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Emerging themes in neuronal activity-dependent gene expression

Ram Madabhushi^{1,2,*} and Tae-Kyung Kim^{2,*}

¹Department of Psychiatry, University of Texas Southwestern Medical Center, Dallas, TX, USA

²Department of Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX, USA

SUMMARY

In this review, we attempt to discuss emerging themes in the regulation of neuronal activity-regulated genes, focusing primarily on an important subset of immediate-early genes. We first discuss earlier studies that have illuminated the role of *cis*-acting elements within the promoters of immediate-early genes, the corresponding transcription factors that bind these elements, and the roles of major activity-regulated signaling pathways. However, our emphasis is on new studies that have revealed an important role for epigenetic and topological mechanisms, including enhancer-promoter interactions, enhancer RNAs (eRNAs), and activity-induced DNA breaks, that have emerged as important factors that govern the temporal dynamics of activity-induced gene transcription.

A fundamental question in neurobiology concerns understanding how adaptive behaviors are developed in response to cues from the environment. While this question has been investigated from numerous perspectives, early studies conducted more than fifty years ago demonstrated that the formation of lasting behavioral adaptations, including long-term memories, requires new protein synthesis to occur during a brief window immediately following the exposure to a stimulus (Flexner, Flexner, & Stellar, 1963). For instance, transcriptional inhibitors were found to be effective in blocking the late phase of long-term potentiation (L-LTP) only when they were administered either prior to or immediately after the application of a stimulus (Frey et al., 1996; Messaoudi et al., 2002; Nguyen et al., 1994). These observations suggested that by identifying the proteins that are synthesized during this short window and characterizing their functions, one could obtain significant insights into how experience exerts its profound influence on behavior.

It was in the backdrop of these developments that Greenberg and Ziff (Greenberg and Ziff, 1984) reported that stimulation of quiescent fibroblasts with serum triggers a very rapid induction of the proto-oncogene, *c-fos*. This finding was significant for at least two reasons: first, it demonstrated that rapid changes to gene expression are, in fact, an important

*To whom correspondence should be addressed: 5323 Harry Hines Blvd, Dallas, TX 75390-9070, Tel: 214-648-6508, Ram.Madabhushi@UTSouthwestern.edu, Taekyung.Kim@UTSouthwestern.edu.

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component of the cellular response to stimuli from the external environment. Second, it paved the way for subsequent studies, which identified that *c-fos* and other so-called immediate-early genes are also rapidly induced in the brain in response to various paradigms of neuronal stimulation (Lanahan and Worley, 1998; Morgan et al., 1987). With advances made in next-generation sequencing technologies, it is now known that neuronal stimuli trigger the induction of hundreds of activity-regulated genes, many of which facilitate changes to neural circuits by modulating dendritic growth, synaptic remodeling, and excitatory/inhibitory balance (West and Greenberg, 2011). For example, one of the activity-induced genes, *Arc* (activity-regulated cytoskeletal), plays a key role in various forms of synaptic plasticity and behavior (Korb and Finkbeiner, 2011). *Arc* levels are rapidly elevated in response to neuronal activity evoked by sensory stimulation or experience, and regulates both Hebbian and non-Hebbian forms of synaptic plasticity by promoting the endocytosis of AMPA receptors. *Arc* knock-out mice exhibit a specific deficit in the late phase of LTP and LTD (long-term depression), which is strongly dependent on activity-dependent gene expression. Likewise, although *Arc* knock-out mice can learn new behavioral tasks as efficiently as wild-type mice, they show defects in memory consolidation (Guzowski et al., 2000; Plath et al., 2006; Ploski et al., 2008). Furthermore, mutations in neuronal activity-regulated genes and disruptions in activity-dependent gene transcription networks manifest in various neurological disorders (Ebert and Greenberg, 2013). A proper understanding of the signaling pathways and molecular features that regulate neuronal activity-dependent transcription is therefore likely to be enormously significant.

Promoter regulatory elements and transcription factors in neuronal activity-dependent gene expression

The observations that external stimuli could rapidly induce gene expression changes led to investigations into identifying the *cis*-acting regulatory elements that are important for stimulus-coupled transcription. In an elegant study involving deletion analysis of upstream sequences of a cloned human *c-fos* gene, Treisman first identified an element called the serum response element (SRE) that was essential for serum stimulation of *c-fos* induction (Treisman, 1985). From HeLa cell nuclear extracts, he then identified the protein, the serum response factor (SRF), that binds to the SRE of the *c-fos* gene (Treisman, 1986). Separate DNA affinity purification experiments resulted in the isolation of a ternary complex consisting of SRE, SRF, and another protein, called Elk-1, that confers serum inducibility of *c-fos* (Hipskind et al., 1991; Shaw et al., 1989). Subsequent to these studies, it was shown that the SRE and SRF also contribute to *c-fos* induction in neuronal cells (Misra et al., 1994).

Meanwhile, it was discovered that calcium influx through voltage-gated calcium channels is the key initiating event that drives *c-fos* expression in neuronal cells (Morgan and Curran, 1986). Furthermore, the ability of calmodulin inhibitors to block stimulus-dependent *c-fos* induction led to the hypothesis that calcium influx could activate a calmodulin-kinase-dependent cascade that ultimately modifies the activity of a transcriptional activator of the *c-fos* gene and drives its expression (Morgan and Curran, 1986). By again resorting to promoter deletion analysis, a distinct *cis*-acting element that was necessary for calcium-

dependent activation of *c-fos* transcription was identified (Sheng et al., 1988). This element proved to be identical to the cyclic AMP response element (CRE) that had been described by within the rat *somatostatin* gene promoter (Montminy et al., 1986). In fact, using DNA affinity chromatography, the transcription factor that binds to the CRE element was identified and named CREB (for cAMP-responsive element binding protein), and it was further described that phosphorylation of CREB at serine-133 was crucial for CREB-dependent gene activation (Gonzalez and Montminy, 1989). These features were subsequently shown to be important for calcium-dependent induction of *c-fos* transcription in neuronal cells (Sheng et al., 1991, 1990).

While these and other studies in cultured cells seemed to suggest that individual *cis*-acting elements could act independently to regulate the expression of *c-fos* and other immediate-early genes in a stimulus-specific manner, analysis of *Fos-LacZ* reporter constructs demonstrated that mutation of either the SRE or the CRE element was sufficient to prevent *c-fos* induction in response to stimulation of neuronal activity in the mouse brain (Robertson et al., 1995). These observations indicate that the regulation of immediate-early genes in response to physiological stimuli requires the collaborative actions of multiple *cis*-acting elements (Misra et al., 1994; Robertson et al., 1995).

The identification of *cis*-acting promoter elements and transcription factors that bind these elements cleared the path for elaborating the signaling cascades that transduce extracellular signals to the nucleus and activate gene transcription. Following early studies, it became clear that calcium influx specifically through N-methyl-D-aspartate receptors (NMDARs) and L-type voltage-sensitive calcium channels (LVSCCs) initiates multiple signal transduction pathways that transmit information regarding an external stimulus to the nucleus and affect activity-dependent gene transcription in neurons (Deisseroth et al., 2003). For instance, calcium influx through NMDARs and LVSCCs allows for the recruitment of the calcium-binding protein, calmodulin (CaM), which translocates to the nucleus and activates the calcium/CaM-dependent protein kinases, CaMKII and CaMKIV. In response to stronger stimulation, calcium/calmodulin also causes the activation of the Ras-mitogen-activated protein kinase (MAPK) pathway. Both these pathways ultimately converge on the transcription factors, CREB, SRF, and Elk-1. Phosphorylation of CREB at serines-133, -142, and -143, SRF at serine-103, and Elk-1 at serine-383 have been shown to stimulate transcription in numerous ways, such as by stabilizing protein-protein interactions, recruiting transcriptional activators, and altering the chromatin environment within the promoters of immediate-early genes (Deisseroth et al., 2003; Esnault et al., 2017; Misra et al., 1994; Rivera et al., 1993; Wang et al., 2005; Xia et al., 1996).

In addition to protein kinases, calcium/calmodulin signaling also activates the phosphatase calcineurin, which targets a distinct set of transcription factors in response to neuronal activity. For instance, calcineurin-mediated dephosphorylation activates the myocyte enhancer factor 2 (MEF2) family transcription factors that control synapse development by regulating the expression of important neuronal activity-regulated genes, including *Nr4a1*, *Arc*, *Homer1a*, and *Bdnf* (Flavell et al., 2008, 2006). Calcineurin also dephosphorylates a member of the nuclear factor of activated T cells (NFAT) family of transcription factors, NFATc4, in response to neuronal activity. Under basal conditions, NFATc4 is predominantly

cytosolic in hippocampal neurons, however, calcineurin-mediated dephosphorylation causes NFATc4 to translocate to the nucleus, where it targets genes essential for neuronal plasticity (Crabtree and Olson, 2002). Taken together, these and other studies elaborated a model in which exposure to an external stimulus activates dedicated signaling cascades that modulate either the binding or the activity of sequence-specific transcription factors within the promoters of neuronal immediate early genes, and drives gene induction.

Epigenetic mechanisms in neuronal activity-dependent gene expression

In an effort to understand how activity-dependent phosphorylation of CREB leads to transcription activation, Goodman and colleagues screened a human thyroid cDNA library with radiolabeled CREB and isolated a 265 kDa protein that specifically bound to serine-133 phosphorylated CREB, which they named CREB-binding protein (CBP) (Chrivia et al., 1993). In subsequent studies, CBP was shown to be a crucial co-activator of CREB-dependent transcription (Arias et al., 1994; Kwok et al., 1994; Parker et al., 1996). The prevailing model during this time was that DNA-binding transcription factors work primarily by recruiting and stabilizing the basal transcription machinery at promoters, and initial studies involving CBP interaction with RNA polymerase II (RNAPII) suggested that CBP could function as a transcriptional adaptor protein for CREB and other transcription factors (Kee et al., 1996; Kwok et al., 1994). However, it was soon discovered that CBP and its closely related protein, p300, have potent histone acetyltransferase (HAT) activity (Ogryzko et al., 1996).

The idea of histone acetylation as a potential mechanism of transcriptional activation originated as early as the discovery of histone acetylation itself (Allfrey et al., 1964); however, it was the purification of histone acetyltransferases and their similarity to previously identified transcriptional co-activators/adapters that provided the first direct evidence linking histone acetylation to gene activation (Brownell et al., 1996; Brownell and Allis, 1996; Ogryzko et al., 1996; Yang et al., 1996). Together with the demonstration that CBP/p300 was able to cooperatively stimulate transcription only on chromatin templates (Kraus and Kadonaga, 1998), these studies suggested that the important functions of CBP/p300 in transcriptional activation could arise from their ability to acetylate histones and render chromatin more permissive to transcription.

These observations coincided with major advancements in the understanding of epigenetic mechanisms, including DNA methylation, histone post-translational modifications, and ATP-dependent chromatin remodelers, in transcriptional regulation. These developments triggered a shift in emphasis from sequence-specific factors to delineating chromatin regulatory mechanisms of neuronal activity-dependent transcription (Qiu and Ghosh, 2008a). In this regard, initial observations that neuronal stimulation causes CBP phosphorylation and activation in a calcium and CaMKIV-dependent manner, and that CBP phosphorylation is necessary for transcriptional activation first linked neuronal activity-dependent signaling pathways with chromatin-modifying activities (Chawla et al., 1998; Hu et al., 1999; Impey et al., 2002). Meanwhile, a screen for activators of calcium-dependent gene expression in neurons led to the identification of a novel transactivator named CREST that is expressed in the developing brain and associates with CBP (Aizawa et al., 2004).

Interestingly, CREST is also present in a chromatin remodeling complex composed of the ATP-dependent chromatin remodeler, BRG1, which plays an essential role in dendritic development (Qiu and Ghosh, 2008b; Wu et al., 2007). Analysis of the *c-fos* promoter revealed that under basal conditions, the association of BRG1-CREST complex with the retinoblastoma protein (Rb) and histone deacetylases (HDACs) correlates with transcriptional repression. Neuronal stimulation and calcium influx then induce calcineurin-mediated Rb dephosphorylation and the release from HDAC-mediated repression, while also causing CREST-dependent CBP recruitment, histone acetylation, and gene transcription (Qiu and Ghosh, 2008b). Together, these studies illuminated a paradigm for how a stimulus-dependent switch in chromatin-modifying activities could mediate neuronal activity-induced transcription.

RNAPII pausing and the control of neuronal activity-dependent transcription

It is noteworthy that up to this point, the elaboration of mechanisms that facilitate the transcription of neuronal activity-regulated genes was influenced in a large part by classical models of transcriptional regulation, which emphasized RNAPII recruitment to promoters and transcription initiation as the key regulatory events in the production of RNA (Ptashne and Gann, 1997). However, the discovery of widespread promoter-proximal RNAPII pausing following transcription initiation in various metazoan model systems has upended this view, and has revealed that events downstream of transcription initiation, such as RNAPII pause-release and transcription elongation, are important rate-limiting steps that are subject to regulatory control (Adelman and Lis, 2012; Jonkers and Lis, 2015).

Although pausing of prokaryotic RNA polymerase had been described *in vitro* (Kassavetis and Chamberlin, 1981; Maizels, 1973), pausing of RNA polymerase *in vivo* was first observed by Gariglio, Bellard, and Chambon while studying the transcription of β -globin genes in mature and immature hen erythrocytes (Gariglio et al., 1981). In nuclear transcription run-on assays, they unexpectedly found RNA polymerase to be preferentially clustered at the 5' end of the β -globin genes in mature erythrocytes (Gariglio et al., 1981). Based on these observations, they insightfully proposed that such clustering of RNA polymerase could provide a mechanism to regulate transcription at the elongation step, and predicted that if this were indeed the case, it should be possible to detect clustering of RNA polymerase at 5' ends of genes that "have the potential to be transcribed" (Gariglio et al., 1981). Similar observations were indeed reported by Gilmour and Lis when they utilized UV crosslinking to assess the distribution of RNAPII on the *hsp70* heat shock gene in *Drosophila* (Gilmour and Lis, 1986). Subsequently, it was determined that promoter-associated RNAPII at the uninduced *hsp70* gene initiates RNA synthesis but is arrested in the early stages of transcript elongation (Rougvie and Lis, 1988). Over the next decade, these findings were extended to a number of promoters, including human *c-myc* and *cfos* genes (Collart et al., 1991; Krumm et al., 1992; Mechti et al., 1991; Plet et al., 1995; Schneider-Schaulies et al., 1987).

While these studies provided the first significant insights into RNAPII pausing as a regulated rate-limiting step in the transcription cycle, it was still unclear whether RNAPII pausing was limited to a small number of loci or whether this was a more widespread phenomenon. This issue was addressed with the advancements in chromatin immunoprecipitation (ChIP)-based methods, such as ChIP-chip (ChIP combined with microarrays) and ChIP-seq (ChIP coupled to next-generation sequencing), that allowed for genome-wide profiling of RNAPII binding (Glover-Cutter et al., 2008; Muse et al., 2007; Zeitlinger et al., 2007). These studies revealed RNAPII pausing to be a widespread phenomenon, and found that RNAPII pausing was particularly enriched in genes that are rapidly induced in response to developmental or environmental cues (Muse et al., 2007; Zeitlinger et al., 2007). Further studies using global run-on sequencing verified that promoter-associated RNAPII was indeed in a paused state, and suggested that almost a third of all genes in human, mouse, and *Drosophila* display promoter-proximal RNAPII pausing (Adelman and Lis, 2012; Core et al., 2008).

Meanwhile, utilizing an immobilized template-based transcriptional system, Marshall and Price identified the biochemical determinants of transcription elongation control in *Drosophila* K_c cell nuclear extracts (Marshall and Price, 1992). These studies revealed that a predominant fraction of RNAPII molecules *in vitro* are incapable of producing long transcripts, but instead entered a paused state that is followed by transcription termination (Marshall and Price, 1992). Elongation competent RNAPII complexes could be derived from paused complexes by the addition of a factor that was sensitive to the transcription inhibitor, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) (Marshall and Price, 1992). This factor was named p-TEF (for positive transcription elongation factor) and the existence of a negative transcription elongation factor that determines the tendency of RNAPII to enter a paused state was postulated (Marshall and Price, 1995, 1992). Working with HeLa cell nuclear extracts and a slightly different line of reasoning, Wada, Handa, and colleagues noted that in contrast to the scenario in intact cells, transcription systems reconstituted from nuclear extracts *in vitro* were insensitive to DRB. This led them to purify the factor that confers DRB sensitivity from HeLa cell nuclear extracts, which they called DSIF (for DRB sensitivity-inducing factor) (Wada et al., 1998). DSIF was shown to consist of two subunits that are homologs of the yeast transcription factors *Spt4* and *Spt5* (Wada et al., 1998), and it was demonstrated that in addition to mediating RNAPII pausing, DSIF is also essential for transcription elongation (Wada et al., 1998). In subsequent studies, Handa and colleagues purified a second factor that conferred DRB sensitivity and named it NELF (for negative elongation factor) (Yamaguchi et al., 1999).

The identification of these biochemical activities allows for the construction of a model in which core promoter features, promoter-bound transcription factors, nucleosome architecture collaborate with DSIF, NELF, and other factors to recruit and stably maintain RNAPII in a paused configuration following transcription initiation. Subsequently, the recruitment of the p-TEFb complex mediates RNAPII pause-release and the transition to productive elongation through phosphorylation of NELF, DSIF, and the RNAPII carboxy-terminal domain (CTD) at serine-2 (Jonkers and Lis, 2015; Yamaguchi et al., 2013).

From the perspective of neuronal activity-dependent transcription, the discovery of RNAPII pausing as a rate-limiting step in transcription is enormously significant for several reasons:

genome-wide analysis of RNAPII binding in mouse cortical neurons using ChIP-seq revealed that the promoters of many neuronal activity-regulated genes, particularly the immediate early genes, including *c-fos*, *Arc*, *Npas4*, *Egr1*, and *Nr4a1*, are already bound by RNAPII under basal conditions (Kim et al., 2010). In addition to this, studies in a number of systems have shown that the transcriptional induction of neuronal immediate early genes, such as *c-fos* and *Arc* is regulated at the level of RNAPII pause-release (Collart et al., 1991; Mechti et al., 1991; Saha et al., 2011; Schaukowitz et al., 2014; Schneider-Schaulies et al., 1987). Most importantly, these observations suggest that mechanisms that govern RNAPII pausing and pause-release are likely targeted by neuronal activity-dependent signaling pathways, and the elaboration of the interactions between neuronal activity-dependent signaling and RNAPII pausing mechanisms is likely to provide new mechanistic insights into the principles that govern activity-dependent transcription in neurons.

Enhancers in neuronal activity-dependent transcription

In addition to promoter-proximal RNAPII pausing, recent discoveries regarding the nature of enhancers have also dramatically altered our understanding of neuronal activity-dependent transcription. The term “enhancer” was first employed by Banerji, Rusconi, and Schaffner to describe the effects of a 72 bp repeat sequence from SV40 on the transcription of the rabbit β -globin gene in HeLa cells (Banerji et al., 1981). They found that when linked to the β -globin gene in *cis*, this repeat sequence caused a 200-fold stimulation of β -globin gene transcription. Moreover, they observed that the enhancing activity occurred independently of the orientation and position of the repeat sequence with respect to the β -globin gene (Banerji et al., 1981). Together, these observations established the framework for defining enhancers. In a subsequent landmark study, Banerji, Olson, and Schaffner identified the first eukaryotic enhancer within the mouse immunoglobulin heavy chain locus (E μ enhancer) (Banerji et al., 1983). Importantly, they observed that E μ enhancer activity was tissue-specific and was only functional in lymphocyte-derived cells (Banerji et al., 1983). These observations were extended to enhancer elements in a variety of systems, and suggested that while promoters mediate accurate transcription initiation, spatiotemporal patterns of gene expression are largely dependent on activities of enhancers (Kim and Shiekhhattar, 2015).

Although putative enhancer elements that could regulate *c-fos* expression were defined in early studies (Deschamps et al., 1985; Treisman, 1985), the difficulty of mapping enhancers proved to be a major impediment to understanding their roles in neuronal activity-dependent transcription. This issue was remedied with the advent of genome-wide profiling methods, particularly, ChIP-seq, which allowed for the annotation of gene regulatory elements based on the chromatin signatures of surrounding nucleosomes. For instance, enhancers were shown to typically be associated with nucleosomes containing higher levels of histone H3 lysine 4 monomethylation (H3K4me1) and lower levels of H3K4me3, and active enhancers were also shown to possess higher levels of H3K27 acetylation (H3K27ac), which, as described above, is deposited by CBP/p300 (Kim and Shiekhhattar, 2015). These developments set the stage for the global characterization of neuronal enhancers.

Subsequently, genome-wide profiling of CBP, H3K4me1, and H3K4me3 binding patterns in cultured neurons led to the identification of thousands of activity-regulated enhancers (Kim

et al., 2010). Interestingly, this study showed that neuronal stimulation causes RNAPII recruitment and pervasive bidirectional transcription from an important subset of these putative enhancers, yielding non-coding RNAs, called enhancer RNAs (eRNAs) (Kim et al., 2010). Simultaneously, and using a similar strategy to map enhancers, de Santa, Natoli, and colleagues also reported widespread transcription from enhancers in macrophages that were stimulated with endotoxin (de Santa et al., 2010). Following these reports, numerous studies in various cell and tissue types have verified the generation of eRNAs in response to diverse stimuli, and have suggested that eRNA synthesis could be a reliable indicator of enhancer activity (Andersson et al., 2014; Kim and Shiekhataar, 2015; Li et al., 2016).

The discovery of eRNAs has led to an important debate about whether transcriptional activity at enhancers is significant for the biological function of enhancer, and whether this function emerges merely from the act of enhancer transcription or whether eRNA transcripts themselves have specific roles. In this regard, the specific pattern of eRNA production within a subset of mapped enhancers (Kim et al., 2010) suggests that eRNA synthesis might not simply be a by-product of stimulus-driven transcriptional activity. A comprehensive analysis of transcription start sites illustrated that promoters and enhancers share remarkably similar architectures of the initiation sites, such as bidirectional transcription from independently assembled transcription complexes and similar frequencies of canonical core promoter elements (Core et al., 2014). Furthermore, kinetic measurements suggested that eRNA transcription precedes the expression of target mRNAs and knockdown of several eRNAs caused a decrease in expression of mRNAs from their target genes in a number of systems, including neurons (Arner et al., 2015; Lam et al., 2013; Li et al., 2013; Melo et al., 2013; Schaukowitch et al., 2014). These results suggest that although the act of enhancer transcription itself could underlie some aspects of enhancer function (Kim and Shiekhataar, 2015), eRNA transcripts have important regulatory roles in stimulus-dependent transcription.

Several mechanisms have been proposed to explain the role of eRNAs in regulating transcription from their target genes. For instance, in postmitotic neurons, eRNAs generated from enhancers of immediate-early genes cause the release of NELF from paused RNAPII at the promoters of these genes (Schaukowitch et al., 2014). These results are exciting because, as mentioned above, recent studies suggest that immediate-early gene expression is largely regulated at the level of RNAPII pausing and pause-release, and eRNA-mediated NELF release provides some of the first mechanistic insights into how activity-dependent signaling might interact with the RNAPII pausing machinery, and thereby regulate neuronal activity-dependent gene transcription. Interestingly, androgen receptor-regulated eRNAs were shown to promote RNAPII pause-release by activating pTEFb (Zhao et al., 2016). Distinct from this, studies on eRNAs generated during transcription induced by estrogen receptor signaling in MCF-7 breast cancer cells, and by androgen receptor signaling in prostate cancer cells have suggested that eRNAs facilitate enhancer-promoter looping interactions through interactions with either the cohesion complex or with subunits of the mediator complex (Hsieh et al., 2014; Li et al., 2013). These results suggest that eRNAs could be utilized differently in different contexts to achieve transcriptional activation (Rajarajan et al., 2016).

Notably, although eRNAs generated from neuronal immediate-early genes had no effect on enhancer-promoter looping interactions, the importance of enhancer-promoter interactions in regulating neuronal activity-dependent transcription is highlighted by several lines of evidence: first, the effect of eRNA knockdown of target gene expression and the role of eRNAs in NELF release from target promoters itself suggests that stable enhancer-promoter interactions would be crucial for activity-dependent gene expression in neurons. Second, recent analysis of the chromatin architecture at *c-fos* revealed that its expression is not only governed by multiple enhancers, but also that distinct subsets of enhancers respond to different stimuli to confer both stimulus specificity and robust inducibility of *c-fos* (Joo et al., 2015). These effects are likely conferred through stimulus-specific interaction of distinct enhancer combinations with the *c-fos* promoter. Finally, analysis of the immediate early gene, *Arc*, has revealed that while eRNAs generated from the *Arc* enhancer are dispensable for enhancer-promoter interactions (Schaukowitch et al., 2014), an intact *Arc* gene promoter is essential for eRNA synthesis from the *Arc* enhancer (Kim et al., 2010). Although precisely how elements within the *Arc* promoter guide eRNA synthesis remains unknown, these observations suggest a sequence of events in which neuronal activity triggers enhancer-promoter interactions at activity-regulated genes. These interactions then facilitate eRNA synthesis, which in turn, activates expression of mRNA from the promoter by orchestrating the escape of paused RNAPII.

Activity-induced breaks as novel regulators of neuronal activity-induced gene expression

The description of the above model implies that the ultimate trigger for neuronal activity-dependent transcription could be provided by mechanisms that facilitate rapid enhancer-promoter communication at immediate-early genes in response to neuronal activity. In this regard, recent observations describing the formation of activity-induced DNA breaks within the promoters of immediate-early genes raise the possibility that these DNA breaks could constitute such a triggering mechanism. Several studies have reported that various paradigms of neuronal stimulation both *in vitro* and *in vivo* result in the formation of DNA double strand breaks (DSBs) (Crowe et al., 2006; Madabhushi et al., 2015; Suberbielle et al., 2013). However, the significance of these activity-induced DSBs was initially unclear because the mechanisms underlying their formation were unknown, and because the formation of DNA DSBs is generally thought to be a cytotoxic event that perturbs genomic stability, and could lead to the development of cancer and various neurological disorders (Crowe et al., 2006; Jackson and Bartek, 2009; Madabhushi et al., 2014; Rass et al., 2007; Suberbielle et al., 2013).

The formation of DNA DSBs elicits an elaborate signaling response that includes pathways that regulate DNA repair, transcriptional regulation, and apoptosis (Jackson and Bartek, 2009). Major aspects of the cellular response to DSB formation are coordinated by the transducer kinase, ataxia-telangiectasia mutated (ATM), and an important target of ATM is the histone variant, H2AX, which is rapidly phosphorylated in the vicinity of sites that incur DSBs. This feature was exploited in an effort to determine whether activity-induced DSBs are preferentially formed at certain loci within the genome of neurons (Madabhushi et

al., 2015). Surprisingly, these and other experiments revealed that neuronal activity-induced DSBs form within the promoters of immediate-early genes, such as *Fos*, *Egr1*, *Npas4*, and *Nr4a1*, and that DSB formation within these promoters is sufficient to drive gene induction (Madabhushi et al., 2015). Stimulus-induced DSB formation and gene induction has now been described in a number of systems, including in the promoters of genes that are activated in response to stimulation with serum, estrogen, insulin, and glucocorticoids (Bunch et al., 2015; Ju et al., 2006; Trotter et al., 2015; Wong et al., 2009), indicating that stimulus-specific DSB formation could be a widely-utilized mechanism to stimulate gene expression.

Further insights into the association between DSBs and transcriptional induction have come from the discovery that activity-induced DSBs are generated through the actions of the topoisomerase, topoisomerase II β (Top2B) (Bunch et al., 2015; Ju et al., 2006; Madabhushi et al., 2015; Trotter et al., 2015; Wong et al., 2009). Cellular topoisomerases are remarkable enzymes that resolve torsional strain that accumulates during various activities on the DNA, including transcription, DNA replication, and recombination (Wang, 2002). The key step in the resolution of topological strain by topoisomerases is that this process involves the generation of transient DNA breaks that are utilized to pass DNA strands to each other. Normally, these DNA breaks are quickly resealed by the topoisomerase itself and are not detected by the cellular DNA damage response pathways. However, this process is somehow modified in response to neuronal activity to generate more lasting DNA breaks that cannot be resealed by the enzyme, and instead requires the action of classical DNA DSB repair pathways, such as nonhomologous end-joining (Madabhushi et al., 2015). These features are also conserved in other systems involving stimulus-induced DSB formation (Bunch et al., 2015; Ju et al., 2006; Trotter et al., 2015).

Together, these observations raise intriguing questions about how the activity of topoisomerases is regulated in response to neuronal activity, and how DSB formation stimulates gene expression. Although these questions remain largely unanswered, it was recently shown that inhibition of Top2B activity increases promoter-proximal RNAPII pausing at serum19 inducible genes, including *c-fos* and *Egr1* (Bunch et al., 2015). These results suggest that Top2B-mediated DSBs could be important for the release of paused RNAPII at specific immediate-early genes. Separately, several recent studies have now shown that Top2B interacts with the architectural protein, CTCF, which regulates interactions between distinct chromatin elements, including enhancer-promoter interactions, and that Top2B-mediated DNA DSBs are enriched at sites that are occupied by CTCF (Canela et al., 2017; Madabhushi et al., 2015; Uusküla-Reimand et al., 2016). These observations suggest an intriguing model in which CTCF-mediated chromatin architecture imposes a topological barrier to enhancer-promoter interactions under basal conditions, and also contributes to the stabilization of paused RNAPII at the promoters of immediate early genes (Figure 1). This topological constraint is relieved through the formation of Top2B-mediated DSBs, which stabilizes enhancer-promoter interactions that culminate ultimately in RNAPII pause-release and transcriptional induction. Future studies that are directed at understanding how DSB formation could affect enhancer-promoter interactions should provide crucial insights into the validity of this model (Figure 1).

Conclusions

The mechanisms that control neuronal activity-dependent gene expression have been studied for more than thirty years. The early phase of these investigations focused on the identification of the required *cis*-acting elements within promoter regions, the corresponding transcription factors that bind these elements, and the modulation of these transcription factors by signaling pathways in response to neuronal activity. However, two major developments have triggered a paradigm shift in our understanding of how neuronal activity-dependent gene expression could be controlled: the first is the discovery that immediate-early gene induction (and the induction of many other genes) is regulated not at the level of RNAPII recruitment, but largely at the level of RNAPII pausing and pause-release. The second major development has been the recognition that spatiotemporal control of gene expression is regulated in a major part through activities at enhancers. The findings that enhancers also bind transcription factors, RNAPII and also produce transcripts suggest that many activities initially thought to be restricted to promoters also occur at enhancers. Together, these observations suggest that stimulus-coupled transcription in neurons and other systems could be governed by mechanisms that modulate the communication of enhancers with promoters. The formation of activity-induced DNA breaks within promoters of immediate-early genes could constitute one such mechanism. These developments raise many interesting questions for future investigations. For instance, further elaboration of the requirements for establishing RNAPII pausing and the mechanisms that govern enhancer-promoter interactions at neuronal activity-regulated genes could provide new clues about how pause-release is coordinated in response to neuronal activity. Similarly, a deeper understanding of how activity-induced DNA breaks are formed, and how break formation facilitates gene induction is also needed. Finally, the utilization of DNA breaks as a strategy to stimulate gene expression suggests that changes in the ability to form or repair activity-induced DNA breaks could have significant pathophysiological implications. Genomic instability has been linked to the development of various neurological disorders, and it would be intriguing to test whether defects in the ability to repair neuronal activity-induced DNA breaks could underlie the development of these disorders.

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Highlights

- Historical overview of the major developments in the discovery of immediate-early genes, including the identification of cis-acting elements within immediate-early gene promoters, signaling pathways that govern gene induction, and the role of chromatinmodifying enzymes
- A review of emerging perspectives on the control of neuronal activity-dependent gene expression, including the role of enhancer RNAs, RNAPII pausing, enhancer-promoter communication, and activity-induced DNA breaks

Figure 1. Mechanisms controlling neuronal activity-regulated gene induction

(Left) Under basal conditions, RNAPII is held in a paused state through the actions of DSIF and NELF, and enhancer-promoter interactions are precluded through the imposition of a topological constraint by the architectural protein, CTCF. (Right) Upon activity stimulation, Top2B-mediated DNA breaks override the CTCF-enforced constraint, and allow for enhancer-promoter interaction. In neurons, this interaction allows for the synthesis of eRNAs at enhancers, which in turn, mediate the release of NELF. The actions of pTEFb then allow for the escape of RNAPII from the promoter and trigger gene induction. eRNAs have also been shown to stimulate gene expression by stabilizing enhancer-promoter interactions through their interaction with the cohesin and/or mediator complexes, and by modulating chromatin structure at promoters. For convenience of illustration, other activities that have been shown to be essential for gene induction are described in the text, but not shown here.