Kim *et al.*, 2010; Rada-Iglesias *et al.*, 2008). However, other factors including SRF, Npas4, and CaRF have far more diverse binding patterns in which they associate with both extragenic and intragenic (exonic and intronic) regions as well as proximal gene promoters (Kim *et al.*, 2010; Pfenning *et al.*, 2010). A second theme that has emerged is that transcription factors do not always bind to strict consensus sites. The binding site for the transcription factor can be inferred by examining the sequences within the co-immunoprecipitated peak. Some transcription factors such as the neuronal repressor RE1-silencing transcription factor (REST) show high enrichment for a specific motif (Johnson *et al.*, 2007). Some other transcription factors such as SRF have multiple subsets of binding sites (Valouev *et al.*, 2008). Others such as CREB and MEF2 bind both consensus and non-consensus sites (Flavell *et al.*, 2008; Impey *et al.*, 2004), whereas some such as E2F show no sequence specificity whatsoever (Rabinovich *et al.*, 2004). Although the mechanisms of these interactions are not known, this knowledge increases the diversity of potential regulatory mechanisms.

Studying the sites of transcription factor binding can yield new insights into transcription factor function. For CREB, the observation that CREB was binding near annotated positions of microRNAs led to the understanding of the role of CREB in regulation of non-coding as well as coding RNA sequences in the genome (Cheng *et al.*, 2007; Impey *et al.*, 2004; Vo *et al.*, 2005). For the unique transcription factor CaRF, for which prior to ChIP-Seq only a single target gene (*Bdnf*) was known, the distribution of CaRF binding sites suggested a role for this factor in coordinating the expression of a number of important neuronal signaling

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pathways including a number of proteins that regulate calcium signaling (Pfenning *et al.*, 2010).

However, ChIP-Seq still has several important limitations that remain areas for development in the future. One concern is to develop techniques to resolve the cell-type specificity of transcription factor binding profiles. Current methods for ChIP-Seq require large amounts of chromatin that are usually derived from pooled sources of cells and from heterogeneous brain tissue. Interestingly, the BacTRAP technology pioneered by Nathaniel Heintz and colleagues may offer a solution to this problem. The BacTRAP system was developed to isolate the full complement of actively translating mRNAs from specific sets of cells (Heiman *et al.*, 2008). In these transgenic mice, BACs are used to drive cell type-specific expression of a ribosomal protein fused with enhanced green fluorescent protein (eGFP). Because ribosomal proteins are assembled in the nucleolus of all cells, the eGFP fluorescence can also be used for fluorescence-activated cell sorting of specific subsets of nuclei. Although this technique has not yet been applied to ChIP-Seq, it has been used for the isolation of Purkinje cell nuclei that led to the identification of the novel chromatin modification 5-hydroxymethylcytosine (Kriaucionis and Heintz, 2009). Another significant issue that need to be resolved is that of target gene assignment for ChIP peaks. Most often it is assumed that a site of transcription factor binding will allow that factor to modulate transcription of the nearest gene. However since genomic DNA is wound into a complex secondary and tertiary structure, transcription factor-gene interactions could be brought together across large expanses of primary sequence. Future insight into these long-distance interactions is likely to come from detailed studies of the spatial organization of chromosomes using techniques such as chromosome conformation capture (3C) and its genome-wide variant Hi-C (Dekker *et al.*, 2002; Lieberman-Aiden *et al.*, 2009). Advances in this area will be aided by close collaboration between neurobiologists, biochemists, genome biologists, and computational biologists.

6.3. Imaging approaches to neuronal activity-regulated transcription

Transcription studies are most often conducted using large-scale biochemical techniques like the RNA analyses and ChIP studies described in the section above. These are very effective techniques for the identification of molecular mechanisms that transduce synaptic activity into new gene transcription. However, as biochemical methodology yields static snapshots of transcriptional activity averaged across a cell population, these studies provide little insight into the temporal response properties of activity-regulated genes and no information about the variation in the transcriptional response between individual cells. Understanding the kinetic parameters of induced transcription is of particular importance in neurons, where tightly regulated patterns of synaptic activity are the key source of information that drives plasticity. Furthermore, studies in single living cells have revealed that the expression of individual genes is highly variable, even within a clonal population of cells (Chubb *et al.*, 2006; Golding *et al.*, 2005; Janicki *et al.*, 2004; Raj *et al.*, 2006; Raser and O'Shea, 2004). Since many of the genes induced by neuronal activity feed back to alter synaptic function, if in the nervous system there is similar randomness and variation in transcriptional activation from cell to cell this would have direct phenotypic consequences for synaptic plasticity within interconnected neuronal networks.

Developments in live imaging technologies have been particularly useful for studying the processes by which synaptic signals reach the nucleus. The refinement of these techniques through their application to new imaging platforms along with the development of genetic tools for imaging continues to open new avenues of understanding. For example, Ryohei Yasuda and colleagues are using a two-photon technique called fluorescence lifetime imaging microscopy (FLIM) to study the temporal and spatial dynamics of synaptic signaling cascade activation following stimulation of single dendritic spines (Yasuda, 2006).

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Their results suggest that single spine activation of CaMKII drives transient activation of this pathway that is restricted to a single spine (Lee *et al.*, 2009), whereas Ras activated by the same stimulus spreads into the neighboring dendritic shaft (Yasuda *et al.*, 2006). The Ras pathway drives transcription by activating the Erk1/2 MAPKs, which have long been known to translocate to the nucleus in response to many stimuli that lead to their activation including neural activity (Impey *et al.*, 1998). Tagging Erk1/2 with the photoactivatable compound Dronpa, Weigert and colleagues examined the translocation of Erk1/2 in different parts of cultured hippocampal neurons (dendrites versus soma) in response to BDNF stimulation or action potential bursting (Wiegert *et al.*, 2007). Their results indicate that propagation of Erk1/2 in dendrites is passive and not signal-inducible. By contrast, translocation of somatic Erk1/2 to the nucleus is induced by stimulation and occurs by facilitated diffusion. Imaging techniques can also be applied to study intranuclear signaling. Hilmar Bading and colleagues capitalized on the development of genetically encoded calcium indicators (Inverse Pericam and GCaMP2.0) to study the specificity of stimuli that induce nuclear calcium transients by targeting these reporters to the nucleus (Bengtson *et al.*, 2010). In acute hippocampal slices, they found that although a burst of synaptic activity elicits a nuclear calcium rise, repetition of the action potential burst (using classical late-LTP-inducing stimuli such as high-frequency stimulation and theta-burst stimulation) greatly amplified the magnitude of the nuclear calcium increase. These data may help to explain why repeated stimulation is required to induce the transcription-dependent phase of late-LTP. Taken together, these data help to define distinct cellular compartments where different stages in the synapse-nucleus signaling pathway may occur to complete information transfer.

Imaging has also been used to identify the specific cells where transcription has occurred, facilitating study of the cellular consequences of activity-regulated transcription (Barth, 2007). Barth and colleagues were the first to develop mice for this kind of experiment, by expressing a Fos-GFP fusion protein from a transgene under the control of the activity-regulated proximal Fos promoter (Barth *et al.*, 2004). GFP expression is robustly induced in neurons that have had a history of elevated activity, permitting the exploration of the physiological properties of these highly active neurons (Barth *et al.*, 2004; Yassin *et al.*, 2010). Wang and colleagues extended this methodology by generating knockin mice in which GFP replaces the coding sequence of the Arc gene (Wang *et al.*, 2006). In mice heterozygous for the knockin mutation, Arc is expressed at normal levels and GFP can again be used to mark populations of neurons in which the neural activity levels and/or patterns have been sufficient to induce *Arc* transcription. However, homozygous knockin mice lack all Arc protein expression, thus in these mice GFP can be used to focus studies of cellular physiology on the time points and in the specific cells in which Arc expression should have been induced but was not.

It has been more challenging to develop live imaging reagents that directly get at the process of transcription itself. Perhaps the closest reagents have been those that allow the fluorescent detection of the stimulus-induced interaction between CREB and its co-activator CBP. The direct physical interaction of these two proteins can be monitored by fluorescence-resonance energy transfer (FRET) between compatible fluorophores fused to the KID domain of CREB and the KIX domain of CBP. The challenge for FRET is optimizing the development of the interacting partners to give a maximal signal to noise ratio. One recent study used such a CREB-CBP FRET system as a readout to study the role of the PKA anchoring protein AKAP79 in the L-VGCC-dependent activation of CREB (Friedrich *et al.*, 2010). Because this FRET pair allows dynamic and reversible imaging of CREB-related transcription in live cells, it may have great usefulness for studying the molecular components and cell-type specificity of activity-regulated gene expression. Alternatively, transcription itself can be monitored in single cells in *fixed* tissue using the cellular compartment analysis of temporal

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activity by fluorescent in situ hybridization (catFISH) technique (Guzowski *et al.*, 1999, 2001). This method relies on the fact that newly synthesized RNA first appears in the nucleus before being transported to the cytoplasm. By using oligonucleotide-based FISH, which has subcellular resolution for localizing RNA, it is possible to derive information about the temporal profile of transcription induction after a stimulus and prior to tissue fixation.

A fascinating challenge that remains for the field of neuronal activity-regulated transcription is to develop ways to visualize transcription itself as it occurs in single cells in real time. Imaging techniques based on fluorescent proteins have been successfully applied for this purpose in other cell types, yielding substantial information about the kinetics of transcription and the consequences of stochasticity for transcriptional regulatory processes (Darzacq *et al.*, 2009). One of the most useful technologies for imaging transcription relies on the tagging of the target RNA with multiple copies of a stem-loop binding site for the phage RNA-binding protein MS2 (Chubb *et al.*, 2006; Janicki *et al.*, 2004) (FIGURE 9). When nuclear-targeted MS2 is fused to a fluorophore such as YFP and expressed in cells in the absence of a tagged target gene, it is found diffusely throughout the nucleus. However, when transcription of the tagged gene is induced, MS2-YFP binds to the stem-loop sequence in the newly synthesized RNA and it clusters in the nucleus at the site of active transcription, causing the appearance of a bright spot of nuclear fluorescence. Important properties of transcription can then be inferred by studying the timing of spot appearance after cell simulation, the frequency of spot formation and disappearance, the intensity of spot fluorescence (which correlates with RNA copy number), and the likelihood that spots appear in any single cell within a cell population. Furthermore, since MS2-YFP stays bound to the RNA as it is processed and exported from the nucleus, this technique has been widely used to study cytoplasmic RNA trafficking, and would be well applied to the study of dendritic RNA targeting in neurons (Grünwald and Singer, 2010; Querido and Chartrand, 2008). Application of the MS2-based technology to the study of one or more of the activity-regulated genes in neurons would represent an important new path for inquiry into the mechanisms of neuronal activity-regulated transcription.

7. Concluding remarks

Substantial progress has been made in the identification of molecular mechanisms that mediate neuronal activity-dependent changes in gene transcription. As described here, these data have shown that neurons use a complex array of signaling pathways and transcriptional mechanisms to orchestrate the expression of diverse gene expression programs in response to a wide range of extracellular stimuli. Among the key principles of activity-dependent transcriptional regulation are the following: 1) transcription factors can be regulated at the level of post-translational modifications that either activate or repress transcription, nuclear versus non-nuclear localization, protein-DNA binding, or expression; 2) regulation of chromatin structure adds an additional layer of context that can modulate the outcome of transcription factor activation; and 3) different neurotransmitter receptors and ion channels in the plasma membrane – both at the synapse and outside the synapse – activate distinct signaling molecules that differentially regulate the transcriptional machinery in the nucleus. The net outcome of these pathways is the tight and appropriate control of cellular responses to extracellular signals, as exemplified by the ability of distinct transcriptional regulatory pathways to drive opposite effects of synaptic scaling under conditions of high versus low synaptic activity.

Under optimal conditions, this dynamic and flexible network permits the brain to rapidly initiate physiologically relevant cellular and behavioral responses to a constantly changing environment. By contrast, disruption of this transcriptional network by genetic mutations

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that impair the function of activity-regulated transcription factors is all too often associated with mental retardation and neuropsychiatric illness (Hong *et al.*, 2005; Tsankova *et al.*, 2007). The challenge for the future is to harness new experimental techniques in genetic, genomics, and imaging that will allow for an enhanced understanding of the functional importance and biological consequences of fine-tuning transcriptional regulation in the CNS.

Abbreviations

NGF	Nerve Growth Factor
CNS	central nervous system
IEG	immediate-early gene
Egr	early growth response factor
Nr	nuclear receptor
TSS	transcription start site
SRE	Serum Response Element
CRE	Calcium/cAMP-Response Element
Rb	retinoblastoma tumor suppressor
RCE	Retinoblastoma Control Element
SRF	Serum Response Factor
TCF	Ternary Complex Factors
Ets	E-twenty six domain
Elk-1	Ets like gene 1
CREB	Calcium-Response Element Binding protein
CREST	Calcium-Responsive Transactivator
HDAC	histone deacetylase
HAT	histone acetyltransferase
CBP	CREB-binding protein
MEF2	Myocyte Enhancer Factor 2
eRNA	enhancer RNA
FIRE	*Fos* intragenic regulatory element
ChIP	chromatin immunoprecipitation
BDNF	Brain-Derived Neurotrophic Factor
CaRE	calcium-response element
CaRF	Calcium-Response Factor
USF	upstream stimulatory factor
PAS	Per-Arnt-Sim
Npas4	Neuronal PAS Domain Protein 4
NF-κB	Nuclear Factor κB

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bHLH	basic helix-loop-helix
NFAT	Nuclear Factor of Activated T Cells
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
Arc	activity-regulated cytoskeletal-associated protein
SARE	Synaptic Activity Response Element
LTD	long-term depression
PKA	protein kinase A
MAPK	mitogen-activated protein kinase
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-proprionate
AMPAR	AMPA receptor
PSD	postsynaptic density
CREM	cAMP Response Element Modulatory Protein
ATF1	activating transcription factor 1
Ser133	serine 133
CaM	calmodulin
CaMK	Ca^{2+}/CaM-dependent protein kinase
Akt or PKB	protein kinase B
Crtc	CREB-regulated transcription coactivator
KID	kinase-inducible domain
KIX	KID-interacting domain
AMPK	AMP-activated protein kinase
SIK2	salt-inducible kinase 2
LTP	long-term potentiation
MKL1	myocardin-related transcription factor A
MKL2	myocardin-related transcription factor B
Sap-1	SRF accessory protein 1
SUMO	small ubiquitin-like modifier
Erk5	extracellular signal-regulated kinase 5
IκB	Inhibitor of NF-κB
IKK	IκB kinase
NEMO	NF-κB essential modulator
L-VGCC	L-type voltage-gated calcium channel
CaMKII	Ca^{2+}/CaM-dependent protein kinase II
NFATn	NFAT nuclear component
DYRK1A	dual specificity tyrosine phosphorylation-regulated kinase 1A

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GSK3	glycogen synthase kinase 3
FOXO3	forkhead box O3
CaMKIV	Ca^{2+}/CaM-dependent protein kinase IV
CCAT	calcium channel associated transcription regulator
GAD65	glutamic acid decarboxylase 65
GABA	γ-aminobutyric acid
PKC	protein kinase C
ATF4 or CREB2	activating transcription factor 4
DREAM	Downstream Repressor Element Antagonist Modulator
NCS	Neuronal Calcium Sensor
Pdyn	prodynorphin
PI3K	phosphatidylinositol 3-kinase
AP-1	activator protein 1
ChIP-Seq	whole genome ChIP sequencing
RNAi	RNA interference
ICER	inducible cAMP early repressor
EGF	epidermal growth factor
Rsk2	ribosomal S6 kinase 2
MSK1	mitogen- and stress-induced kinase 1
MSK2	mitogen- and stress-induced kinase 2
FGF	fibroblast growth factor
Dnmt1	DNA methyltransferase 1
Dnmt3a	DNA methyltransferase 3a
Dnmt3b	DNA methyltransferase 3b
AID	activation-induced deaminase
APOBEC	apolipoprotein B mRNA-editing enzyme complex
shRNA	short hairpin RNA
MBD	methyl-DNA binding domain
MBD1, MBD2, and MBD4	MBD proteins 1, 2, and 4
MeCP2	methyl CpG binding protein 2
Ras-GRF	Ras-specific guanine nucleotide-releasing factor
CaMKK	CaMK kinase
CaMKI	Ca^{2+}/CaM-dependent protein kinase I
TM	transmembrane domain
Clca1	calcium-activated chloride channel regulator 1

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MAGUK	membrane-associated guanylate kinase
PSD-95	postsynaptic density-95
SAP-97	synapse-associated protein-97
PSD-93	postsynaptic density-93
SAP-102	synapse-associated protein-102
MEK	Erk/MAPK kinase
GAP	GTPase activating protein
GEF	GTP exchange factor
SynGAP	synaptic Ras GTPase activating protein
DAPK1	death-associated protein kinase 1
PP1	protein phosphatase 1
EphB2	Ephrin type-B receptor 2
PP2A	protein phosphatase 2A
Rheb	small GTPase Ras homologue enriched in brain
VGCCs	voltage-gated calcium channels
eIF3E	eukaryotic initiation factor 3 subunit E
PIKfyve	phosphatidylinositol 3-phosphate 5-kinase
P_O	open probability
AKAP15	A kinase-anchoring protein 15
N-VGCCs	N-type voltage-gated calcium channels
ADAR2	RNA-dependent adenosine deaminase 2
ER	endoplasmic reticulum
PLC	phospholipase C
IP_3	inositol trisphosphate
CRAC channels	calcium release-activated calcium channels
Stim1	stromal interaction molecule 1
Stim2	stromal interaction molecule 2
nAChRs	nicotinic acetylcholine receptors
YFP	yellow fluorescent protein
eYFP	enhanced YFP
TTX	tetrodotoxin
ActD	actinomycin D
ChR2	channel rhodopsin 2
SCN	suprachiasmatic nucleus
BAC	bacterial artificial chromosome
RNA-Seq	whole transcriptome RNA sequencing

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PCR	polymerase chain reaction
REST	RE1-silencing transcription factor
GFP	green fluorescent protein
eGFP	enhanced GFP
3C	chromosome conformation capture
FLIM	fluorescence lifetime imaging microscopy
FRET	fluorescence-resonance energy transfer
catFISH	cellular compartment analysis of temporal activity by fluorescent in situ hybridization
ZRE	Zeste-like response element
RFP	red fluorescent protein

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Research Highlights

In this review we cover the following:

- The classes of genes and transcription factors that are regulated by neuronal activity.
- How calcium-signaling pathways confer specificity upon cellular responses to stimuli.
- Importance of activity-regulated transcriptional pathways for synaptic homeostasis.
- Technological developments that are advancing studies in this field.

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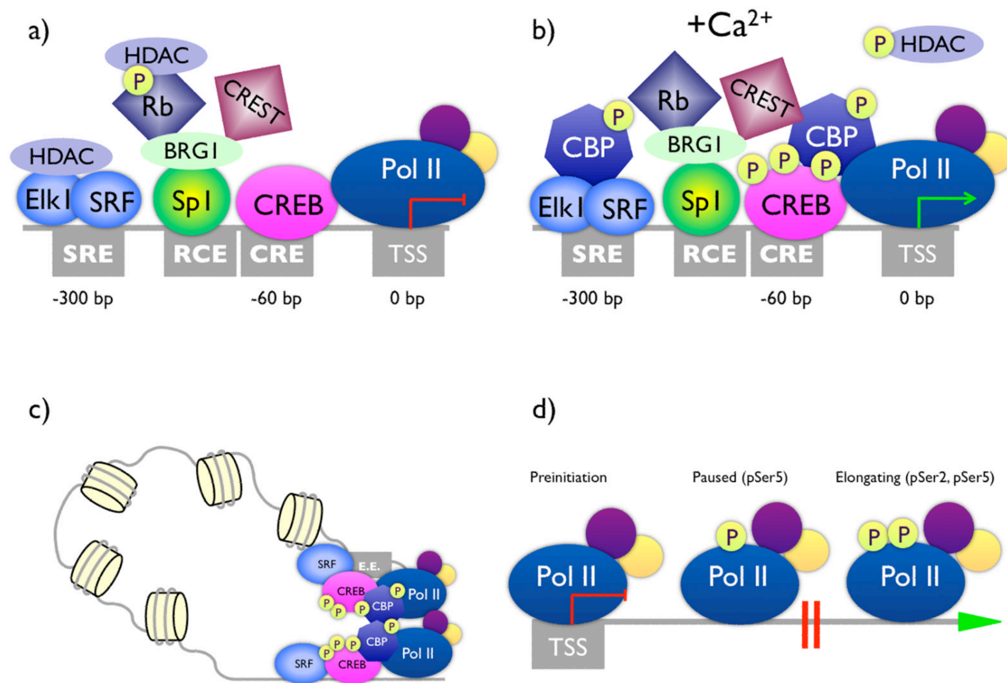

FIGURE 1. Mechanisms of neuronal activity-induced transcription

The diagrams represent four steps in the process of *Fos* transcription that are regulated by neural activity. **a)** Prior to neuronal activity, the *Fos* promoter is primed for response by the association of sequence-specific DNA binding transcription factors with stimulus-response elements (gray boxes) in the proximal promoter. RNA polymerase II (Pol II) is also pre-associated with the *Fos* promoter prior to activation. Numbers show base pair distances of each element from the transcription start site (TSS). The promoter is kept off in part by the local recruitment of HDACs. Key sites of calcium-regulated phosphorylation are represented by the circled letter P. **b)** Calcium induces a switch in cofactors present at the *Fos* promoter, with recruitment of the co-activator and histone acetyltransferase CBP and loss of the repressive HDACs. **c)** Distal enhancer elements (E.E.) contribute to activity-dependent transcription. These elements are bound by transcription factors including SRF and CREB, and show calcium-dependent recruitment of CBP and Pol II binding. Chromatin looping may bring the enhancer into physical proximity of the proximal promoter and *Fos* TSS. **d)** Transcription elongation is regulated by stimulus-dependent phosphorylation of two sites in the C-terminal domain of the large subunit of RNA polymerase II. The pre-initiation form of RNA Pol II is bound to the *Fos* promoter but is not competent to drive RNA synthesis. Phosphorylation of serine 5 (pSer5) is sufficient to promote engagement but results in polymerase stalling within the transcribed region of many genes. Productive elongation requires additional phosphorylation of RNA Pol II at serine 2 (pSer2).

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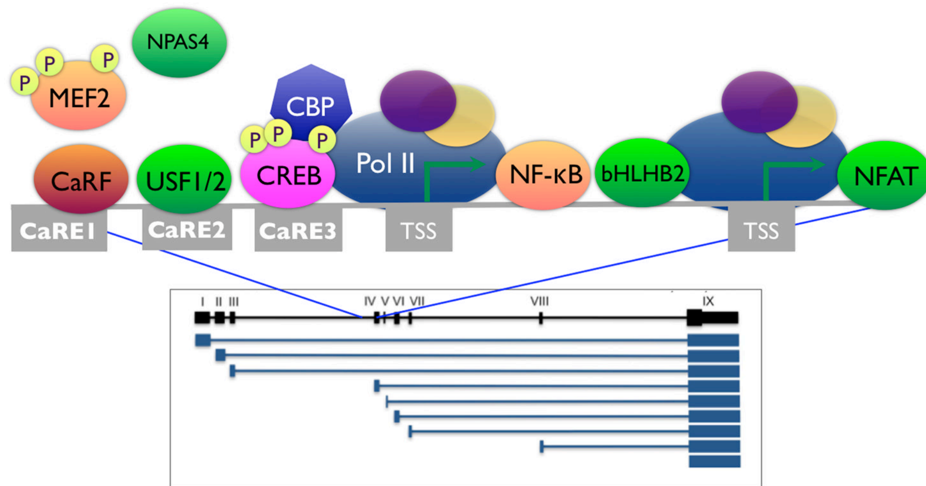

FIGURE 2. Multiple activity-regulated transcription factors contribute to inducibility of *Bdnf* promoter IV

The box shows spliced mRNAs (blue) encoding *Bdnf* transcripts driven by the eight alternative *Bdnf* promoters mapped onto chromosome 2 of *Mus musculus* (black). Boxes represent the nine exons that comprise the *Bdnf* gene and the thicker region of exon IX indicates the coding sequence. The gray line shows an expansion of the region just upstream of exon IV. Three calcium-response elements (CaREs) and two transcription start sites (TSSs) are indicated by the gray boxes. Transcription factors demonstrated to regulate *Bdnf* promoter IV are shown at their binding sites. Npas4 binding has been localized to a PAS response element just 5′ to CaRE1 in human *BDNF* promoter IV (Pruunsild *et al.*, 2011) and to a region near the CaRE2 element by ChIP-Seq in mouse neurons (Kim *et al.*, 2010). Although MEF2 has been localized to *Bdnf* promoter IV by chromatin immunoprecipitation, its binding elements have not yet been reported.

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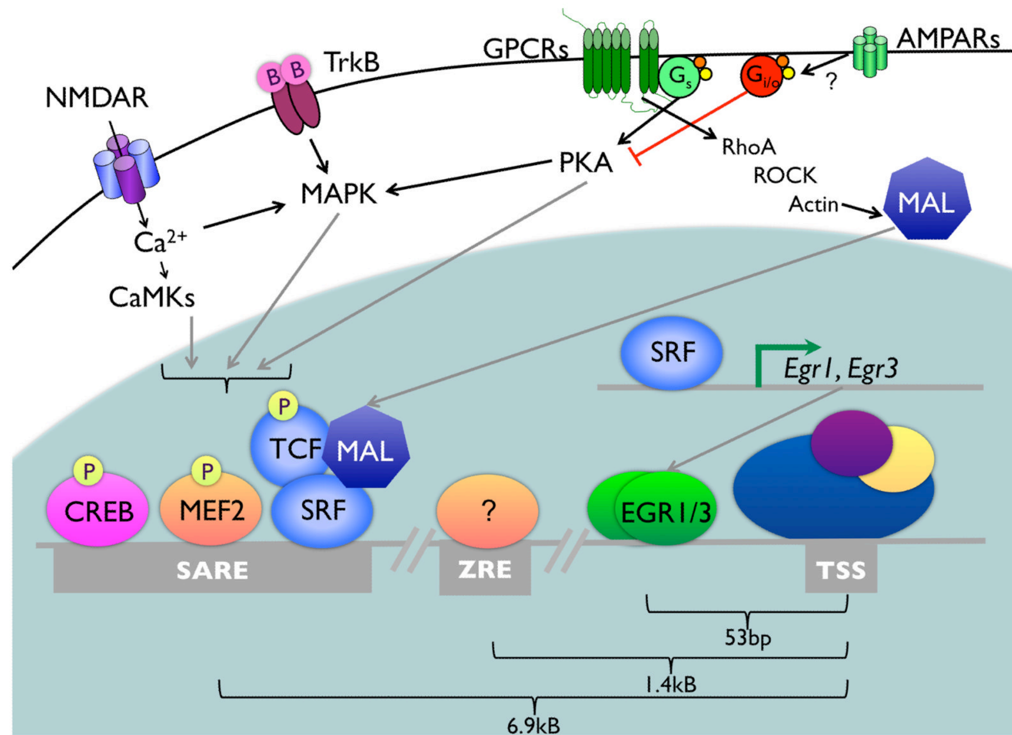

FIGURE 3. Convergent signaling cascades regulate *Arc* transcription

Signaling cascades that promote (gray arrows) and inhibit (red bars) the transcription of *Arc* are shown. The light blue region indicates the cell nucleus. B, BDNF; TrkB, the BDNF receptor TrkB; GPCRs, G-protein coupled receptors; $G_{i/o}$, heterotrimeric G proteins negatively coupled to adenylate cyclase; G_s, heterotrimeric G proteins positively coupled to adenylate cyclase; ROCK, Rho kinase. Three genetic regulatory regions have been identified in the proximal and distal *Arc* promoter regions. In addition to the distal SARE element (bound by CREB, SRF, and MEF2) and the proximal promoter (bound by EGR1/3), a region of open chromatin 1.4kB upstream of the TSS has been identified that has homology to a Zeste-like response element (ZRE) and that contributes to activity-dependent regulation of an *Arc* reporter gene (Pintchovski *et al.*, 2009). The transcription factor(s) that regulate this element in mammalian neurons remain to be identified.

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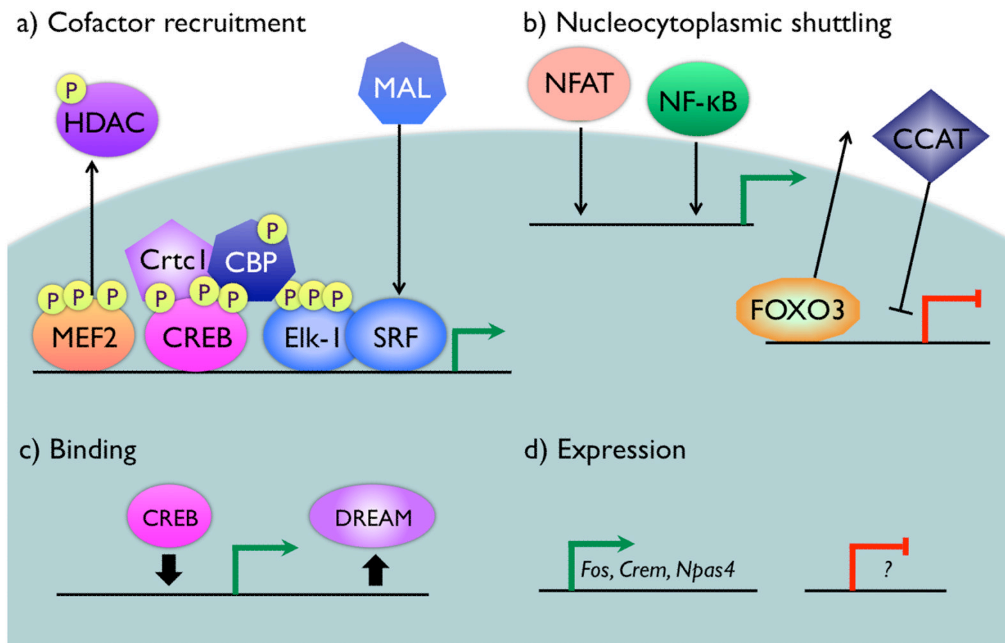

FIGURE 4. Mechanisms of transcription factor regulation by neuronal activity

Transcription factor function can be regulated by neuronal activity through at least four different mechanisms. Green arrows represent genes that show activity-induced transcriptional activation, red bars represent genes that undergo activity-induced transcriptional repression. **a)** Recruitment of transcriptional coactivators and corepressors is an important mechanism to regulate the function of pre-bound transcription factors such as CREB, MEF2, and SRF. **b)** Nuclear translocation of the transcription factors NFAT and NF-κB is induced by a wide variety of stimuli including neuronal activity, allowing these factors to bind to their target gene promoters. By contrast, neuronal activity drives the nuclear export of the forkhead box transcription factor FOXO3 and prevents nuclear translocation of CCAT. **c)** Regulated binding of transcription factors is controlled through different mechanisms. In the case of CREB, signal-dependent regulation of histone modifying enzymes may lead to a change chromatin structure that alters the accessibility of CREB binding sites. For DREAM, direct binding of calcium to the EF-hands in this repressor protein changes its affinity for DNA, releasing it from its binding site. **d)** In addition to the classic IEGs transcription factors, such as *Fos*, *Jun*, and *Egr* family members, additional transcription factors are subject to activity-dependent regulation of expression. These transcription factors include ICER, a repressor form of the CREB family member CREM, and the bHLH-PAS domain transcription factor NPAS4. Expression of other transcription factors is likely to be repressed by neuronal activity, however these remain to be identified.

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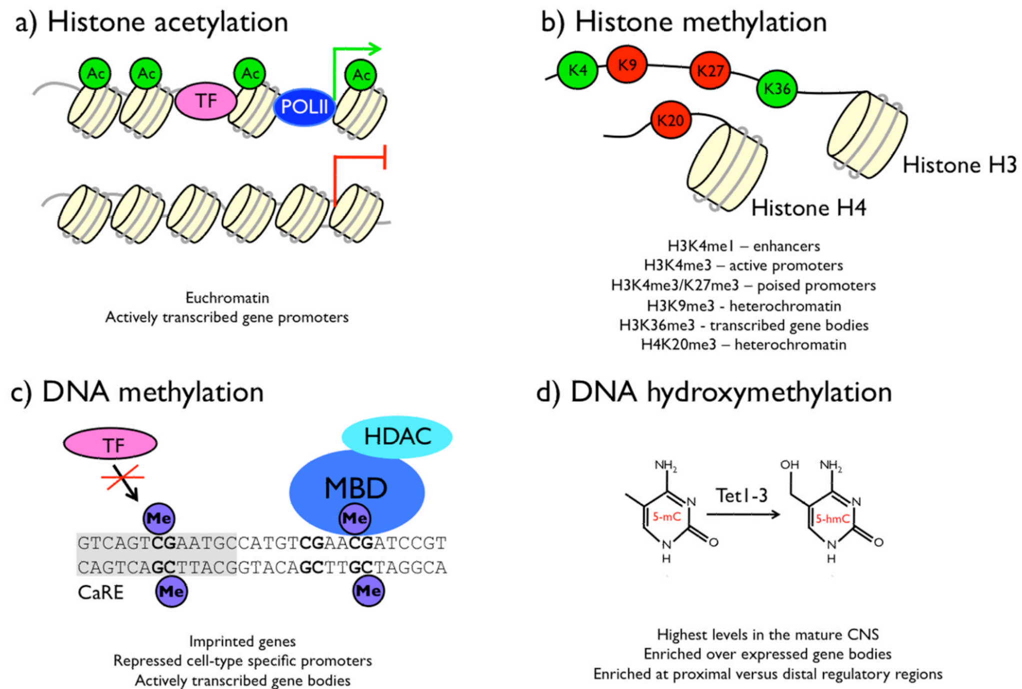

FIGURE 5. Chromatin modifications that regulate transcription

Post-translational modifications of histones and covalent modifications of genomic DNA regulate transcription. **a)** Acetylation (Ac) can be added to multiple lysine residues in the N-terminal tail domain of histones H3 and H4. This modification is preferentially associated with transcriptionally active genes. **b)** Methylation (me) of histones H3 and H4 occurs at specific lysine (K) and arginine residues. The functional consequence of each of these modifications depends on the specific amino acid modified (K4, K9, K27, and K36 in histone H3, K20 in histone H4) (Kouzarides, 2002). Sites of methylation associated with transcriptional activation are shown in green, sites associated with transcriptional repression are shown in red. Each lysine can be mono- (me1), di- (me2), or trimethylated (me3), and the global distribution of these methyl marks varies across different kinds of genetic regulatory elements (Hon *et al.*, 2009; Mikkelsen *et al.*, 2007). **c)** In differentiated mammalian cells, DNA methylation occurs on cytosines at a subset of CpG dinucleotides. Methylation can regulate transcription by sterically blocking the association of a transcription factor (TF) with its binding site (the gray box represents a CaRE), or by recruiting the association of a protein with a methyl-DNA binding domain (MBD), which can act as a scaffold for additional chromatin regulatory enzymes. **d)** 5-hydroxymethylation of cytosine (5-hmC) is catalyzed by the enzymes Tet1, Tet2, and Tet3, which add a hydroxyl group to methylated cytosine bases (5-mC) (Ito *et al.*, 2010; Ko *et al.*, 2010; Tahiliani *et al.*, 2009). Little is known about the functional relevance of this modification of DNA, but new chemical methods are beginning to allow its genome-wide distribution to be described (Song *et al.*, 2011).

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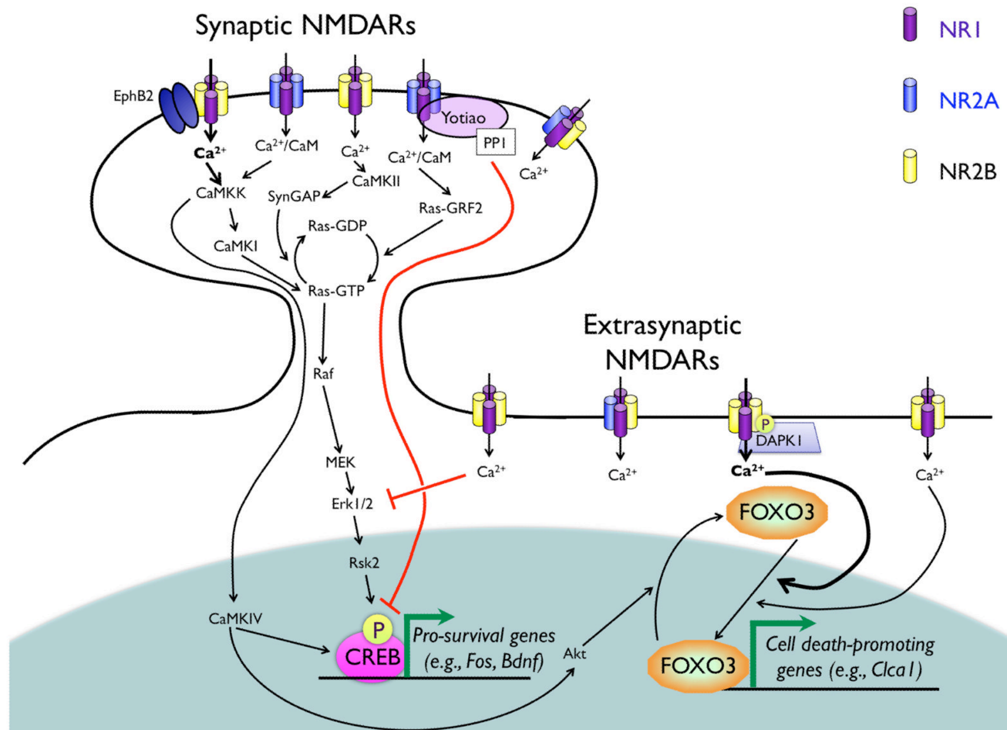

FIGURE 6. Different pools of NMDARs are capable of effecting distinct changes in gene transcription

Synaptic NMDARs are capable of signaling through the CaMKs and the Ras/Raf/MEK/Erk/Rsk2 pathway to phosphorylate CREB and initiate transcription of a variety of pro-survival genes. Additionally, synaptic NMDAR activation of CaMKK and CaMKIV leads to the activation of Akt and subsequently the phosphorylation and nuclear export of the transcription factor FOXO3, thereby inhibiting transcription of various cell death-inducing genes. In contrast, activation of extrasynaptic NMDARs opposes the effects of synaptic NMDAR activation. Activation of extrasynaptic NMDARs inhibits the activation of Erk1/2, thereby reducing CREB phosphorylation. Additionally, extrasynaptic NMDARs induce the nuclear import of FOXO3 and the subsequent transcription of pro-death genes; this is potentiated by DAPK1. Subunit composition can also lend specificity to synapse-to-nucleus signaling by NMDARs. NR2A-containing NMDARs are capable of interacting with and selectively activating Ras-GRF2; activation of NR2B-containing NMDARs leads to the activation of CaMKII and the subsequent phosphorylation of SynGAP. While NR2B-containing NMDARs have also been shown to interact with Ras-GRF1 (not shown), how that signal is integrated with SynGAP activation remains to be determined. The EphB2 receptor has been shown to interact with the NR1 subunit and potentiate NMDAR-mediated calcium influx and downstream signaling cascades by phosphorylating the NR2B subunit. Lastly, the scaffolding protein Yotiao can bind to NR1 splice variants containing the C1 cassette and tether PKA and PP1 to the channel complex. While it is not known if this tethering is important for PKA-mediated NMDAR signaling (not shown), this interaction could mediate PP1-dependent signaling to the nucleus and dephosphorylation of CREB.

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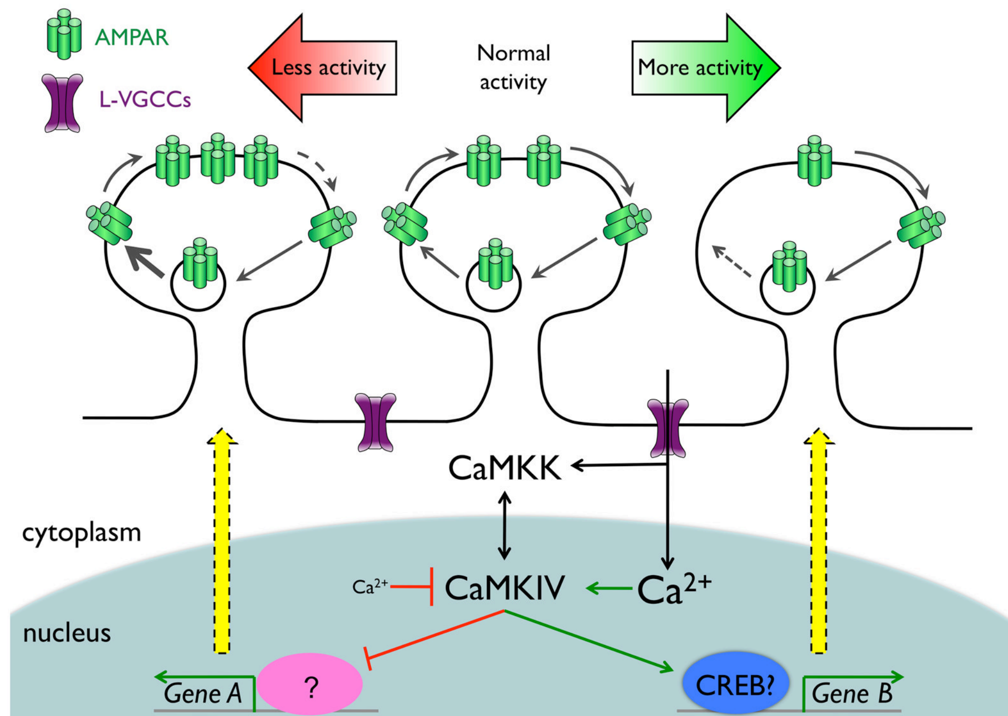

FIGURE 7. Transcriptional regulation of homeostatic synaptic scaling

Changes in synaptic activity drive homeostatic compensatory changes in the number of surface AMPARs at synapses. Downregulation of synaptic AMPARs in response to increased activity requires the influx of calcium (Ca^{2+}) through L-VGCCs and activation of a CaMKIV-dependent transcriptional pathway (green arrow). Upregulation of synaptic AMPARs in response to decreased synaptic activity is driven by reduced calcium influx through L-VGCCs, and a decrease in activity of the CaMKK/CaMKIV pathway (red bar). Because transcriptional activity is required for scaling induced by either increases or decreases in synaptic activity, these data that suggest that distinct classes of calcium-regulated transcription factors (one group activated by CaMKIV and one repressed) and distinct sets of target genes (*Gene A* and *Gene B*) mediate the two sides of the pathway.

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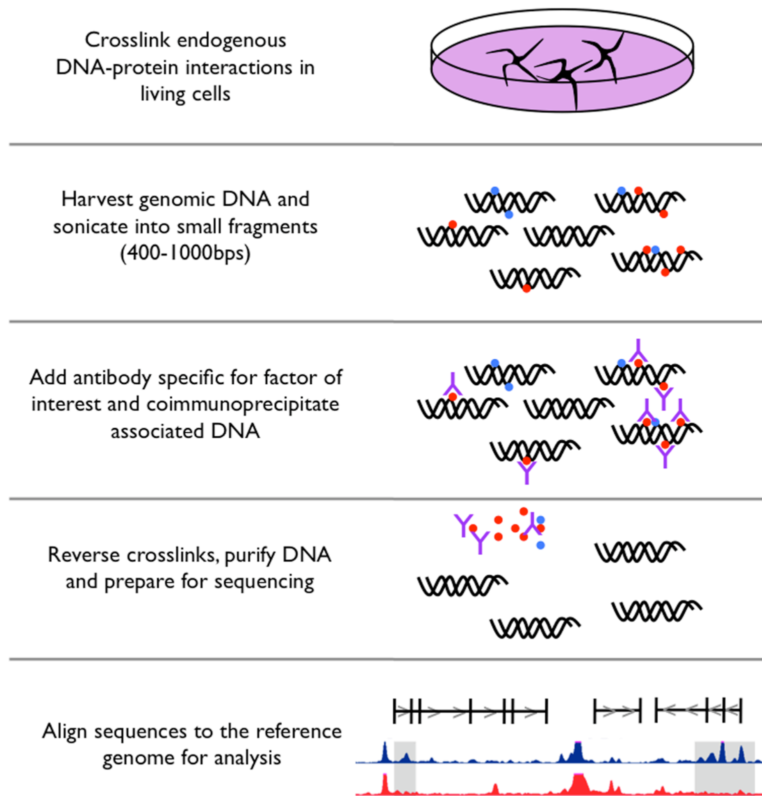

FIGURE 8. ChIP-Seq defines transcription factor binding sites genome-wide

The steps in a ChIP-Seq protocol are diagrammed. Blue and red circles represent two different DNA binding proteins. An antibody specific for one of the transcription factors selectively co-immunoprecipitates the subset of DNA fragments to which that factor is crosslinked. For the final step, the black line diagrams represent genes visualized across a region of a chromosome. Vertical lines represent exons and arrows show the direction of transcription. The blue and red diagrams represent the distribution of sequences obtained after co-immunoprecipitation with either the red or blue transcription factors. In this example, the red protein is preferentially bound immediately upstream of the TSS for each of the three genes shown, whereas the blue protein shows additional binding sites outside of proximal promoters (gray boxes).

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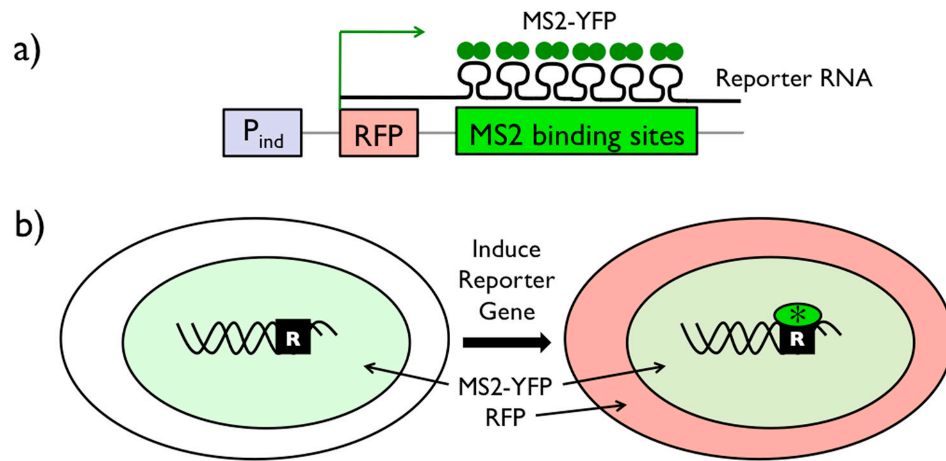

FIGURE 9. Imaging RNA synthesis with the RNA binding protein MS2

a) A representative reporter gene for MS2-based visualization of new RNA synthesis. P_{ind}, stimulus-inducible promoter; RFP, red fluorescent protein. The black line represents the RNA transcribed from the reporter locus. A dimer of the phage protein MS2 fused to the fluorescent protein YFP binds each stem loop made by one of the series of MS2 binding sites encoded at the reporter locus. **b)** Representation of MS2-based transcriptional imaging. When reporter gene expression is induced, MS2-YFP binds to the cluster of stem-loop sequences in the new RNA synthesized at the site of reporter gene integration (R). Relocalization of MS2-YFP in the nucleus to this binding site is detected as a bright spot of nuclear fluorescence (asterisk).