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DIFFERENT NEURONAL ACTIVITY PATTERNS INDUCE DIFFERENT GENE
EXPRESSION PROGRAMS

A dissertation presented
by
Kelsey Marie Tyssowski
to
The Division of Medical Science
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
in the subject of
Biological and Biomedical Sciences

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Cambridge, Massachusetts
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Different Neuronal Activity Patterns Induce Different Gene Expression Programs

ABSTRACT

Neurons induce hundreds of activity-regulated genes (ARGs) in response to extracellular stimuli, suggesting that a vast number of different stimuli could each be coupled to a distinct ARG expression profile. Such coupling could explain how neurons induce different types of plasticity in response to different stimuli. In this dissertation, I focus on one aspect of extracellular stimuli: its temporal pattern. Few studies have compared ARG induction between different neuronal activity patterns, especially on a genomic scale.

Using RNA-sequencing, I show—both in cultured cortical neurons and in the mouse visual cortex—that neurons stimulated for different durations induce different ARGs. Specifically, brief activity selectively induces a small subset of ARGs that corresponds to the first of three temporal waves of ARGs induced by sustained activity. I formally demonstrate that I can use this differential ARG induction to infer neurons' activity duration history. I then apply this inference to single-cell RNA-sequencing data from individual neurons in stimulated cortex, demonstrating the potential for pattern-dependent ARG expression to be used as a tool for inferring neuronal stimulation histories.

I further show that the first-wave genes induced by brief activity uniquely require MAPK/ERK signaling for their induction. MAPK/ERK signaling likely regulates first-wave genes by promoting eRNA production but not histone acetylation at the enhancers near first-wave genes. These mechanistic findings provide a molecular handle for testing the role of first-wave genes in plasticity. Furthermore, they demonstrate that the same mechanisms that establish the multi-wave temporal structure of gene induction also enable different gene sets to be induced by different durations of stimulation.

In memory of my friend and bay-mate, Sam D. Rendall.

Thank you for your infectious curiosity.

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CHAPTER I.

INTRODUCTION: THE STIMULATION-TRANSCRIPTION COUPLING MAP

This chapter is adapted from an as-yet-unpublished review I wrote with guidance from Jesse Gray.

INTRODUCTION

Neurons transcribe different subsets of neuronal-activity-regulated genes (ARGs) in response to different extracellular stimuli (Chawla and Bading, 2001; Douglas et al., 1988; Elliott et al., 2016; Fields et al., 1997; Greenberg et al., 1986; Grehl et al., 2015; Gunther et al., 2005, 2003; Lee et al., 2017; Schaukowitch et al., 2017; Sheng et al., 1995, 1993; Worley et al., 1993; Zhang et al., 2007). Thus, a vast number of different stimuli could each be coupled to a unique transcription program. We have known since the 1980s that the nature of neuronal stimulation, including its intensity and temporal pattern, determines the magnitude of induction of a few individual ARGs, such as *Fos* and *Egr1* (Chawla and Bading, 2001; Douglas et al., 1988; Fields et al., 1997; Greenberg et al., 1986; Sheng et al., 1995, 1993; Worley et al., 1993), and recent genomic studies of hundreds of ARGs have begun to expand such findings to reveal how different stimuli are coupled to the ARG program as a whole (Chen et al., 2016; Grehl et al., 2015; Gunther et al., 2005, 2003; Lee et al., 2017; Schaukowitch et al., 2017; Zhang et al., 2007).

Understanding this coupling between stimulation and transcription could be a new and powerful route toward understanding how ARGs orchestrate neuronal-activity-dependent plasticity. ARG transcription contributes not only to neuron-wide plasticity like homeostatic synaptic scaling (Ibata et al., 2008), but also to synapse-specific plasticity (e.g., long-term potentiation (Nguyen et al., 1994), which may occur via synaptic tagging (Steward et al., 1998)). Thus far, this ARG-dependent plasticity has primarily been investigated using single-gene knockouts and total blockades of transcription (Ibata et al., 2008; Nguyen et al., 1994). For example, knockout mice have revealed that *Arc* and *Homer1a* are important for homeostatically tuning synaptic strength (Diering et al., 2017; Shepherd et al., 2007), that *Npas4* and *Igf1* regulate inhibition onto excitatory neurons (Hartzell et al., 2018; Lin et al., 2008; Mardinly et al., 2016;

Spiegel et al., 2014), and that *Nptx2* regulates excitation onto inhibitory neurons (Chang et al., 2010). However, hundreds of ARGs are induced in response to stimulation, making it laborious to use single-gene manipulation to link each gene to a specific type of plasticity. Because different stimuli induce different types of plasticity (Nicoll and Schmitz, 2005), one alternative to single-gene manipulation is to test the function of ARG modules that are defined by the stimuli that induce them. This approach will require identifying stimulus-specific modules and manipulating those modules through manipulation of ARG-module-specific regulatory mechanisms. Thus, to enable this approach and reveal new roles for ARG modules in plasticity, it will be important to understand (1) how each type of stimulation is coupled to an ARG expression program and (2) the transcriptional regulation that establishes such coupling.

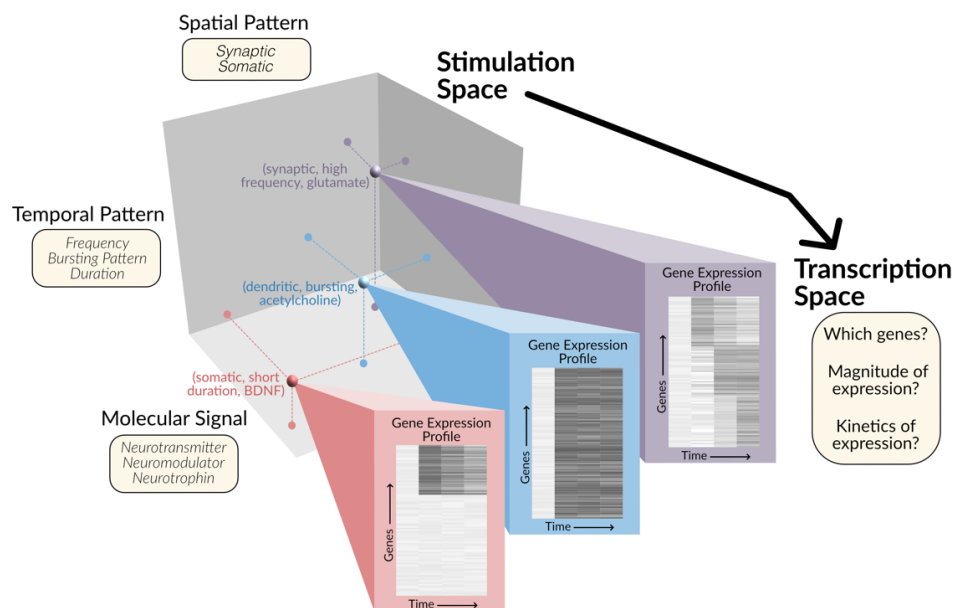


FIGURE 1.1. THE STIMULATION-TRANSCRIPTION COUPLING MAP

We propose creating a stimulation-transcription coupling map that would link stimulation space (all possible different stimuli) to transcription space (all possible transcription programs). Here we show an example of three theoretical couplings between stimulation space and transcription space, depicted on a simplified three-dimensional stimulation space defined by the spatial pattern (x) molecular identity (y), and temporal pattern (z) of the stimulus.

To systematically assess stimulation-transcription coupling, I propose creating a stimulation-transcription coupling map (Figure 1.1). This map will describe how stimulation space, which encompasses all possible variation in stimulation, maps onto transcription space, which encompasses all possible ARG expression programs. Specifically, stimulation space includes the neurotransmitter or neurotrophin stimulating the neuron, the valence of this stimulus (excitatory or inhibitory), and its temporal or spatial pattern. Transcription space includes which genes are regulated by stimulation as well as the magnitude and kinetics of their expression. While we are still far from a complete stimulation-transcription coupling map, genomic experiments—which allow measurement of all of transcription space in a single experiment—have the potential to dramatically improve our map.

EARLY STUDIES OF STIMULATION-TRANSCRIPTION COUPLING IN NEURONS

Early studies of individual genes revealed the first principle of the coupling map: increased neuronal activation results in increased transcription. In cultured neurons, transcription of the ARG *Fos* increases with increasing frequency of electrical stimulation (Sheng et al., 1993), longer durations of membrane depolarization (Chawla and Bading, 2001), and greater concentrations of nicotine (Greenberg et al., 1986). The magnitude of *Fos* transcription also depends on the bursting pattern of electrical stimulation: it is greater in neurons stimulated with short bursts and short inter-burst intervals compared to long bursts with long inter-burst intervals, even when both patterns have an equal number of total spikes (Fields et al., 1997; Sheng et al., 1993). Thus, even at the level of the single gene, gene expression contains information about the temporal pattern or strength of stimulation.

Early studies of a few genes revealed another principle of the coupling map: different genes are induced in response to differences in stimulation. For instance, longer durations of stimulation lead to induction of more genes: a train of 100 25-ms pulses induces expression of

Egr1 and *Junb*, whereas a train of 400 25-ms pulses also induces *Fos*, and *c-Jun* (Worley et al., 1993). Varying the method of stimulation can also lead to differences in gene induction: potassium chloride and kainic acid stimulation induce different combinations of *Fos*, *Jun*, and *Egr1* in individual neurons (Sheng et al., 1995). Thus, pre-genomic studies in neurons revealed that the frequency, intensity, and duration of stimulation, as well as its temporal organization, determines which ARGs are transcribed and the magnitude of their transcription.

GENOMIC STUDIES OF STIMULATION-TRANSCRIPTION COUPLING IN NEURONS

In the last decade, genomic studies have begun to confirm the principles identified in early studies and have revealed more principles of stimulation-transcription coupling in neurons (Figure 1.2). First, neuronal excitation and inhibition regulate partially, but not entirely, reciprocal gene programs (Garay et al., 2019; Schaukowitz et al., 2017; Yu et al., 2015). Inhibition might be expected to induce an entirely reciprocal program to excitation, where all genes up-regulated (or down-regulated) by excitation are down-regulated (or up-regulated) by inhibition. Instead, the inhibition and excitation gene programs are only partially reciprocal: In cultured neurons, ~45% of the genes differentially regulated in response to inhibition via sodium channel blockade are reciprocally regulated in response to excitation via synaptic stimulation (Garay et al., 2019; Schaukowitz et al., 2017). These same reciprocally-regulated genes make up only 7-32% of the excitation-regulated gene program (because excitation regulates more genes than inhibition) (Garay et al., 2019; Schaukowitz et al., 2017; Yu et al., 2015). This leaves a substantial fraction of genes that are uniquely, rather than reciprocally, regulated by either inhibition or excitation. These uniquely-regulated genes may be important for the plasticities that occur specifically in response to inhibition (e.g., homeostatic synaptic strengthening) or excitation (e.g. homeostatic synaptic weakening) (Turrigiano, 2008). For example, a gene up-regulated by elevations in activity, *Homer1a*, is required for homeostatic synaptic weakening (Diering et al., 2017), whereas

a gene up-regulated by reductions in activity, *Nptx1*, is required for homeostatic synaptic strengthening (Schaukowitch et al., 2017). In contrast, reciprocal regulation of a single gene has yet to be functionally implicated in opposite homeostatic plasticities.

Genome-wide experiments have also revealed that neurons stimulated by excitatory neurotransmitters, neurotrophins, or neuromodulators transcribe overlapping gene programs. Excitatory neurotransmitters induce transcription primarily via membrane depolarization, whereas neuromodulators and neurotrophins instead mainly act through metabotropic or tyrosine kinase receptors (Flavell and Greenberg, 2008). Thus, these distinct classes of stimuli might be expected to regulate widely different gene programs. Instead, I calculate from separately-published gene lists from cultured cortical neurons that 43% (16/37) of genes induced in response to the neurotrophin BDNF (Korb et al., 2015) are also induced in response to membrane depolarization (our data included here). Similarly, 27% (30/111) of genes induced in cultured cortical neurons by synaptic glutamate stimulation are also induced by stimulation with forskolin, a proxy for neuromodulator stimulation (Benito et al., 2011). Thus, many different non-inhibitory stimuli likely regulate stimulus-specific gene modules drawn from a common set of ARGs.

Finally, different spatial patterns of stimulation are coupled to different gene expression programs. Pharmacological separation of synaptic and cell-body NMDA-receptor stimulation in cultured neurons revealed that stimulation of synaptic NMDA receptors induces transcription of far more genes than stimulation of cell-body receptors (Zhang et al., 2007). The genes uniquely induced by synaptic NMDA receptor stimulation are pro-survival genes, whereas those uniquely induced by somatic NMDA receptor stimulation are cell-death genes, revealing a potential mechanism for glutamate-driven excitotoxicity (Zhang et al., 2007). Furthermore, synaptic depolarization from excitatory post-synaptic potentials (EPSPs) and somatic depolarization from

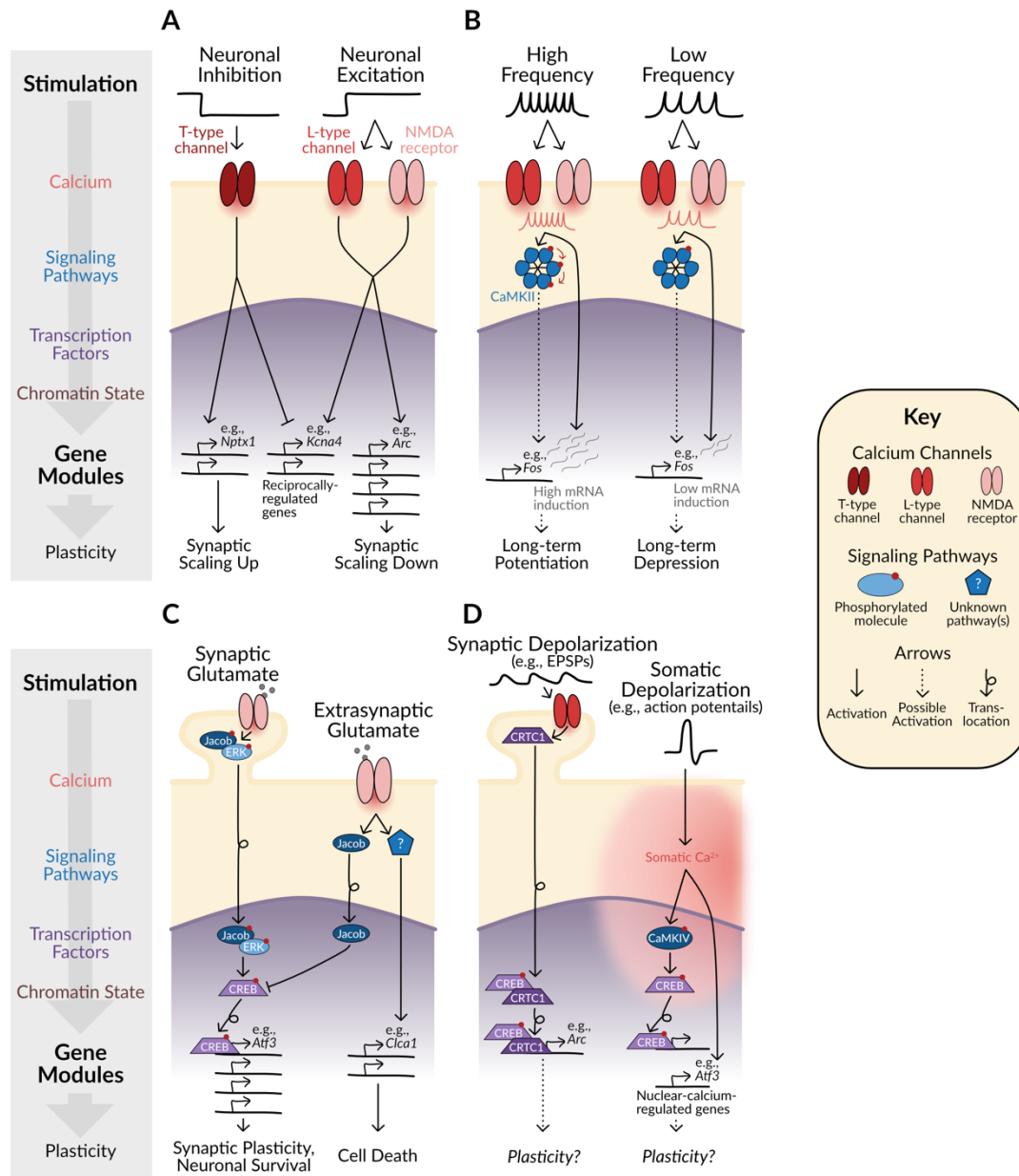


FIGURE 1.2. PRINCIPLES OF STIMULATION-TRANSCRIPTION AND ITS TRANSCRIPTIONAL REGULATION.

A. Neuronal excitation and inhibition regulate different, partly reciprocal gene programs, with excitation regulating more genes than inhibition. **B.** Neurons stimulated at higher frequencies transcribe ARGs at a greater magnitude. **C.** Stimulation of synaptic NMDA receptors induces transcription of synaptic plasticity and pro-survival genes, whereas stimulation of extrasynaptic NMDA receptors induces transcription of cell-death genes. **D.** EPSPs are better than action potentials at activating L-type calcium channels.

action potentials in the soma differentially regulate the binding partners and binding sites of the activity-regulated transcription factor, NPAS4 (Brigidi et al., 2019). This differential binding suggests, but does not confirm, that EPSPs and action potentials also regulate different ARG programs.

MECHANISMS OF STIMULATION-TRANSCRIPTION COUPLING

The neuron likely implements stimulation-transcription coupling by transforming each stimulus into a transcriptional output through multiple layers of regulation, including calcium channels, cell-signaling pathways, and transcription factors. While there are no examples that directly link stimulation, regulatory mechanisms, and transcription, there is evidence that each layer of transcriptional regulation is involved in coupling.

First, the stimulus determines the location of elevated calcium within the neuron (Hagenston and Bading, 2011; Hardingham et al., 2001a; Liu et al., 2003; Mermelstein et al., 2000; Watanabe et al., 2006) and its temporal pattern of influx (Eshete and Fields, 2001; Fujii et al., 2013), which can influence transcription (Schaukowitch et al., 2017; Zhang et al., 2009). Activating stimuli, such as membrane depolarization or synaptic glutamate, drive gene induction via calcium influx through L-type calcium channels or NMDA receptors (Flavell and Greenberg, 2008). The temporal pattern of this calcium influx reflects the temporal pattern of spiking or glutamate stimulation (Eshete and Fields, 2001; Fujii et al., 2013). Similarly, the spatial pattern of calcium influx also is dependent on the spatial pattern of membrane depolarization, as L-type calcium channels are preferentially activated by synaptic EPSPs rather than action potentials (Liu et al., 2003; Mermelstein et al., 2000). Unlike activating stimuli, neuronal silencing through sodium channel blockade appears to drive gene induction through T-type calcium channels rather than L-type calcium channels or NMDA receptors (Schaukowitch et al., 2017). Finally, stimulation of metabotropic receptors results in release of calcium from intracellular stores

rather than an influx of extracellular calcium (Hagenston and Bading, 2011). Compared to extracellular calcium influx through channels, calcium from intracellular stores spreads further throughout the neuron (Hagenston and Bading, 2011; Watanabe et al., 2006). The extent of calcium spread is important for gene induction, as some genes and transcription factors, like CREB, require elevated nuclear calcium for their induction (Hardingham et al., 1997; Zhang et al., 2009). Thus, in the first step of stimulation-transcription coupling, stimuli are translated into a temporal and spatial pattern of calcium within the neuron.

Next, temporal and spatial patterns of calcium determine signaling pathway activation. First, the temporal pattern of stimulation, and the resulting calcium influx, influences the extent of phosphorylation of CaMKII and AKT, as well as activation of calcineurin (Eshete and Fields, 2001; Fujii et al., 2013; Zhu et al., 2015). Rather than influencing the extent of pathway activation, the source and cellular location of calcium affects which signaling pathways are activated. Calcium influx through NMDA receptors and L-type channels activates signaling pathways that reside near the channel, such as MAPK/ERK and CaMKII (Deisseroth et al., 1996; Dolmetsch et al., 2001; Hardingham et al., 2001b, 1999; Wheeler et al., 2012), whereas calcium released from intracellular stores activates not only MAPK/ERK but also PKC (Hagenston and Bading, 2011; Roberson et al., 1999). Furthermore, calcium influx into as few as three dendritic spines activates nuclear transport of synapse-residing signaling molecules and transcription factors, including ERK, Jacob, CRTCL, CREB2, and NF κ B (Ch'ng et al., 2012; Karpova et al., 2013; Lai et al., 2008; Meffert et al., 2003; Zhai et al., 2013), whereas other signaling molecules, such as CaMKIV, reside in the nucleus (Hardingham et al., 1999) and therefore are less likely to be activated by synaptic signals. Thus, spatial and temporal calcium patterns throughout the cell determine which signaling molecules reach the nucleus and their degree of activation upon arrival.

Once in the nucleus, different signaling pathways activate different transcription factors (Flavell and Greenberg, 2008), and different transcription factors regulate different subsets of ARGs (Kuzniewska et al., 2016; Lösing et al., 2017; Malik et al., 2014; Taniguchi et al., 2017). For example, MAPK/ERK signaling activates SRF (Flavell and Greenberg, 2008), which binds to a subset of activity-regulated genes (Kim et al., 2010), whereas MEF2 is activated by calcineurin and binds to the promoters and enhancers of a subset of genes that overlaps, but is not entirely equivalent to those bound by SRF (Flavell and Greenberg, 2008). Thus, some genes are regulated both SRF and MEF2. Such combinatorial regulation, where each gene is regulated by multiple transcription factors, allows genes to be transcribed in response to multiple types of stimuli. Indeed, the ARG *Fos* is regulated by five different enhancers that each bind different transcription factors and respond to different types of stimulation, including membrane depolarization, BDNF, and forskolin (Joo et al., 2015). Globally, different activity-regulated enhancers bind different combinations of transcription factors (Kim et al., 2010; Telese et al., 2015), suggesting that perhaps enhancers establish stimulation-transcription coupling by specifying which stimuli regulate each ARG.

STIMULATION-TRANSCRIPTION COUPLING IN NON-NEURONAL CELLS

Different stimuli are also coupled to different transcription outcomes in non-neuronal cells. Immune and muscle cells induce different genes in response to different frequencies of stimulation. In T-lymphocytes, *IL-8* is induced in response to lower-frequency calcium influx than *IL-2* (Dolmetsch et al., 1998). Muscle cells induce a fast-twitch gene program in response to high-frequency activity and a slow-twitch gene program in response to low-frequency activity, allowing them to alter their activity and function based on their stimulation (Pette and Dusterhöft, 1992; Rana et al., 2009). Non-neuronal cell types are also sensitive to the duration of stimulation. Cancer cell lines transcribe a slowly-induced gene *RANTES* in response to sustained

TNF-alpha stimulation, but not in response to a 5-minute pulse of TNF-alpha, which only induces faster genes such as *MCP-1* (Ashall et al., 2009). Furthermore, pancreatic beta cells have a different transcriptional response to a one and a four-hour stimulation (Glauser and Schlegel, 2006), and macrophages induce more *Il6* when exposed to longer-duration TLR4 stimulation (Litvak et al., 2009), similar to the effect of stimulus duration on *Fos* induction in neurons. Primary mouse dendritic cells also respond differently to different types of stimulation: bacterial peptides induce different genes, and a different immune response, from viral peptides (Amit et al., 2009). As the transcriptional regulation of inducible genes is similar across cell types (Fowler et al., 2011), it is possible that some of the principles of stimulation-transcription coupling are also shared between neurons and non-neuronal cells.

Unlike in neurons, in non-neuronal cells there are several examples of causal mechanisms that establish stimulation-transcription coupling. These mechanistic links fall into the same layers as in neurons: calcium, signaling pathways, transcription factors, and chromatin modifiers. In lymphocytes, *IL-8* can be induced by slow oscillations of calcium because it is regulated by NF κ B: NF κ B is induced by slow calcium oscillations, unlike the transcription factors NFAT and Oct/OAP (Dolmetsch et al., 1998). Temporal-pattern-dependent transcription factor activation also regulates the expression of slow-twitch muscle genes: NFATc1 translocates to the nucleus and binds to slow-twitch gene promoters in response to low frequency, but not high frequency, stimulation of muscle cells (Rana et al., 2005). NFATc1 knockdown specifically affects the slow-twitch gene program. Next, in macrophages, extended, but not brief, TLR4 stimulation activates the transcription factor CEBP δ , which regulates a subset of the inducible gene program, including the gene *Il6* (Litvak et al., 2009). Finally, in dendritic cells, manipulation of a variety of transcriptional regulators revealed coupling mechanisms that differentiate between bacterial and viral stimulation (Amit et al., 2009). For example, bacterial, but not viral, stimulation induces the chromatin modifier Cbx4, which inhibits the induction of the viral response gene *Ifnb1*. This

regulation specifies induction of this viral response gene to only viral stimulation. Thus, when studying the transcriptional regulation underlying stimulation-transcription coupling in neurons, we may be able to learn from experiments performed in other cell types.

GOALS FOR THIS DISSERTATION

In the project described in this dissertation, I aim to (1) determine whether transcription is coupled to the duration of stimulation and (2) identify transcriptional regulators that establish this coupling. I have chosen to focus on the duration of stimulation because it is simple to control precisely in our neuronal cell culture system and because brief and sustained neuronal activity induce different transcription-dependent neuronal plasticities (Ibata et al., 2008; Nguyen et al., 1994). I use RNA-sequencing to identify a new principle of the coupling map: brief neuronal activity specifically induces rapidly-induced genes. Furthermore, I find that the MAPK/ERK pathway is specifically required for induction of the rapidly-induced genes, but not of other ARGs. This is, as far as I know, the first example of a direct link between stimulation, a transcriptional regulator, and transcriptional output in neurons. This kind of mechanistic insight into stimulation-transcription coupling can be leveraged to test the role of stimulation-specific gene programs in plasticity.

CHAPTER II.

DATA: DIFFERENT NEURONAL ACTIVITY PATTERNS INDUCE DIFFERENT GENE EXPRESSION PROGRAMS

This chapter was published as Tyssowski KM, DeStefino NR, Cho JH, Dunn CJ, Poston RG, Carty CE, Jones RD, Chang SM, Romeo P, Wurzelmann MK, Ward JM, Andermann ML, Saha, RN, Dudek SM, Gray, JM.: Different Neuronal Activity Patterns Induce Different Gene Expression Programs. Neuron 2018, 98:530–546.

Author contributions: *I designed the study and wrote the manuscript with JMG with input from other authors. I did all experiments and analysis except: rat neuron experiments (CJD, RGP, PR, MKW, JMW, RNS, SMD), photometry (CEC, MLA); Pol2 ChIP-seq (NRD); luciferase assays (RDJ); 11e inhibitor experiments (SMC); Elk-1 western blotting (NRD). I worked with J-HC on design, execution, and analysis of in vivo visual cortex experiments. I made all figures with input from other authors.*

ABSTRACT

A vast number of different neuronal activity patterns could each induce a different set of activity-regulated genes. Mapping this coupling between activity pattern and gene induction would allow inference of a neuron's activity-pattern history from its gene expression and improve our understanding of activity-pattern-dependent synaptic plasticity. In genome-scale experiments comparing brief and sustained activity patterns, we reveal that activity-duration history can be inferred from gene expression profiles. Brief activity selectively induces a small subset of the activity-regulated gene program that corresponds to the first of three temporal waves of genes induced by sustained activity. Induction of these first-wave genes is mechanistically distinct from that of the later waves because it requires MAPK/ERK signaling but does not require de novo translation. Thus, the same mechanisms that establish the multi-wave temporal structure of gene induction also enable different gene sets to be induced by different activity durations.

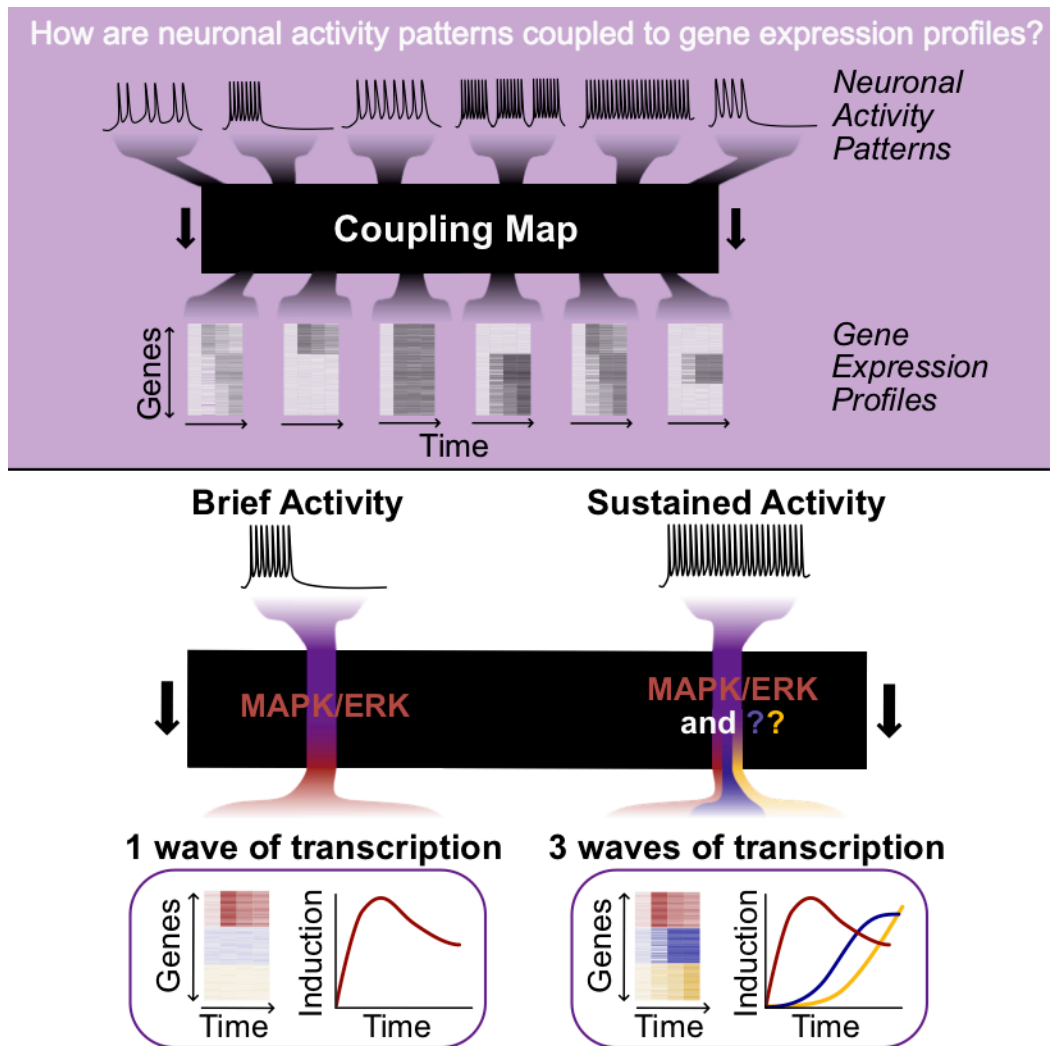


FIGURE 2.0. GRAPHICAL ABSTRACT

INTRODUCTION

Neurons induce hundreds of activity-regulated genes (ARGs) in response to elevations in their activity (Flavell and Greenberg, 2008), suggesting that a vast number of different neuronal firing patterns could each be coupled to a different gene expression profile. Consistent with this idea, distinct neuronal activity patterns differentially induce the expression of several individual genes (Douglas et al., 1988; Greenberg et al., 1986; Sheng et al., 1993; Worley et al., 1993). However, single-gene studies are inadequate for creating a complete coupling map that relates

each neuronal activity pattern to a corresponding gene expression profile. This coupling map would be powerful because it would allow inference of a neuron's activity history from its gene expression profile. This kind of inference could enable single-cell-RNA-sequencing (scRNA-seq)-based assessment of the activity histories of thousands of neurons at a time, far more than can be assessed with electrical recording or calcium imaging (Hrvatin et al., 2017; Hu et al., 2017a; Jun et al., 2017; Mohammed et al., 2016; Wu et al., 2017). To generate a coupling map, it will be necessary to make genome-scale comparisons of the ARGs induced by different activity patterns (Lee et al., 2017).

Transcriptional regulators could establish the coupling map, as they can both define specific ARG subsets and respond differentially to different activity patterns. Regulators that define ARG subsets include transcription factors, such as CREB and SRF, that bind the promoters and enhancers of only some ARGs (Kim et al., 2010). Regulators that respond differentially to different activity patterns include calcium-dependent cell-signaling pathways, such as the MAPK/ERK pathway (De Koninck and Schulman, 1998; Dolmetsch et al., 1998, 1997; Dudek and Fields, 2001; Eshete and Fields, 2001; Fields et al., 1997; Fujii et al., 2013; Ma et al., 2011; Wu et al., 2001). Thus, each of the many inducible signaling pathways could regulate a distinct subset of ARGs, creating gene modules that are each independently coupled to activity patterns. Identifying the regulators of these gene modules would enable manipulation of the coupling map to investigate its contribution to firing-pattern-specific, gene-induction-dependent synaptic plasticity, such as long-term potentiation, long-term depression, and synaptic scaling (Ahn et al., 1999; Ibata et al., 2008; Nguyen et al., 1994).

One example of a regulatory mechanism that could couple stimulation patterns to induction of different gene modules comes from non-neuronal cells, where it has been proposed that brief and sustained stimulation differentially induce two of the best-defined gene modules in inducible systems: primary and secondary response genes (PRGs and SRGs) (Fowler et al.,

2011). These gene modules are defined by their requirement for de novo translation. PRGs can be induced rapidly and do not require de novo translation for their induction, whereas SRGs are induced slowly, require de novo translation for their induction, and are regulated by PRG protein products (Fowler et al., 2011; Herschman, 1991). Brief stimulation is sufficient to induce PRGs, but sustained cell-signaling pathway activation, which is induced by sustained stimulation, is required to stabilize PRG protein products and induce SRGs (Fowler et al., 2011). In neurons, brief activity could similarly induce only PRGs while sustained activity could be required to induce SRGs. Therefore, defining PRGs and SRGs in neurons and determining their responsiveness to different activity durations could reveal a basic principle underlying the coupling map between activity patterns and gene expression.

In a step toward generating this coupling map, we performed genome-scale comparisons of gene induction in response to neuronal activity patterns of varying duration. We found that different durations of activity induce different sets of genes, allowing us to infer neuronal activity duration from gene expression data. We further reveal that the coupling between activity duration and gene expression is determined in part by MAPK/ERK signaling, enabling future manipulation of the coupling map.

RESULTS

Rapid but not delayed PRGs are induced by brief activity

We investigated the possibility that different patterns of neuronal activity induce different subsets of ARGs by varying just one aspect of neuronal activity: its duration. We activated neurons briefly (10s-5min.) or continuously (for up to 6h) using three methods of stimulation that allowed us to precisely control the duration of neuronal firing or calcium influx (Figure 2.1A). We primarily stimulated mouse cultured cortical neurons with KCl-mediated

membrane depolarization and assessed the resulting gene induction using either total RNA-seq, which allowed to assess both mRNA and pre-mRNA transcription (Gaidatzis et al., 2015; Gray et al., 2014), or targeted sequencing of 251 ARG mRNAs (ARG-seq), which allowed us to reduce the number of reads needed per experiment (Supplementary Table A2.1, Supplementary Figure A2.1A, see methods in Appendix I).

We first used ARG-seq to characterize the gene induction in response to sustained activity. We found that sustained activity induces 173 ARGs, 114 of which also show significant induction in at least one of three *in vivo* studies (Cho et al., 2016; Lacar et al., 2016; Spiegel et al., 2014) (significant overlap, $p=0.0002$, Fisher's exact test). We observed that these 173 ARGs are induced in two waves, as expected (Flavell and Greenberg, 2008): a rapid wave that includes 19 genes and a delayed wave that includes 154 genes (Figure 2.1B-C, Supplementary Figure A2.1C-E, see methods for details of classification). We hypothesized that the first wave corresponds to the de-novo-translation-independent PRGs and the second to SRGs, which require PRG protein products for their induction. Indeed, after defining PRGs and SRGs based on their requirement for de-novo-translation, we found that the first wave of gene induction is entirely comprised of PRGs (Figure 2.1B, Supplementary Figure A2.1B). However, the second wave includes both PRGs and SRGs, similar to findings in human cancer cell lines and macrophages (Ramirez-Carrozzi et al., 2006; Tullai et al., 2007). Thus, neurons also induce two kinetically distinct classes of PRGs: rapid PRGs (rPRGs) and delayed PRGs (dPRGs). A finer-grained time course using high-throughput qPCR revealed that dPRGs are actually induced earlier than SRGs, suggesting that rPRGs, dPRGs, and SRGs represent three temporally distinct waves of transcription (Figure 2.1C, Supplementary Figure A2.1G, Supplementary Table A2.3).

We next measured gene induction in response to brief (KCl-mediated) activity using ARG-seq. Remarkably, rPRGs comprise 14 of the 15 genes significantly induced by brief activity

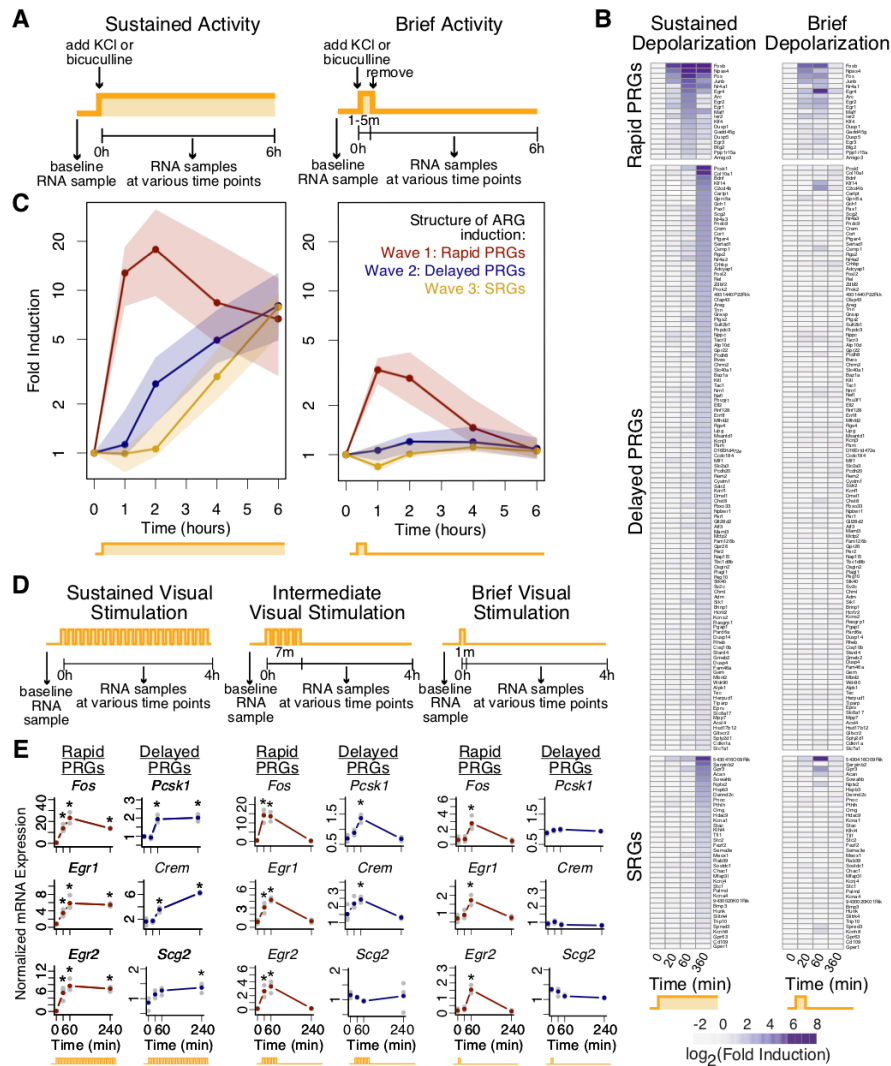


FIGURE 2.1. BRIEF NEURONAL ACTIVATION SELECTIVELY INDUCES THE FIRST OF THREE WAVES OF GENE INDUCTION

A. Experimental system for comparing sustained and brief neuronal activation in vitro. Except where indicated otherwise, neuronal activation is accomplished with brief (1-min) or sustained KCl depolarization of cortical neurons silenced 14–16 hr before stimulation with APV and NBQX. **B.** Comparison of gene induction upon sustained or brief neuronal activation using activity-regulated gene-capture-based RNA sequencing (ARG-seq) (means, $n = 3$ –6 biological replicates). Only induced genes are shown. Gene categories are defined based on kinetics of gene induction as well as induction in the presence of the translation inhibitor cycloheximide (Supplementary Figure A2.1B). Genes induced by brief neuronal activation are enriched for rPRGs ($p < 10^{-13}$, Fisher's exact test). PRG, primary response gene; SRG, secondary response gene; rPRGs, rapid PRGs. **C.** Three kinetically distinct temporal waves of gene induction as detected by high-throughput microfluidic qPCR. Points represent the mean expression of the median gene for each class. Shading covers the middle quartiles of mean expressions (25%–75%) ($n = 6$ biological replicates). *continued overleaf*

Figure 2.1 (Continued) Each wave is kinetically distinct from the other waves (rPRG versus dPRG/SRG induction at 1 hr, dPRG versus SRG induction at 2 hr, $p < 0.003$, rank-sum test). Plotted are 15, 37, and 9 genes from waves 1–3, respectively. dPRG, delayed PRG. **D.** Experimental system for comparing the duration of neuronal activation in the visual cortex in vivo. Mice were dark housed for 3 days prior to visual stimulation consisting of lights flashing in a repeated pattern: 60 s on, 20 s off. (E) Gene induction in the visual cortex following visual stimulation as measured by qPCR. Colored points are means of $n = 3$ biological replicates. Gray points are values from individual biological replicates. Gene categories defined as in (B). *significant induction compared to 0 hr time point, $p < 0.05$ unpaired, two-sided t test, fold induction > 1.5 .

(FDR <0.05 , mean fold change >1.5) (Figure 2.1B-C, Supplementary Figure A2.1E,G). Pre-mRNA expression assessed in total RNA-seq data recapitulated these mature mRNA findings (Supplementary Figure A2.1C), suggesting that the differential responsiveness to brief activity between rPRGs and dPRGs is due to transcriptional rather than post-transcriptional mechanisms. The selective induction of rPRGs but not dPRGs by brief activity is not specific to KCl-mediated depolarization, as it also occurs following brief (5-min) bicuculline-induced activity in rat primary cortical neurons, as detected by NanoString (Supplementary Figure A2.1F, Supplementary Table A2.4). rPRGs are also induced by just ten seconds of bicuculline-induced synaptic activity (Supplementary Figure A2.1H), equivalent to a single burst of firing (Yu et al., 2017). These findings indicate that de-novo-translation-independence is not the only requirement for induction in response to brief activity. Instead, rPRGs in neurons may be distinguished from dPRGs by transcriptional mechanisms that allow them to respond both rapidly and to brief activity.

To confirm that rPRGs but not dPRGs are induced in response to brief activity in vivo, we assessed gene induction in the visual cortex in response to a visual stimulus consisting of bright, flashing lights (Figure 2.1D). Using photometry-based *in vivo* recordings of calcium activity, we first confirmed that neuronal activity in primary visual cortex increases with the onset of each flash of light, even for repeated flashes presented for several hours (Supplementary Figure

A2.2A-B). We assessed mRNA induction using qPCR with primers for four rPRGs and eight dPRGs, as classified using our in vitro data. The rPRGs are all induced rapidly and in response to one minute of visual stimulation, consistent with in vitro findings (Figure 2.1E, Supplementary Figure A2.2C). Most of the dPRGs (7/8) have delayed induction kinetics and no induction in response to one minute of stimulation, again consistent with our in vitro results. The exception, *Nr4a3*, is induced rapidly and by brief activity, thus behaving as a rPRG in vitro but a dPRG in vivo. The concordance between our in vitro and in vivo results suggests that activity duration is coupled to gene expression similarly in primary cortical neurons and in the cortex.

Our finding that dPRGs are induced in response to sustained but not brief activity suggests that there is a minimum activity duration required to induce dPRGs. To determine whether this minimum is the same for every dPRG, we assessed PRG expression in response to an intermediate duration of visual stimulation. This intermediate (7-min) stimulus is sufficient to induce only a subset (five) of the seven dPRGs (Figure 2.1E, Supplementary Figure A2.2C), indicating that different dPRGs have different minimum-activity-duration thresholds. The observation that there are three distinct ARG induction profiles for one-minute, seven-minute, and sustained activity suggests that ARG induction has a graded response to the duration of activity and hints at the potential complexity of the coupling between activity pattern and ARG induction.

We next investigated whether the genes in each of the three waves of ARG induction differ in their known or annotated gene function (Supplementary Table A2.5). Most (17/19) rPRGs that we identified in mouse cortical neurons are directly or indirectly involved in regulating transcription. rPRGs are also more likely than dPRGs or SRGs to be stimulus-induced in macrophages ($p = 0.0004$, Fisher's exact test) (Escoubet-Lozach et al., 2011) and human cancer cell lines ($p=0.0001$, Fisher's exact test) (Tullai et al., 2007), consistent with the idea that transcription factors are re-used in many cell types. Therefore, most (112/114) of the effector

(i.e., non-transcription-regulating) ARGs, which are thought to orchestrate transcription-dependent neuronal plasticity, are dPRGs or SRGs. A major exception is the rPRG effector gene *Arc* (Shepherd and Bear, 2011). We found that brief activity induces ARC protein in a de-novo-transcription-dependent manner (Supplementary Figure A2.1I), consistent with the idea that ARC could mediate the synaptic changes driven by brief activity. These results suggest that any transcription-dependent synaptic changes caused by brief activity are driven by the protein products of only a few genes, including *Arc*.

Neuronal activity history is encoded in gene expression profiles

Given that brief and sustained activity induce different gene sets, we asked whether we could infer neurons' past activity duration from their ARG expression profiles. Indeed, a nearest-neighbor classifier correctly identified in vitro samples as having been stimulated with brief or sustained KCl-mediated depolarization, using normalized expression values from all significantly induced genes or all captured genes but not constitutively active control genes (Figure 2.2A). For such classification to be broadly useful, it should be robust to the method of stimulation. We therefore aimed to classify our in vivo visual stimulation samples using our in vitro KCl-mediated depolarization data as a training set. A classifier using 11 ARGs that have similar expression profiles between in vitro and in vivo experiments was able to correctly classify 100% of visual cortex samples as having undergone either brief, sustained, or no stimulation (Figure 2.2B). Thus, the duration of past neuronal activity is indeed encoded in the ARG expression profile, and this information can be used to infer in vivo activity histories.

We therefore considered the possibility of using scRNA-seq data to infer the activity histories of thousands of individual neurons in a single experiment (Hrvatin et al., 2017; Hu et al., 2017a; Wu et al., 2017). We asked whether we could use scRNA-seq-based detection of ARG expression to identify a population of visual cortex neurons that are activated only briefly in

response to sustained visual stimulation. We analyzed published data collected one hour after the onset of visual stimulation (Hrvatin et al., 2017). We found that both rPRGs and dPRGs are robustly induced by one hour when compared to control mice left in the dark (Supplementary Figure A2.3A). We classified neurons that induced rPRGs but not dPRGs as having been putatively briefly active (“BRIEF neurons”), whereas those that induced dPRGs were predicted to have had a history of sustained activity (“SUSTAINED neurons”) (Figure 2.2C, Supplementary Figure A2.3B). We found that the majority (52%) of neurons were putative SUSTAINED neurons. However, we found a small (13%), but significant, population of putative BRIEF neurons (Figure 2.2D). The remaining 35% of neurons showed no PRG induction and were therefore classified as putatively inactive. We therefore predict that a subset of neurons in the mouse visual cortex undergoes brief activity in response to sustained visual stimulus.

To determine the identity of these BRIEF neurons, we performed differential gene expression analysis comparing BRIEF and SUSTAINED neurons. We found that the genes expressed significantly more in BRIEF neurons include deep layer (5 and 6) markers such as *Tmem91*, *Gabra5*, *Rprm*, and *Crym*. In contrast, genes with greater expression in SUSTAINED neurons included upper layer (2/3 and 4) markers, such as *Calb1*, *Cux1*, and *Rasgrf2* (fold change > 2, FDR < 0.1, Figure 2.2E) (layer markers from Hrvatin et al., 2017; Tasic et al., 2016). Impressively, almost all of the genes differentially expressed between BRIEF and SUSTAINED neurons show similar layer-specific trends in expression, suggesting that the major genetic differences between BRIEF and SUSTAINED neurons arise from their layer positions (Figure 2.2F). We therefore directly asked whether deep layers of the cortex have a greater enrichment for BRIEF neurons than upper layers, using gene-expression-based layer definitions (Hrvatin et al., 2017). We indeed found that deep layers of the cortex have more BRIEF neurons than upper layers, with only deep layers having a statistically significant population of BRIEF neurons (Figure

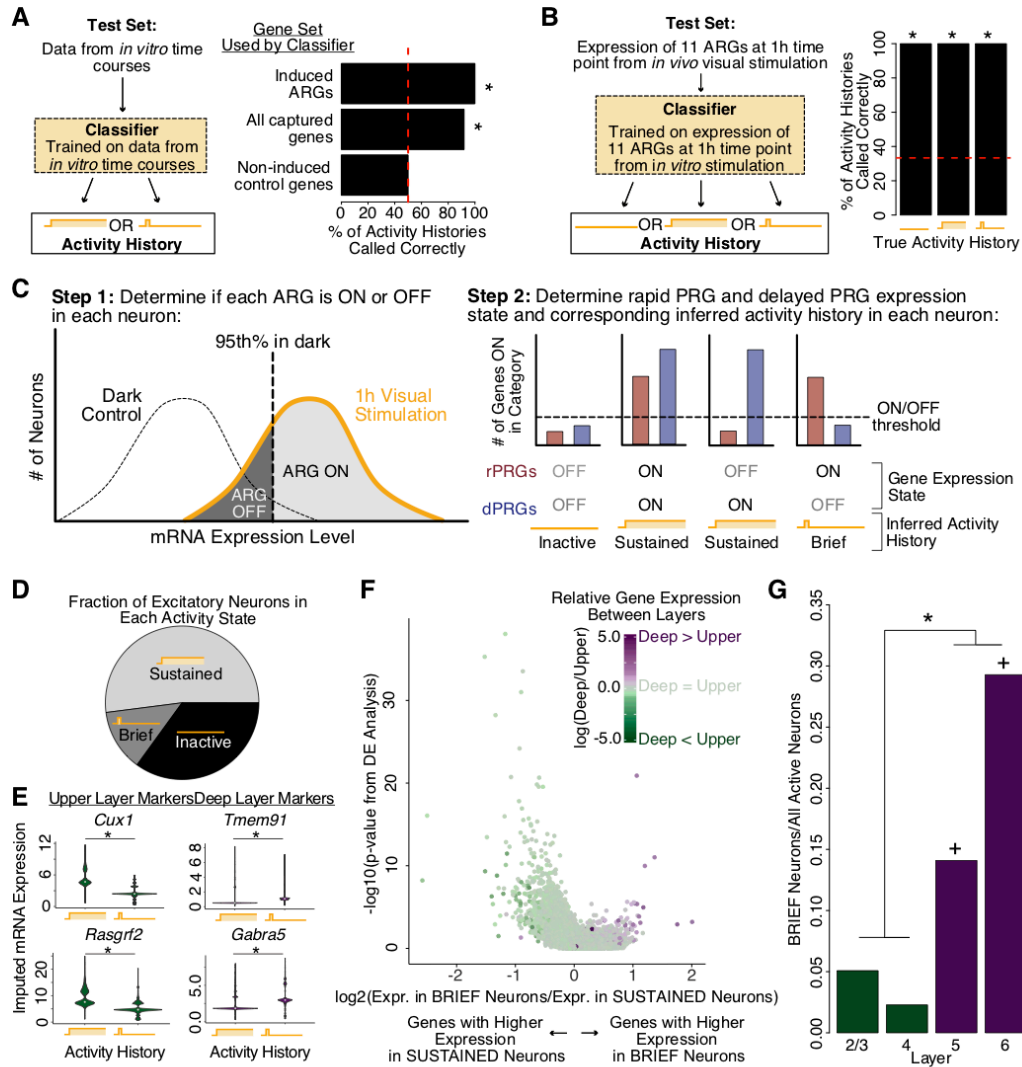


FIGURE 2.2. NEURONAL ACTIVITY PATTERNS CAN BE INFERRED FROM ARG EXPRESSION

A. A classifier trained on *in vitro* gene expression data to infer activity histories of 12 *in vitro* samples (6 brief, 6 sustained). The classifier identified test samples as having undergone either brief or sustained activity based on based on Euclidean distance to training samples. * $p = 0.007$, exact binomial test. **B.** A similar (*in vitro* trained) classifier used to infer the activity histories of 12 *in vivo* visual cortex samples (3 brief, 3 sustained, and 6 unstimulated). * $p < 0.04$, exact binomial test. **C.** Method for scRNA-seq-based inference of BRIEF and SUSTAINED activity histories of individual visual cortex excitatory neurons from mice exposed to 1 hr of sustained visual stimulation. scRNA-seq data from Hrvatin et al. (2018). **D.** 1 hr of visual stimulation significantly increased the fraction of excitatory neurons with BRIEF and SUSTAINED inferred activity states ($p < 10^{-15}$, Fisher's exact test). **E.** Expression of four layer markers in BRIEF and SUSTAINED neurons in scRNA-seq data. Data plotted are imputed mRNA reads after using DECENT (Ye et al., 2017) to account for the presence of technical zeroes. *FDR < 0.1, rank-sum test. **F.** Differential expression (DE) of all genes (excluding ARGs) Color of the points represent the log of the ratio of gene expression in deep layers (layers 5 and 6) to that in upper layers (layers 2/3 and 4). **G.** Fraction of

Figure 2.2 (Continued) stimulated neurons in each layer that are BRIEF. *More BRIEF neurons in deep versus upper layers, $p < 10^{-15}$, Fisher's exact test. *Significant population of brief neurons, $p < 0.001$ based on a Fisher's exact test comparing the number of rPRG-ON neurons among dPRG-OFF neurons in the stimulated cortex to the number of rPRG-ON neurons in unstimulated cortex.

2.2G, Supplementary Figure A2.3C). We were concerned about being biased toward detecting BRIEF neurons in deep layers if upper layer neurons induce more of the dPRGs on our in-vitro-defined list than deep layers, which is possible given that different layers of cortex induce different dPRGs (Hrvatin et al., 2017). To control for this alternative possibility, we used the scRNA-seq data to define dPRGs for each layer individually and confirmed that we still found an enrichment of BRIEF neurons in deep layers using the layer-specific dPRGs (Supplementary Figure A2.3D). This analysis therefore predicts that upon sustained visual stimulation, a population of neurons in layers 5 and 6 of the primary visual cortex exhibits only brief elevations in activity.

Rapid PRG promoters are distinguished by open, active chromatin and the presence of pre-bound transcription regulators

We next investigated what might enable rPRGs both to be induced rapidly and by brief activity. The faster mRNA induction of rPRGs could be facilitated in part by their shorter gene length compared to dPRGs and SRGs (median ~13 kb shorter, Supplementary Figure A2.4A). However, we found that rPRG first exons are induced before those of dPRGs or SRGs (Supplementary Figure A2.4B), indicating that rPRG promoters are also activated more rapidly. We hypothesized rPRG promoters might be primed for faster promoter activation due to an open chromatin state prior to stimulation. To assess this hypothesis, we evaluated three marks of open chromatin: high DNaseI hypersensitivity (data from ENCODE Project Consortium et al., 2012), high CpG (and GC) content, and high levels of active chromatin marks, including H4K16ac,

H3K4me2, and H3K27ac (data from Kim et al., 2010; Telese et al., 2015). We found that by all three of these criteria, unstimulated rPRG promoters have more open chromatin than unstimulated dPRG or SRG promoters (Figure 2.3A, Supplementary Figure A2.4C-D). Importantly, the histone acetylation signals extend across a wider promoter-proximal region and are more bimodal at rPRG promoters, indicative of reduced nucleosome occupancy at or near transcription start sites prior to stimulation (Figure 2.3A, Supplementary Figure A2.4C-D). These differences in average DNase hypersensitivity and histone marks could be due to the greater number of neuronal and non-neuronal brain cell types that induce rPRGs compared to dPRGs and SRGs (Hrvatin et al., 2017) rather than to differences in chromatin accessibility in the neurons that actually induce each class. However, the observation that that rPRGs have more open chromatin than dPRGs and SRGs in homogenous non-neuronal cell populations (Hargreaves et al., 2009; Ramirez-Carrozzi et al., 2009, 2006) leads us to favor the idea that rPRG promoters in neurons are also distinguished by a relatively open chromatin state, potentially poising them for rapid activation in response to brief activity.

The open chromatin state at rPRG promoters in unstimulated neurons prompted us to ask whether these promoters might be selectively pre-bound to transcriptional regulators prior to neuronal activation. We found that RNA Polymerase 2 (Pol2) occupancy in unstimulated neurons is higher at the promoters of rPRGs and constitutively active genes compared to dPRGs and SRGs (Supplementary Figures A2.4F, A2.7E) data, despite the finding that rPRGs, dPRGs and SRGs have similar levels of transcription in unstimulated neurons (Supplementary Figure A2.4G). Furthermore, we found greater binding of the neuronal activity-regulated transcription factors SRF and MEF2, as well as the Mediator subunits MED23 and MED1 (Figure 2.3B, Supplementary Figure A2.4E), at rPRG promoters compared to dPRG or SRG promoters in unstimulated neurons (data from Kim et al., 2010; Telese et al., 2015). In contrast, the transcription factor CREB is pre-bound to a similar extent to rPRG and dPRG promoters but is not pre-bound to SRG promoters

(Figure 2.3B, Supplementary Figure A2.4E). Interestingly, the NCoR repressor complex also binds preferentially to rPRG promoters compared to dPRG or SRG promoters (Supplementary Figure A2.4H) and could prevent them from being transcribed despite their open state. These data suggest that in addition to an open chromatin state, pre-binding of transcriptional activators may uniquely poise rPRGs for rapid induction in unstimulated neurons.

The MAPK/ERK pathway is required for the first wave of gene induction

We next asked whether rPRGs are targeted by a rapidly-activated signaling pathway that endows them with the ability to respond quickly and to brief activity. In evaluating this possibility, we compared rPRGs and dPRGs but excluded SRGs to eliminate the confounding possibility of altered PRG induction affecting SRG induction. We first hypothesized that the CaMKIV pathway might mediate rPRG induction due to its role in rapid phosphorylation of the transcription factor, CREB (Hardingham et al., 2001b; Wu et al., 2000). Using immunocytochemistry, we observed phospho-CaMKIV in the nucleus within just five minutes of membrane depolarization, indicating rapid pathway activation (Supplementary Figure A2.5A). However, when we blocked CaMKIV phosphorylation using an inhibitor for the upstream kinase, CaMKK (Supplementary Figure A2.5A), we found no effect on induction of rPRGs or dPRGs in response to either brief or sustained activity, despite a small effect on ARG expression in unstimulated neurons (Supplementary Figure A2.5B-C, Supplementary Table A2.3). Therefore, the rapid induction and sensitivity to brief activity of rPRGs is not explained by a dependence on CaMKIV signaling.

We next asked whether another canonical neuronal signaling pathway, the MAPK/ERK pathway (Thomas and Huganir, 2004), is activated rapidly and in response to brief activity, which would be consistent with selective regulation of rPRGs. We assessed MAPK/ERK pathway activation by western blotting for the pathway's terminal kinase, phospho-ERK (pERK). In

response to both brief and sustained activity, pERK levels reach the same peak magnitude by five minutes after the start of activity (Figure 2.3C-D,F), suggesting that the MAPK/ERK pathway is rapidly and fully activated by brief activity. Because pERK can activate transcription via phosphorylation of nuclear proteins (Thomas and Huganir, 2004), we confirmed that the MAPK/ERK target transcription factor, Elk-1, is phosphorylated rapidly and MAPK/ERK-dependently in response to sustained depolarization (Supplementary Figure A2.6A). In further support that the MAPK/ERK pathway signals rapidly to the nucleus, we detected increased pERK in the nucleus by two minutes following both brief and sustained neuronal activity (Figure 2.3E). Interestingly, upon brief stimulation, ERK activity remains elevated for at least ten minutes after the removal of stimulus, which is more than sufficient time for activation of rPRG transcription.

We therefore hypothesized that the MAPK/ERK pathway is required for rPRG induction. To test this hypothesis, we measured ARG induction using ARG-seq in the presence of MAPK/ERK pathway inhibition (Supplementary Figure A2.6B) using the potent and highly specific allosteric MEK inhibitor, U0126 (Favata et al., 1998). We found that MEK inhibition dramatically blunts induction of rPRGs but not dPRGs in response to sustained activity (Figure 2.3G-I). 95% of rPRGs but only 17% of dPRGs are sensitive to MEK inhibition (based on >40% decrease in maximum expression, Figure 2.3H). We also confirmed that MEK inhibition blocks induction of rPRG but not dPRG pre-mRNAs, suggesting that the MAPK/ERK pathways acts at the level of transcription (Supplementary Figure A2.6C). This blunting of gene induction is unlikely to be due to off-target effects of U0126, since the MEK inhibitor PD184352 and the ERK inhibitor 11e have similar effects (Supplementary Figure A2.6G-H). Most rPRGs are partially induced in the presence of MEK inhibition, but with delayed kinetics, indicating that MAPK/ERK activity is most important for the early stages of gene induction (Figure 2.6E-F).

We next asked whether MAPK/ERK signaling is also required for gene induction in response to brief activity. Impressively, MEK inhibition substantially decreases mRNA and pre-mRNA induction in response to brief activity (Figure 2.3J-L, Supplementary Figure A2.6D,I), blunting mRNA induction of all but one of the induced rPRGs. Again, we observed similar results using the ERK inhibitor 11e (Supplementary Figure A2.6G). Therefore, the MAPK/ERK pathway

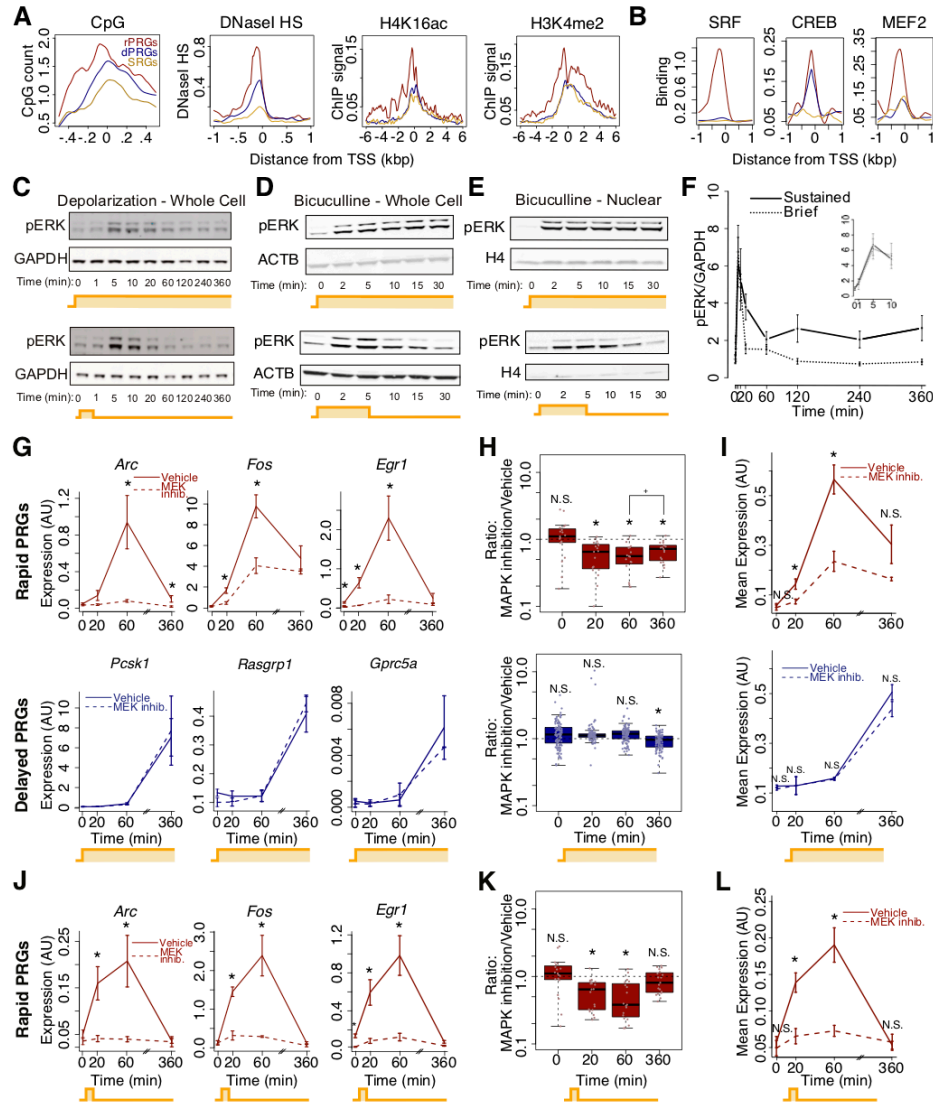


FIGURE 2.3. REQUIREMENT FOR MAPK/ERK SIGNALING AND AN OPEN CHROMATIN STATE TO DISTINGUISH FIRST AND SECOND WAVES OF GENE INDUCTION

A. Chromatin state in unstimulated neurons shown in metaplots of the geometric mean signal for all genes in each category. All measures of chromatin state are significantly different *continued overleaf*

Figure 2.3 (Continued) between rPRGs and dPRGs or SRGs ($p < 0.009$, rank-sum test on the area under the curves shown). ChIP-seq data are from cultured cortical neurons (Telese et al., 2015). DNaseI hypersensitivity data are from the 8w cerebrum (ENCODE Project Consortium, 2012). **B.** Transcription factor binding in unstimulated neurons from ChIP-seq shown in metaplots as in (A). SRF and MEF2: significantly different between rPRGs and dPRGs or SRGs; CREB: not significantly different between rPRGs and dPRGs ($p = 0.2$) but is different between rPRGs and SRGs ($p < 0.009$, rank-sum test). Data from cultured cortical neurons (Kim et al., 2010; Telese et al., 2015). **C.** ERK activation kinetics with KCl-mediated depolarization. Representative (1 of $n = 3$) western blot for phosphorylated ERK (pERK). Phosphorylation of ERK paralogs, p44 and p42 (upper and lower bands), is kinetically similar ($r^2 = 0.97$, Pearson correlation). **D.** Similar to (C), but rat cortical neurons were treated with sustained or brief bicuculline/4AP. One of $n = 3-4$ representative biological replicates is shown. **E.** Same as (D) but from isolated nuclei. **F.** Quantification of (C), $n = 3$ biological replicates. The inset is a magnified version of the first 10 min. pERK induction at its peak (5 min) is not different between brief and sustained stimulus ($p = 0.3$, paired, two-sided t test). Error bars represent $\pm$ SEM. **G.** rPRG, but not dPRG, induction in response to sustained activity is dependent on MAPK/ERK. ARG-seq-based gene expression of three representative rPRGs and three representative dPRGs following sustained KCl depolarization of mouse neurons with and without 10 mM of the MEK inhibitor U0126. $n = 3-7$ biological replicates. Error bars are $\pm$ SEM. * $p < 0.01$, rank-sum test. **H.** Data from the same experiment as (G) showing all ARGs. *significantly different from 1, $p < 0.01$, rank-sum test; + $p = 0.02$, rank-sum test. Expression of rPRGs is more affected by MEK inhibition than expression of dPRGs ($p = 0.002$; rank-sum test on 17 rPRGs versus 110 dPRGs using the mean for each gene across $n = 3-7$ biological replicates at its most induced time point). **I.** Data the same as in (H) but showing the geometric mean of gene expression. Error bars are $\pm$ SEM from each of $n = 3-7$ biological replicates of all genes in the category. * $p < 0.03$, rank-sum test. **J.** rPRG, but not dPRG, induction in response to brief activity is dependent on MAPK/ERK. Same as (G), top row, but with 1 min KCl depolarization. **K.** Same as (H), top row, but with 1 min KCl depolarization. **L.** Same as (I), top row, but with 1 min KCl depolarization.

is required for rapid ARG induction and induction in response to brief activity, thus establishing the first wave of ARG induction in vitro.

We next investigated whether the MAPK/ERK pathway is required for rapid gene induction in vivo. We exposed dark-housed mice to brief (1-min) or sustained (up to 2.5-h) visual stimulation, consisting of turning on the room lights, in the presence or absence of MEK inhibition (Supplementary Figure A2.6J). ARG-seq of the visual cortex revealed that MEK inhibition has a larger effect on rPRG compared to dPRG expression in cortices from mice exposed to sustained visual stimulation (Figure 2.4A-B), and it blocks nearly all ARG induction in mice exposed to brief visual stimulation (Figure 2.4C-D). Most of the ARG induction we

observed appears to be due to the visual stimulation itself rather than stress from the lights or handling, as we did not observe induction of the rPRG, *Fos*, in the prefrontal cortex of mice exposed to visual stimulus (Supplementary Figure A2.6K). We also confirmed that for the room-light visual stimulation used for this experiment, brief stimulation induces rPRGs better than dPRGs and SRGs ($p=0.03$, Fisher's exact test, Supplementary Figure A2.6L). We therefore conclude that both in vivo and in vitro, the MAPK/ERK pathway is a fast pathway necessary rapid ARG induction and induction in response to brief activity.

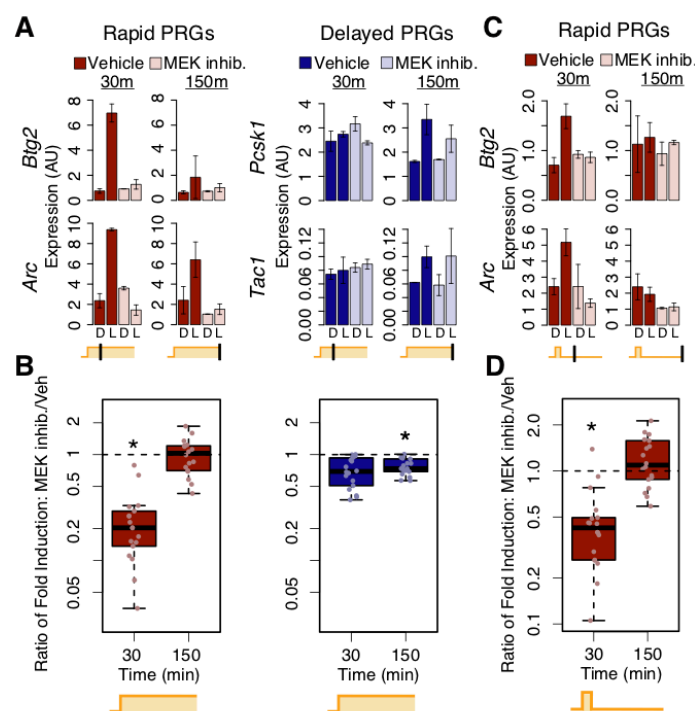


FIGURE 2.4. MAPK/ERK IS REQUIRED FOR THE FIRST WAVE, BUT NOT THE SECOND WAVE, OF GENE INDUCTION IN VIVO

A. Visual-stimulus-mediated gene induction of representative genes in the visual cortex upon sustained stimulation in mice injected intraperitoneally with corn oil vehicle or the MEK inhibitor SL327 (100 mg/kg), based on ARG-seq. D, dark, no visual stimulation; L, light, with visual stimulation. Error bars are 95% confidence intervals across $n = 2-3$ mice. **B.** Same experiment as (A), but showing all rPRGs or dPRGs detected by ARG-seq from $n = 2-3$ biological replicates. * $p < 0.01$ from rank-sum test, significant difference from 1. Induction of rPRGs is more affected by MEK inhibition than induction of dPRGs ($p = 0.02$; rank-sum test, 16 rPRGs versus 14 dPRGs using the mean for each gene at its most induced time point across $n = 2-3$ biological replicates). **C.** Same as (A) but with brief visual stimulation. **D.** Same as (B) but with brief visual stimulation.

The MAPK/ERK pathway mediates fast Pol2 recruitment to rapid PRG promoters

We next sought to understand how the MAPK/ERK pathway mediates rapid induction of rPRG promoters. Because ARG induction is accompanied by Pol2 recruitment to ARG promoters within the first two hours of activity (Kim et al., 2010), we hypothesized that the rapidity of rPRG induction could be mediated by fast, MAPK/ERK-dependent Pol2 recruitment. Indeed, using Pol2 ChIP-seq, we observed a rapid increase in Pol2 occupancy at rPRG promoters by one to ten minutes of activity (Figure 2.5A-B, Supplementary Figure A2.7A-B, Supplementary Figure A2.7G). We also found that pharmacological blockade of new transcription initiation completely abolishes rPRG mRNA induction (Supplementary Figure A2.7F), suggesting that initiation of transcription by newly recruited Pol2 is essential for rPRG induction. To ask if MAPK/ERK signaling is required for the rapid recruitment of Pol2 to rPRG promoters, we performed Pol2 ChIP-seq in a time course of neuronal stimulation in the presence and absence of MEK inhibition. MEK inhibition reduces Pol2 occupancy at rPRG promoters at ten and 30 minutes of activity (Figure 2.5A-C, Supplementary Figure A2.7A-B), indicating that MAPK/ERK signaling is required for rapid recruitment of Pol2 to these promoters. However, MEK inhibition has no effect at later time points, suggesting that other pathways mediate slower Pol2 recruitment to rPRG promoters. Because pre-bound, paused Pol2 may facilitate faster recruitment of Pol2 by maintaining an open chromatin state (Gilchrist et al., 2010), we next asked whether MAPK/ERK signaling might enable rapid Pol2 recruitment by mediating the pre-binding and pausing of Pol2 at rPRG promoters in unstimulated neurons (Saha et al., 2011). We found that MEK inhibition does not change the Pol2 occupancy at ARG promoters in unstimulated neurons (Figure 2.5A, Supplementary Figure A2.7A), indicating that MAPK/ERK signaling is required specifically for rapid, activity-dependent Pol2 recruitment.

We next assessed the effect of MAPK/ERK signaling on Pol2 recruitment to dPRG promoters. Surprisingly, despite the slow transcriptional induction of dPRGs, we observed

recruitment of Pol2 to many of their promoters by ten minutes of neuronal activation (Figure 2.5D-E, Supplementary Figure A2.7C-D). However, in contrast to rPRGs, recruitment of Pol2 to dPRG promoters is not affected by MEK inhibition at early or late time points, either for the full set of dPRGs (Figure 2.5D-F, Supplementary Figure A2.7C-D) or a restricted set with greater Pol2 occupancy (FDR>0.01, rank-sum test, see methods). These results are consistent with a model in which MAPK/ERK signaling is required for rapid Pol2 recruitment to rPRG promoters, which are primed by pre-bound transcriptional machinery, but not for recruitment to dPRG promoters, which may require chromatin remodeling.

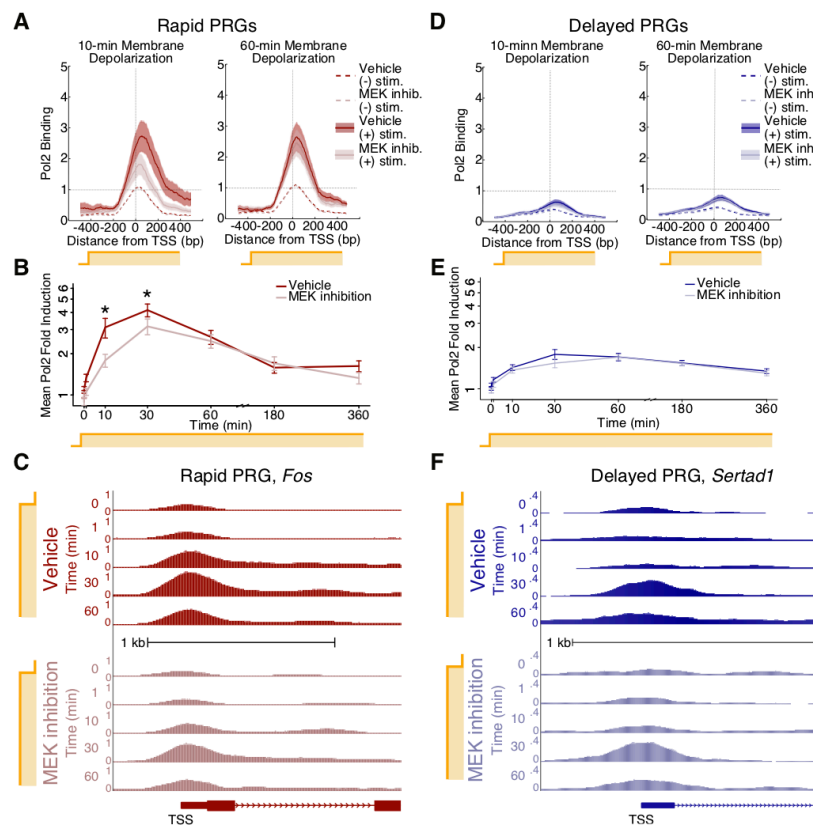


FIGURE 2.5. MAPK/ERK MEDIATES FAST RECRUITMENT OF POL2 TO RAPID PRG PROMOTERS

A. RNA polymerase 2 (Pol2) binding (ChIP-seq) at the promoters of rPRGs. Lines represent the mean and shading the SEM across loci. Data shown are from $n = 1$ of 2 biological replicates. Pol2 binding to rPRG promoters is blunted by MEK inhibition (see B). The KCl-dependent fold increase in mean Pol2 density (-300 bp to +300 bp) is significant under both vehicle and MEK inhibitor treatments (FDR < 0.001 in each of two biological replicates, paired rank-sum test). MEK inhibition *continued overleaf*

Figure 2.5 (Continued) does not affect Pol2 occupancy in unstimulated neurons (FDR > 0.05 in each of two biological replicates, paired rank-sum test). **B.** ChIP-seq-based time course of fold change in Pol2 occupancy at rPRG promoters (-300 bp to +300 bp). Shown are mean fold change values across genes, with $\pm$ SEM error bars. *FDR < 0.01 in each of two replicates, paired rank-sum test on fold change values. **C.** Pol2 binding at the promoter of the representative rPRG *Fos* upon sustained neuronal activation. Data normalized prior to visualization. **D.** Plotting and statistics same as (A) but showing dPRG promoters. **E.** Plotting and statistics as in (B) but showing dPRG promoters. **F.** Plotting as in (C) but showing representative dPRG *Sertad1*.

The MAPK/ERK pathway is required for eRNA transcription but not H3K27 acetylation at rapid enhancers

Pol2 could be recruited to the promoters of rPRGs in a MAPK/ERK-dependent manner via delivery from genomic enhancers (Szutorisz et al., 2005). We therefore asked whether enhancer activation might be dependent on MAPK/ERK signaling using H3K27 acetylation (H3K27ac) as a proxy for enhancer activity (Creyghton et al., 2010; Rada-Iglesias et al., 2011). We performed H3K27ac ChIP-seq throughout a time course of neuronal activation and analyzed H3K27ac levels at 940 putative ARG enhancers. We hypothesized that enhancers near rPRGs would have rapid, activity-dependent activation and require MAPK/ERK signaling whereas enhancers near dPRGs would be activated slowly and be MAPK/ERK-independent. Surprisingly, most activity-regulated enhancers rapidly gain H3K27ac within ten minutes of activity, regardless of the kinetics of their nearby promoters (Figure 6A-C). Furthermore, accumulation of H3K27ac does not require MAPK/ERK signaling, as MEK inhibition has no effect on activity-dependent H3K27ac at these enhancers, including those near MEK-dependent rPRGs (Figure 2.6D, Supplementary Figure A2.8A). Thus, H3K27ac is neither MAPK/ERK-dependent nor kinetically distinguishes enhancers near rPRGs versus dPRGs.

We next assessed another proxy of enhancer activity, enhancer RNA (eRNA) transcription (Kim et al., 2010). Surprisingly, total RNA-seq revealed that eRNA is induced more rapidly at enhancers near rPRGs than at those near dPRGs, thus mirroring mRNA expression

kinetics more closely than H3K27ac (Figure 2.6E). Furthermore, in contrast to our finding that H3K27ac is unaffected by MEK inhibition, MEK inhibition attenuates eRNA induction at enhancers near rPRGs (Figure 2.6F, Supplementary Figure A2.8A). These results indicate that rPRGs are distinguished by their proximity to rapidly activated enhancers whose eRNA induction but not H3K27ac is MAPK/ERK-dependent.

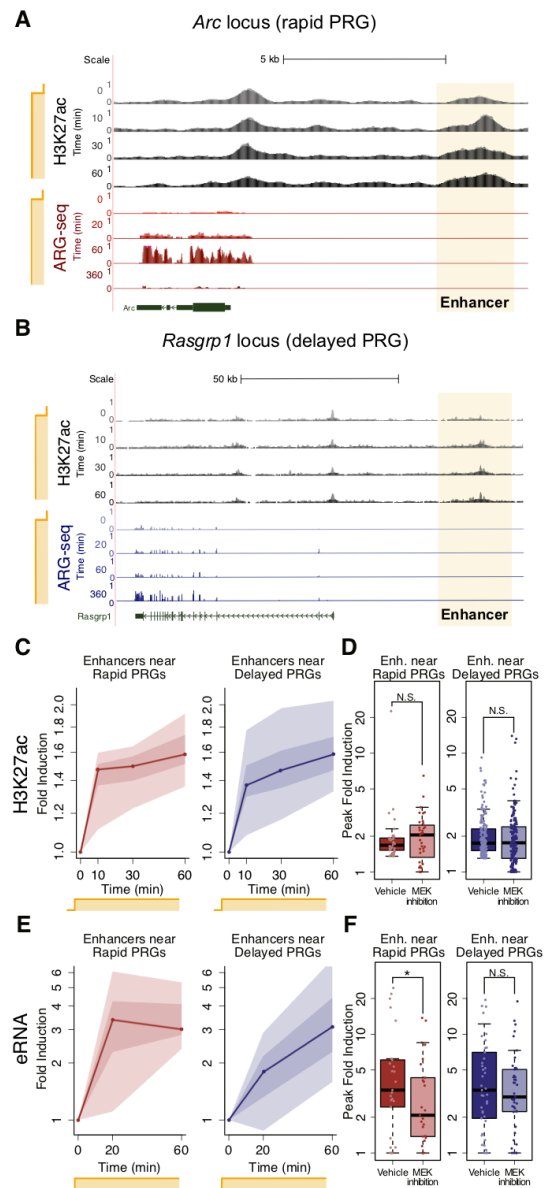


FIGURE 2.6. MAPK/ERK IS REQUIRED FOR RAPID eRNA INDUCTION, BUT NOT H3K27 ACETYLATION, AT ENHANCERS

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Figure 2.6 (Continued) **A.** H3K27ac accumulation (ChIP-seq) at the rPRG *Arc* locus upon sustained KCl depolarization. The gene expression of *Arc* based on ARG-seq is shown for comparison. Data normalized by read depth prior to visualization. **B.** Same as (A) but for the dPRG *Rasgrp1*. **C.** H3K27ac accumulation (ChIP-seq) at enhancers upon sustained KCl depolarization. Plotted are means from $n = 2$ biological replicates. Lines represent the median across enhancers, dark shading the two middle deciles, and light shading the upper and lower quartiles. The increase from 0 to 10 min is significant for both enhancers near rPRGs and those near dPRGs ($p < 0.00001$, rank-sum test). **D.** H3K27ac accumulation at enhancers near rPRGs and dPRGs is not significantly affected by MEK inhibition ($p > 0.2$, rank-sum test). Data as in (C). The y axis shows the induction at each enhancer's most-induced time point (10, 30, or 60 min) in each condition. **E.** eRNA induction (total RNA-seq) upon neuronal activation. Plotted as in (C). **F.** MEK inhibition blocks eRNA induction at enhancers near rPRGs, but not dPRGs. Plotting as in (D), except showing the maximum eRNA induction at 20 or 60 min. * $p = 0.01$, rank-sum test, using means for each enhancer from $n = 2$ biological replicates; N.S., $p > 0.05$.

We next asked whether the rapidity of eRNA induction near rPRGs is inherent to the enhancers themselves or simply a by-product of activation of nearby promoters. We predicted that if enhancer activation properties are inherent to the enhancers, we should observe a subset of enhancers whose kinetics and sensitivity to brief activity differ from their nearby promoters. To test this prediction, we needed to assess enhancers individually rather than in groups based on the kinetics of nearby promoters (as above). We therefore developed (Figure 2.7A-B, Supplementary Table A2.1) and validated (Supplementary Figure A2.8B) a targeted capture method, eRNA-seq, to enrich RNA-seq libraries for eRNAs by about 500-fold. We then identified and classified activity-regulated enhancers as rapid or delayed based on the kinetics of their eRNA induction (Figure 2.7C). While most activity-regulated enhancers near rPRG promoters are rapid enhancers, a minority (21%) are delayed enhancers (Figure 2.7D-E). Moreover, 50% of activity-regulated enhancers near dPRGs are rapid and 50% are delayed enhancers (Figure 2.7D), supporting the idea that enhancer activation kinetics are inherent to enhancers rather than nearby promoters. In further support of this idea, we found that rapid enhancers are more sensitive to brief activity than delayed enhancers (Figure 2.7H), even when considering only those enhancers near dPRGs ($p < 10^{-4}$, rank-sum test, see methods). This dissociation between the

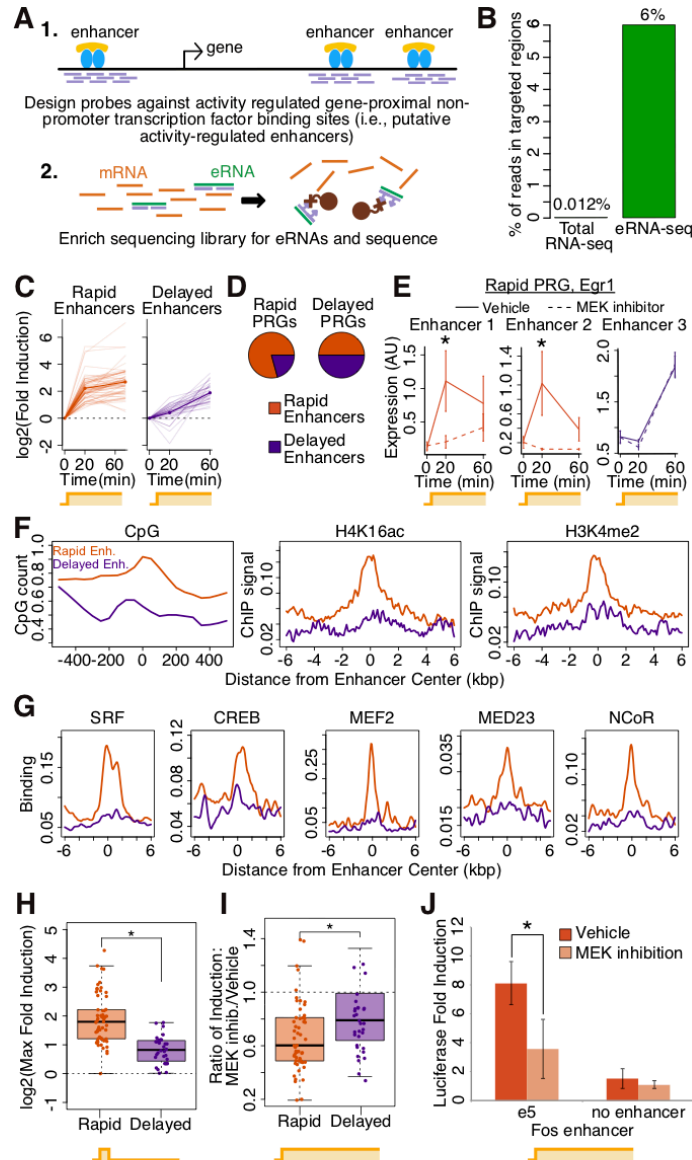


FIGURE 2.7. eRNA-SEQ ENABLES eRNA QUANTIFICATION AT INDIVIDUAL ENHANCERS, REVEALING RAPID AND DELAYED ENHANCERS

A. eRNA-seq methodology. **B.** Reads in target enhancers: eRNA-seq versus total RNA-seq. **C.** eRNA-seq-based eRNA expression at significantly induced (FDR < 0.05) rapid and delayed enhancers upon sustained activation. Rapid enhancers are significantly induced by 20 min and delayed enhancers only by 60 min. Light lines are means for individual enhancers from $n = 4$ biological replicates, and heavy lines are the geometric means for all enhancers shown. **D.** rPRGs compared to dPRGs are enriched for the presence of nearby rapid enhancers ($p = 0.02$, Fisher's exact test), but there are also rapid enhancers near dPRGs. **E.** eRNA-seq-based eRNA expression at three enhancers near the rPRG *Egr1* revealing two rapid and one delayed enhancer. $*p < 0.05$, paired rank-sum test. Error bars are means $\pm$ SEM. **F.** Indicators of open chromatin prior to stimulation at rapid versus delayed enhancers, with metaplots showing the geometric mean of all

continued overleaf

Figure 2.7 (Continued) enhancers in each class. All are significantly different between rapid and delayed enhancers ($p < 10^{-7}$, rank-sum test using area under the curve). Histone mark ChIP-seq data from cultured cortical neurons (Telese et al., 2015). **G.** Binding of transcription factors, the mediator subunit MED23, and NCoR at rapid versus delayed enhancers prior to stimulation, shown as in (F). All are significantly different between rapid and delayed enhancers ($p < 10^{-4}$, rank-sum test on area under the curve). ChIP-seq data from cultured cortical neurons (Kim et al., 2010; Telese et al., 2015). **H.** Rapid enhancers show greater induction in response to brief activity than delayed enhancers based on eRNA-seq ($p < 10^{-9}$, rank-sum test). The y axis shows the mean fold induction from $n = 4$ biological replicates for each enhancer at its most-induced time point (20 or 60 min). **I.** Rapid enhancers are more MAPK/ERK-dependent than delayed enhancers, based on eRNA-seq ($p = 0.006$, rank-sum test, using means for each enhancer from $n = 4$ biological replicates). For each class of enhancers, the earliest time point at which that class exhibits significant eRNA induction is shown (20 min for rapid and 60 min for delayed enhancers). The y axis shows the KCl-dependent fold induction with MEK inhibition divided by the same fold induction with vehicle treatment only (i.e., ratio of fold inductions). **J.** Effect of MEK inhibition on the enhancer function of the *Fos* enhancer e5 using a luciferase reporter assay in which the enhancer drives transcription from a minimal *Fos* promoter. * $p < 0.03$ from t test based on $n = 3$ biological replicates. Error bars represent $\pm$ SEM.

kinetics and brief-activity sensitivity of a subset of enhancers and their nearby promoters

supports the idea that enhancer activation is not merely a by-product of transcription at the promoter.

After identifying individual enhancers as inherently rapidly activated, we asked whether rapid eRNA induction at rapid enhancers might be mediated by an open chromatin state and sensitivity to MAPK/ERK signaling, similar to mRNA induction from rPRG promoters. Indeed, compared to delayed enhancers, we found that rapid enhancers have significantly elevated CpG content. They also have more open, active chromatin in unstimulated neurons, as evidenced by higher DNase hypersensitivity, greater binding of the transcription activators SRF, MEF2 and Mediator, and greater binding of the transcriptional repressor NCoR (Figure 2.7F-G, Supplementary Figure A2.8C-D). However, unlike rPRG promoters, rapid enhancers show little binding of Pol2 in unstimulated neurons (Supplementary Figure A2.8C). The more active chromatin state at rapid enhancers appears to be intrinsic to the enhancers themselves rather than an indirect effect of their associated promoters, since a comparison of just those rapid and

delayed enhancers near dPRGs revealed the same differences in CpG content, active chromatin marks, and transcription factor pre-binding in unstimulated neurons ($p < 0.01$, rank-sum test, see methods). Using eRNA-seq in the presence of a MEK inhibitor, we also found that rapid enhancers are more sensitive to MAPK/ERK inhibition than delayed enhancers (Figure 2.7E,I, Supplementary Figure A2.8E). In the case of at least one enhancer, Fos “e5” (Joo et al., 2015), MAPK/ERK-dependent enhancer activation is required for activity-dependent promoter activation, based on a luciferase reporter assay (Figure 2.7J). These results indicate that rapid enhancers are primed for rapid MAPK/ERK-dependent activation whether they are near first- or second-wave genes.

DISCUSSION

Using genome-scale technology, we demonstrate that a neuron’s activity pattern is encoded in its gene expression profile. Furthermore, we uncover a principle underlying the coupling map that links activity pattern to gene expression: the duration of neuronal activity has a logical relationship to three temporally and mechanistically distinct waves of gene induction. These three waves of gene induction include rPRGs, dPRGs, and SRGs, which are all induced by sustained neuronal activity. In contrast, brief activity induces only the first of these waves, rPRGs, which are uniquely dependent on MAPK/ERK signaling for their induction (Figure 2.8). Abolishing MAPK/ERK signaling not only alters the multi-wave structure of the ARG response by blunting and delaying rPRG induction, but it also abolishes rPRG induction in response to brief activity. In this way, MAPK/ERK both establishes the multi-wave structure of ARG transcription and enables activity-duration-specific gene induction. This shared mechanism suggests that a biological advantage of the multi-wave structure of ARG induction is to enable different activity patterns to induce different subsets of genes.

MAPK/ERK establishes the first wave of gene induction

We identify the MAPK/ERK pathway as a key determinant of the first wave of neuronal ARG induction, enabling first-wave genes to respond rapidly and to brief activity. However, our results suggest that other pathways must establish later waves of ARG induction. In contrast to this idea that multiple different pathways each regulate their own subset of genes, in PC12 cells the MAPK/ERK pathway itself mediates two different cellular outcomes depending on the duration of MAPK/ERK activation (Gotoh et al., 1990; Marshall, 1995; Santos et al., 2007).

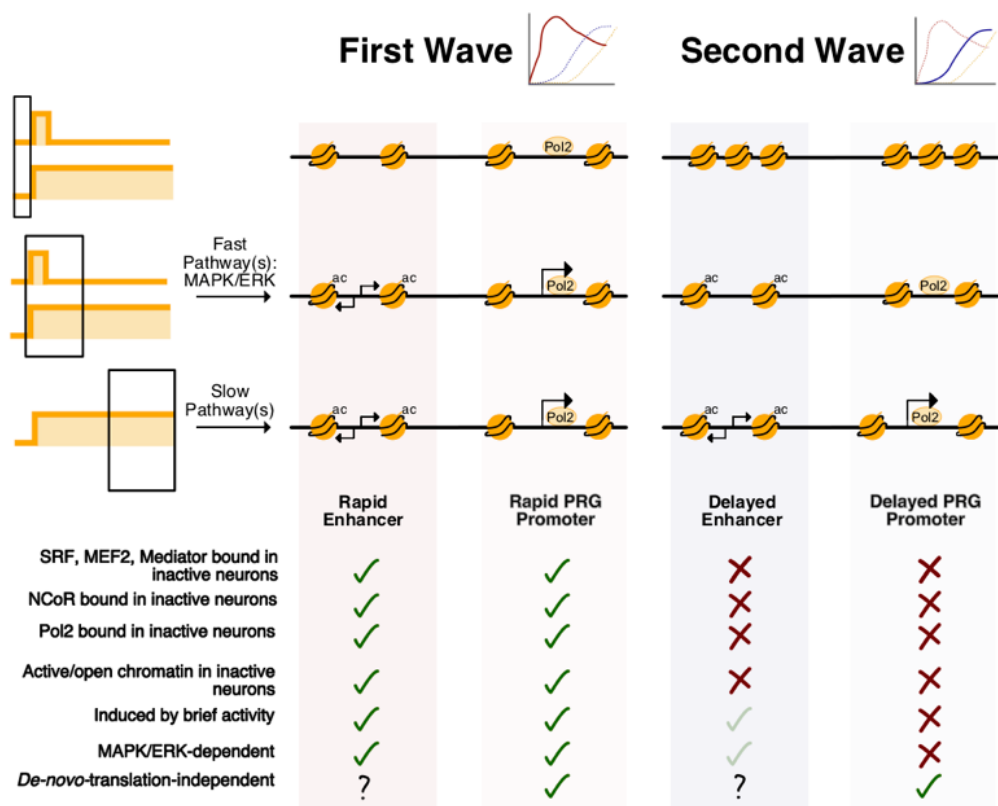


FIGURE 2.8. DISTINGUISHING FEATURES OF FIRST- WAVE GENES AND SECOND-WAVE GENES RPRGs

rPRGs are distinguished by dependence on MAPK/ERK signaling, proximity to rapid enhancers, and an open chromatin state. Light green check marks indicate partial effects.

Our finding that MAPK/ERK pathway is a fast pathway for activating ARG induction also differs from previous studies that suggest it could be a relatively slow regulator of transcription, including those showing it is slow to phosphorylate the transcription factor CREB (Hardingham et al., 2001b; Murphy et al., 1994; Toettcher et al., 2013; Wu et al., 2000). Slow MAPK/ERK-dependent phosphorylation of CREB could be important for regulating SRGs, especially given the persistence of phospho-ERK in response to sustained stimulation. Despite its slow phosphorylation of CREB, others have found that the MAPK/ERK pathway can be rapidly activated in the nucleus in response to brief stimulation (Dudek and Fields, 2001; Zhai et al., 2013) and is required for induction of several genes that we can now classify as rPRGs (Davis et al., 2000; Eriksson et al., 2006; Zheng et al., 2009).

There are at least two ways that MAPK/ERK could specify which genes are included in the first wave. In a passive model, rPRG promoters could be uniquely sensitive to MAPK/ERK signaling solely due to their open chromatin state in inactive neurons. This open chromatin state could prime rPRG promoters to be activated by MAPK/ERK within the first few minutes following neuronal activation, when MAPK/ERK is most active. Our study (Supplementary Figure A2.7E) and previous work (Saha et al., 2011) suggest that this open chromatin state may be maintained in inactive neurons by the paused Pol2 found at rPRG promoters prior to neuronal activation. This function for Pol2 is consistent with the current view of the function of paused Pol2 generally (Gilchrist et al., 2010), as well as our finding that the paused Pol2 is insufficient for appreciable gene induction in the absence of new Pol2 recruitment and initiation. In an active model of how MAPK/ERK specifies first-wave genes, MAPK/ERK signaling could activate rPRGs due to specific binding of MAPK/ERK-dependent transcriptional activators, including SRF (Treisman, 1996). SRF is required *in vivo* for the transcription of rPRGs (Ramanan et al., 2005) and often acts in concert with Elk-1, which is directly phosphorylated by MAPK/ERK (Supplementary Figure A2.6A, Marais et al., 1993; Sgambato et al., 1998; Xia et al., 1996). Elk-1

facilitates Pol2 recruitment via interactions with the Mediator subunit MED23 (Allen and Taatjes, 2015; Wang et al., 2005). We present correlative evidence that rPRGs may be regulated by SRF, Elk-1, MEF2, and MED23 and by activation of nearby rapid enhancers, but further work is required to causally link these mechanisms to rPRG induction.

Separable mechanisms of enhancer activation revealed by MAPK/ERK

Surprisingly, we find that the MAPK/ERK pathway regulates eRNA induction but not H3K27ac accumulation at rapid enhancers, suggesting that enhancer activation occurs in multiple mechanistically separable steps. H3K27ac is a commonly used mark for enhancer activity (Creyghton et al., 2010; Rada-Iglesias et al., 2011), but we find H3K27ac accumulates at enhancers even in the presence of MAPK/ERK inhibition, which blocks eRNA (and mRNA) induction. In other contexts histone acetylation has been shown to accumulate despite blocking eRNA transcription, Pol2 recruitment, or initiation of transcription (Hah et al., 2013; Kaikkonen et al., 2013; Wang et al., 2005). These and other experiments (Zhu et al., 2013) suggest that eRNA transcription may be a better marker for enhancer activation than H3K27ac, more accurately reflecting the extent to which an enhancer is activating transcription at a nearby promoter. Given these findings, our eRNA-seq method may be a particularly useful technique for reliably assaying enhancer activation genome wide.

Role of rapid PRG protein products

The protein products of rPRGs may be required for the cell biological changes that occur following a single occurrence of brief neuronal activity. For example, brief single behavioral trials are sufficient both for Arc induction in hippocampal region CA3 (Miyashita et al., 2009) and for CA3-dependent spatial learning (Nakazawa et al., 2003), suggesting that Arc may be required for this learning. In another example, just seven minutes of bicuculline treatment (similar to our five-

minute treatment) is sufficient to induce transcription- and MAPK/ERK-dependent long-lasting synchronous bursting of primary neurons (Arnold et al., 2005). We hypothesize that the ARG-dependent cell biological effect of brief activity is due in large part to the effect of just a few rPRGs that are not transcription factors (e.g., *Arc*, *Amigo3*). In contrast, physiological responses to prolonged activity, including homeostatic responses like synaptic scaling and firing rate homeostasis (Hengen et al., 2016; Ibata et al., 2008; Turrigiano, 2011), may be mediated by the protein products of dozens to hundreds of dPRGs and SRGs. Our identification of the MAPK/ERK pathway as structural determinant of the first wave of ARG induction now makes it possible to test the specific function of these first wave genes in transcription-dependent plasticity. In other words, by defining the contribution of MAPK/ERK to the coupling map, our work should enable manipulation of the coupling map to investigate its functional significance.

CHAPTER III.

DISCUSSION AND FUTURE DIRECTIONS

This chapter is adapted from an as-yet-unpublished review I wrote with guidance from Jesse Gray.

SUMMARY OF FINDINGS AND SIGNIFICANCE

A new coupling map principle

Here, I identify a new property of the stimulation-transcription coupling map: that different temporal patterns of neuronal activity are coupled to different kinetically-defined gene modules. In both cultured cortical neurons and the cortex in vivo, short durations of neuronal activity selectively induce rapidly-induced rPRGs, including *Fos* and *Arc*, whereas longer durations of activity also induce slowly-induced dPRGs and SRGs, including *Bdnf* and *Nptx2*. Similar kinetically-defined modules may also be differentially regulated in cultured dorsal-root ganglion neurons in response to different action potential bursting patterns. 71% of genes induced by electrical stimulation with long bursts and long inter-burst intervals are induced rapidly, whereas only 4.5% of genes induced by short bursts and short inter-burst intervals are induced rapidly (Lee et al., 2017). More detailed kinetic analysis and transcriptional analysis in the presence of a translation blocker could reveal that the long-burst pattern induces rPRGs and the short-burst pattern induces dPRGs and SRGs. Finding that two different variations in temporal activity pattern, duration and bursting, both differentially regulate the same gene classes would suggest that the neuron makes use of the multi-wave kinetic structure of the ARG program to induce different genes in response to a variety of different temporal patterns of stimulation.

Mechanisms of duration-transcription coupling

The finding that MAPK/ERK signaling couples activity duration to gene induction through regulation of rPRGs is, as far as I know, the first example where a transcriptional regulator is

causally demonstrated to couple differential neuronal stimulation to differential transcription. Our data together with previously-published findings about rPRG versus dPRG and SRG regulation suggests a model for duration-transcription coupling that includes all layers of stimulation-transcription coupling: calcium, signaling pathways, transcription factors, and chromatin (Figure 3.1). First, brief and sustained activity likely induce brief and sustained calcium influx, respectively (Dolmetsch et al., 2001; Evans et al., 2013; Kingsbury et al., 2007). Next, brief and sustained activity both regulate MAPK/ERK signaling, but MAPK/ERK signaling selectively acts on rPRG promoters. This specificity of MAPK/ERK signaling is likely in part due to the fact that rPRG promoters bind the MAPK/ERK-dependent transcription factor, SRF. However, it could also be because rPRGs have a more open, accessible chromatin state in unstimulated neurons. As chromatin remodeling takes time, rPRGs may be primed by this open chromatin state to respond quickly to brief stimulation. On the other hand, induction of dPRGs and SRGs, which have a relatively closed chromatin state in unstimulated neurons, requires histone turnover (Maze et al., 2015) as well as the bromodomain protein Brd4 (Sullivan et al., 2015), which recognizes histone acetylation. Stimulus-dependent chromatin remodeling of these delayed genes is mediated by the AP1 transcription factor complex (Su et al., 2017; Vierbuchen et al., 2017), which is made of rPRG protein products. Thus, dPRGs and SRGs may be slower in their induction, and require long-lasting stimulation, because chromatin remodeling as well as transcription and translation of AP1 proteins occurs relatively slowly. Furthermore, it may be that further stimulation is required following chromatin remodeling to complete transcriptional induction (e.g., to drive Pol2 recruitment) of these genes, thus allowing them to function as persistence detectors.

Using Transcription to Infer Past Activity

I also demonstrated that the duration of neuronal stimulation can be inferred from neuronal gene expression state (i.e., the mRNA in a neuron), demonstrating that neurons encode information about their stimulation history in gene expression. A similar type of inference has

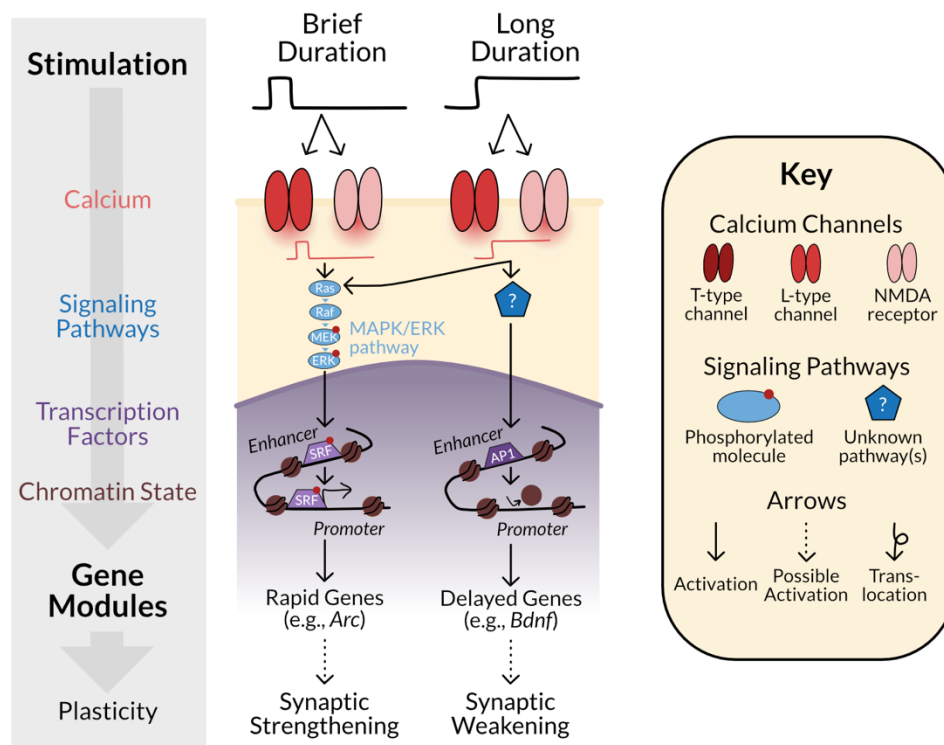


FIGURE 3.1. MECHANISTIC MODEL OF ACTIVITY DURATION-TRANSCRIPTION COUPLING

Neurons stimulated for brief durations selectively transcribe a rapid gene module, whereas neurons stimulated for longer durations also transcribe a delayed gene module. This coupling is mediated by differential calcium influx, signaling pathway activity, transcription-factor binding, and chromatin state.

been demonstrated using bulk RNA-seq to infer the type of psychotropic drug treatment given to cultured neurons (Gunther et al., 2005, 2003). This type of inference could be a powerful tool when paired with scRNA-seq, allowing assessment of the stimulation history of tens of thousands of neurons in a single experiment. My analysis of scRNA-seq data predicts that in

response to sustained visual stimulation, neurons in layer V of the cortex are activated briefly, whereas neurons of layer II/III and IV are activated in a sustained manner. It would be interesting for future studies to confirm this prediction about layer-specific activation with recording or calcium imaging, perhaps by performing photometry in mice with layer-specific GCaMP expression.

In addition to inferring the stimulation history of individual neurons, the coupling map could also be used to infer the past experience of an animal. An experience leaves a unique ARG signature in the brain that encompasses both where in the brain ARGs are expressed (Tischmeyer and Grimm, 1999) and which ARGs are induced (Korostynski et al., 2013; Mukherjee et al., 2018; Piechota et al., 2010; Whitney et al., 2014). A recent study used this ARG signature to infer past experiences that ranged from cocaine exposure to foot shock (Mukherjee et al., 2018). They found that using both components of the ARG signature, which genes are induced and where, allowed better inference than using just one component. Once we better understand which ARG modules are important for which plasticities, these ARG signatures could help reveal where in the brain different forms of experience-dependent plasticity occur.

COUPLING MAP PLASTICITY

Cell-type-specific coupling

An interesting avenue for future study is to determine the extent of plasticity of the coupling map. For example, scRNA-seq experiments have revealed that different neuronal cell types in the brain induce different ARGs, especially different delayed genes, in animals exposed to seizure, visual stimulation, novel environment, or acute stress (Cheadle et al., 2018; Gray and Spiegel, 2019; Hrvatin et al., 2017; Hu et al., 2017b; Jaeger et al., 2018; Lacar et al., 2016; Wu et al., 2017). These scRNA-seq results could either be due to cell-type-specific coupling (i.e., cell-

type specific ARG programs) or cell-type specific responses to experience (e.g., due to different intrinsic firing patterns or different neurotransmitter receptors). However, bulk RNA-seq of cultured, identically-stimulated inhibitory versus excitatory neurons revealed similar cell-type specific ARG programs as those observed in vivo (Spiegel et al., 2014). Thus, differences in stimulation-transcription coupling between cell types is likely due, at least in part, to cell-type-specific ARG programs.

Another question about cell-type-specific coupling is whether different cell types abide by different coupling principles. To describe what I mean, I'll take the example of duration-transcription coupling. Excitatory and inhibitory neurons both induce both rPRGs and dPRGs/SRGs, but the exact genes that fall into each category are cell-type dependent (Hrvatin et al., 2017; Spiegel et al., 2014). Therefore, it is possible that excitatory and inhibitory neurons share the same duration-transcription coupling principle: that brief durations selectively induce rPRGs. Alternatively, it is possible that inhibitory neurons have different coupling-map principles: brief activity could induce all PRGs, for example. I expect that cell types share coupling principles in part because cancer cells appear to follow the same duration-transcription coupling principle that we found in neurons, at least for a few genes: sustained TNF-alpha stimulation is sufficient to induce the SRG, *RANTES*, as well as rPRGs, whereas briefer stimulation is only sufficient to induce rPRGs (Ashall et al., 2009). To determine the extent of cell-type-specific coupling, it will be important to perform experiments that compare the transcriptional output in response to a range of extracellular stimuli across cell types.

Stimulation-transcription coupling over evolution

The stimulation-transcription coupling map has also changed over evolution. Genomic comparisons of stimulated human neurons, either iPSC-derived or fetal, and comparable mouse neurons have identified genes that are induced specifically in human neurons: e.g., *OSTN*, *HIC1*,

ETS2 (Ataman et al., 2016; Pruunsild et al., 2017; Qiu et al., 2016). However, like with cell-type-specific coupling map plasticity, it is still an open question as to whether different species have different coupling map principles or just slightly different gene module compositions. I would predict that coupling principles are preserved across evolution given that many human-specific ARGs likely evolved via gain of an activity-dependent binding site. Several of the human-specific ARGs harbor nearby transcription factor binding sites for stimulus-dependent transcription factors in the human, but not mouse, genome (Ataman et al., 2016; Pruunsild et al., 2017; Qiu et al., 2016). Alternatively, there are no examples of a transcription factor newly evolving to become activity-dependent, suggesting that perhaps gain of an activity-dependent binding site is a more common evolutionary adaptation than gained activity-dependence of a transcription factor. Addition of an activity-dependent binding site, as observed, would likely add genes to existing gene modules, whereas gained activity-dependence of a transcription factor would be more likely to create different gene modules and thus different coupling principles.

Coupling map plasticity based on activity history

The coupling map of a neuron may also be shaped by its past activity over several timescales. In the short term, a second stimulus that follows the first by 45s induces CREB phosphorylation faster and to a greater magnitude than a single stimulus, suggesting that gene induction may be similarly primed by recent past activity. This short-term priming is mediated by calmodulin, which remains in the nucleus following the first stimulation to allow faster CREB activation upon the second (Mermelstein et al., 2001). In the medium term, chromatin at ARGs, especially slowly-induced genes, is opened and acetylated in response to stimulation and this acetylation persists for minutes to hours (Malik et al., 2014; Mews et al., 2017; Su et al., 2017; Telese et al., 2015; Whitney et al., 2014). This open chromatin state that follows activity may prime ARGs for faster, remodeling-independent induction upon a second stimulus minutes-to-

hours later. Finally, in the long-term, stimulation-dependent DNA methylation changes could affect future ARG induction over hours, days, or years. During development, neuronal activity depletes DNA methylation at ARGs (Stroud et al., 2017). This lowered methylation status persists throughout the life of the animal and is predicted to lead to greater gene expression later in life. Given that DNA methylation at ARGs also changes with activity in the adult brain (Day et al., 2013; Halder et al., 2015), DNA methylation could be a mechanism by which neurons fine-tune their coupling maps in response to past activity.

Another interesting possibility is that activity-regulated transcription is important for determining a neuron's stereotyped firing pattern. As demonstrated here and elsewhere, many of the genes induced by activity are channels or potential regulators of synaptic activity, which could affect the neuron's response to future stimulus (Marder and Goaillard, 2006). Indeed, activity regulated genes such as *Nptx2* and *Fn14* have been demonstrated to be important for circuit refinement during development, which affects the activity of the neurons throughout the life of the animal (Cheadle et al., 2018; Gu et al., 2013; Yap and Greenberg, 2018). Given that different activity patterns are coupled to different transcriptional programs, it is possible that different activity patterns differentially alter the intrinsic excitability or synaptic connections of neurons, which, in turn, alters their activity pattern in response to future stimulus. It would be interesting to test whether such a cyclical relationship between activity pattern and transcription exists either in development or in the adult brain by first determining whether various activity patterns can rescue phenotypes associated with the loss of activity (e.g., in ocular dominance plasticity).

FILLING OUT THE COUPLING MAP

Genomic experiments have dramatically improved our ability to create a stimulation-transcription coupling map. In particular, in vitro experiments that systematically vary a stimulus

along a single dimension of stimulation space have been, and will continue to be, helpful for describing these coupling principles. However, in vivo, a single stimulus can vary along multiple dimensions of stimulation space, and a neuron can receive multiple different stimuli at one time. Thus, going forward, it will be important to determine how different dimensions of stimulation interact in their coupling to transcription.

While testing all of stimulation space would be prohibitively laborious, we could instead focus on achieving a full coupling map for a single neuronal subtype, thus limiting the relevant stimulation space to only the stimuli experienced by that subtype in vivo. With such a map in hand, it may eventually be feasible to manipulate the coupling map, that is to change the genes induced by a given stimulation through manipulation of gene-module-specific regulation. Such manipulation has the potential to link gene modules to physiologically-relevant plasticities and to reveal new transcription-dependent mechanisms of neuronal plasticity.

APPENDIX 1.
METHODS FOR CHAPTER II

This appendix includes the supplementary figures and tables related to the results in Chapter II. This Appendix was originally published in: Tyssowski KM, DeStefino NR, Cho JH, Dunn CJ, Poston RG, Carty CE, Jones RD, Chang SM, Romeo P, Wurzelmann MK, Ward JM, Andermann ML, Saha, RN, Dudek SM, Gray, JM.: Different Neuronal Activity Patterns Induce Different Gene Expression Programs. Neuron 2018, 98:530–546.e11.

Author contributions same as in Chapter II.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse primary neuronal cultures

Culturing

Cortical neurons were dissected from embryonic day 16 (E16) CD1 embryos of mixed sex. They were dissociated with papain (Worthington, (L)(S)003126) and plated on plates coated for at least one hour with poly-ornithine (30mg/mL, Sigma) in water and then washed three times with water. They were maintained at 37C at 5% CO₂ in neurobasal media (ThermoFisher) supplemented with B27 (ThermoFisher), Glutamax (ThermoFisher), and penicillin/streptomycin (ThermoFisher).

Stimulation

At 6 or 7 days in vitro (DIV), neurons were silenced with APV (100uM, Tocris) and NBQX (10uM, Tocris) to block NMDA and AMPA receptors. 14-16 hours later neurons were stimulated with a final concentration of 55mM potassium chloride using KCl depolarization solution (170mM KCl, 10mM Hepes pH 7.4, 1mM MgCl₂, 2mM CaCl₂). For sustained stimulation, KCl was left on neurons for up to 6 hours, whereas for brief stimulation, it was added for one minute, and

then removed and replaced with conditioned neurobasal supplemented with APV and NBQX until RNA collection. While sustained KCl-mediated depolarization elevates intracellular calcium for a minimum of 20 minutes and likely indefinitely (Dolmetsch et al., 2001; Evans et al., 2013), brief KCl-mediated depolarization elevates intracellular calcium only during the period of elevated KCl (Kingsbury et al., 2007). 10 μ M U0126 (Tocris), 625nM 11e (Tocris), 3 μ M STO-609 (Tocris), 30 μ M cycloheximide (Cell Signaling) or DMSO (equal volume) were added 30 minutes before stimulation and left on the neurons throughout the experiment. 10 μ g/mL ActinomycinD (Sigma) was added 15 minutes before stimulation. 10 μ M triptolide (Tocris) was added 5 minutes before stimulation.

Rat primary neuronal culture

Culture

Cultures of cortical neurons were prepared from embryonic day 18 Sprague Dawley rats of mixed sex (NIEHS Animal Study Proposal #01-21). Dissociated cortical neurons were plated in Neurobasal medium (Invitrogen) supplemented with 25 mM glutamate (Sigma-Aldrich) and 0.5 mM L-glutamine (Sigma-Aldrich) and either B27 (Invitrogen) or NS21 and maintained in a similar medium without the glutamate. NS21 was prepared in the laboratory (Chen et al., 2008). Cultures were grown at 37C with 5% CO₂.

Stimulation

Neurons were used routinely between 10–14DIV. To induce synaptic stimulation, we triggered neuronal activity by co-treating neurons with 50 μ M Bicuculline (Sigma-Aldrich) and 75 μ M 4-Aminopyridine (Acros Organics) (or a DMSO control). To induce brief activity, activity was ceased at the desired time point (5 min or 10s) using 2 μ M TTX. Neurons were collected at

various time points. 2 μ M PD184352 (Tocris) was added with bicuculline. 10 μ M U0126 was added 30 min before treatment with bicuculline.

Mice

Animal Care

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committees at each institution. Animals were housed with standard mouse chow and water provided *ad libitum*. Male C57BL/6J adult male mice (6-14 weeks old) were used for in vivo experiments in this study.

Visual Stimulation

For the flashing-light visual stimulation used in experiments with qPCR-based gene expression analysis and photometry, adult mice were housed in the dark for three days (for gene expression) or 12 h (for photometry). Bright lights (two GE White 18" Fluorescent Light Fixtures, part # UCF18P and F15T8, 15W/60Hz) were placed on either side of the mouse home cage. Mice were housed with 3 mice per cage (for gene expression) or single-housed (for photometry). Sustained stimulation was achieved by repeated 60s of illumination followed by 20s of darkness for up to 4 hours. For intermediate (7 min) and brief (1 min) stimulation, the cage was illuminated using the same program, but stopping after 7 or 1 minute(s), respectively, followed by waiting for up to 4 hours in the dark before tissue collection. This illumination schedule was achieved using a Raspberry Pi B (Model #756-8308) and relay (Adafruit Controllable Four Outlet Power Relay Module ID#: 2935). For gene expression experiments, at several time points following the start of stimulation, mice were sacrificed using carbon dioxide, eyes were enucleated, both visual cortices were separately dissected and homogenized in Trizol (Invitrogen) for subsequent qPCR.

For in vivo experiments testing the effects of MEK inhibition, mice were singly dark-housed for 3 days. The stimulus consisted of turning on the room lights either continuously or briefly (for one minute). On the day of the experiment, mice were intraperitoneally injected with 100mg/kg of SL327 (Tocris), a blood-brain-barrier-crossing analog of U0126 (Atkins et al., 1998), in corn oil or with a corn oil vehicle. Injections started 30 minutes before visual stimulus and continued once per hour for the duration of the experiment to maintain the effects of the drug. SL327 was solubilized first in 100% ethanol. Then this ethanol mixture was added to corn oil and vortexed for 30 minutes. The ethanol was then removed from the mixture using a speed vac. The vehicle was prepared in the same way using just ethanol and corn oil without any drug. Mice were sacrificed before the stimulus or either 30 min or 2.5 h after turning on the lights using carbon dioxide. After enucleating the eyes, their visual cortices were immediately dissected. One hemisphere from each mouse was homogenized in Trizol (Invitrogen) for subsequent ARG-seq, and the other was homogenized in cold lysis buffer (see Western Blotting) for western blotting to confirm ERK activation.

METHOD DETAILS

RNA extraction and qPCR

Mouse neurons/cortex

Samples were collected in Trizol (Invitrogen), and total RNA was extracted using the RNeasy mini kit (Qiagen) with in-column DNase treatment (Qiagen) according to the instructions of the manufacturer. The RNA was then either used for RNA sequencing (see below) or converted to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). For standard qPCR experiments, we used SsoFast Evagreen supermix (BioRad) with primers in Supplementary Table A2.6. For high-throughput qPCR, we used Taq-man qPCR

probes (designed by Invitrogen) using the Fluidigm microfluidics system (see Supplementary Table A2.3). High-throughput qPCR was performed by the BCH IDDRC, 1U54HD090255 according to the manufacturer's protocol.

Rat neurons

Total RNA was isolated from dissociated neurons using the RNeasy Mini Kit (Qiagen) with in-column DNase (Qiagen) digestion or the illustra RNAspin Mini kit (GE Healthcare) with on-column DNase (GE Healthcare) digestion. cDNA was synthesized using MuLV reverse transcriptase (Promega), random primers (Promega), oligo dT primers (Promega), and RNase inhibitors (Thermo Scientific). qPCR was performed using iTaq Universal Sybr Green Supermix (BioRad) and the BIO-RAD CFX Connect realtime PCR Detection System or the PerfeCTa SYBR Green FastMix (Quantabio). To measure pre-mRNA, primers that target intron-exon borders served for cDNA synthesis and subsequent amplification (14 cycles) using the manufacturer's protocol in the One-Step RT-PCR kit (Qiagen). The amplified product level was quantified by qPCR using the same primers. Pre-mRNA primers are in Supplementary Table A2.6 (Saha et al., 2011).

NanoString

NanoString probes were designed for indicated pre-mRNAs (Supplementary Table A2.4) by NanoString technologies and assays were performed following the manufacturer's protocol.

RNA sequencing

General protocol

Before library preparation, for capture experiments, ERCC spike-in RNA (Ambion) was added to RNA samples according to the instruction of the manufacturer. Libraries were prepared using the High Throughput Total RNA TruSeq kit (Illumina), following the instructions of the manufacturer but scaling down all volumes to 1/3 of the recommended volumes. Libraries were sequenced on a NextSeq 500 (Illumina) to a depth of at least 30 million reads per library for total RNA-seq, 20 million reads per library for eRNA-seq and 3 million reads per library for ARG-seq. We aligned reads to the mm9 genome using the STAR aligner (Dobin et al., 2013), and then made the resulting SAM files into BED files using SAMtools and BEDtools (Li et al., 2009; Quinlan and Hall, 2010). We used UCSC-tools (Kuhn et al., 2013) to make bigWig files for viewing on the genome browser. We used bedtools map to count reads in both exons and introns. We then analyzed the raw count data using R, including edgeR (Robinson et al., 2009).

ARG-seq probe design synthesis

For ARG-seq, capture probes were designed as oligonucleotides tiling activity-regulated exons and control exons. Genes to be captured were 251 ARGs that showed a reproducible 3.5 fold increase in transcription at either 1 or 6 hours of KCl treatment in two replicates of published RNA-seq data (Kim et al., 2010) and 47 genes that showed no change with KCl but spanned a range of expression values (controls). Synthesized probes were 100 base pairs in length, with each probe overlapping the previous probe by 76 base pairs (Supplementary Table A2.1). Probes had PCR primer binding sites and IVT promoters added. These oligonucleotides were ordered from Custom Array, PCR-amplified, and transcribed in vitro into biotinylated RNA baits using the Megascript SP6 In Vitro Transcription kit (ThermoFisher).

eRNA-seq probe design and synthesis

For eRNA-seq, capture probes were designed as oligonucleotides tiling putative activity-regulated enhancers, which were identified based on their location relative to ARGs and their

transcription factor binding. To identify these putative enhancers, we started with all CREB, SRF, CBP, Npas4 or Pol2 binding sites from a previous study (Kim et al., 2010). We then took only those sites that were within 100kb of a transcription start site of one of the ARGs used in our ARG-seq experiment. We chose this threshold because 80% of enhancers regulate transcription start sites (TSSs) within 100kb (Chepelev et al., 2012). We eliminated intragenic enhancers and those located within 1kb from the transcription end site or 500bp from the transcription start site of a gene. We designed probes to span the entire TF-bound putative enhancer, plus 500 bp on each side. Synthesized probes were 99 base pairs in length, with each probe overlapping the previous probe by 73 base pairs (Supplementary Table A2.1). This oligonucleotide library was ordered from Twist Biosciences. We amplified and in vitro transcribed the RNA baits as described above for the ARG-seq baits. We also designed probes to tile the ERCC spike ins (Ambion) that were designed and ordered with our eRNA capture oligonucleotides. ERCC spike in oligonucleotides were made with different PCR adaptors so that they can be amplified and in-vitro-transcribed separately.

Capture

For ARG-seq and eRNA-seq, samples were treated in the same manner as with total RNA-seq, except that after library preparation, 250ng of pooled libraries were heated to 95C to denature DNA and then incubated with 250ng ARG-seq or eRNA-seq RNA baits (plus ERCC baits in a volume to allow for equal molar ratios of all probes) overnight at 65C in hybridization buffer (2.5ug Cot1 DNA (ThermoFisher), 2.5ug Salmon Sperm DNA (ThermoFisher), 15mM p5 blocking primers, 15mM p7 blocking primers, 5X SSPE (ThermoFisher), 5X Denhardt's Solution (ThermoFisher), 0.133% SDS). Blocking primers are: p5-
AATGATACGGCGACCACCGAGATCTACAC,
ACACTCTTTCCCTACACGACGCTCTTCCGATC/3InvdT/ p7-

CAAGCAGAAGACGGCATAACGAGAT, GTGACTGGAGTTCAGACGTGT

GCTCTTCCGATC/3InvdT/ Primers for amplification are: p5-AATGATACGGCGACCACCGAGA, p7-CAAGCAGAAGACGGCATAACGAG.

Hybridized samples were incubated with MyOne Streptavidin T1 Dynabeads (Invitrogen) in binding buffer (1M NaCl, 10mM Tris-HCl pH 7.5, 1mM EDTA). Beads were washed once in 1x SCC, 0.1% SDS at room temperature and three times in 0.1x SCC 0.1% SDS at 65C. Captured libraries were eluted with 0.1M NaOH and neutralized with 1M Tris-HCl pH 7.5. Libraries were then purified using the Qiagen MinElute PCR cleanup kit and re-amplified using Herculase II Fusion polymerase (Agilent).

Capture-seq processing and normalization

Data was normalized by the geometric mean of the reads from control genes or enhancers. Control regions were identified as regions that do not change with KCl in published RNA-seq data (Kim et al., 2010). ERCC spike-ins confirmed that capture occurred with similar efficiency across initial RNA concentrations.

Fiber Photometry

Viral injection and optic fiber placement

To monitor bulk activity of neurons in mouse primary visual cortex (V1), mice were anesthetized with isoflurane in 100% O₂ (induction, 3%–5%; maintenance, 1%–2%), and placed on a heating pad (CWE) in a stereotaxic apparatus (KOPF). Ophthalmic ointment (Puralube) was applied to the eyes. We expressed a genetically-encoded calcium indicator via viral injection (0.2 µL per hemisphere of AAV1.Syn.GCaMP6s.WPRE.SV40, Penn Vector Core) bilaterally into V1

(coordinates relative to Bregma: AP: -3.6 mm; ML: +/- 2.9 mm; DV: 250 μ m and 500 μ m below the pial surface, via a burrhole).

Two weeks after viral injection, mice were again anesthetized with isoflurane in 100% O₂ (induction, 3%–5%; maintenance, 1%–2%), and optic fibers (400 μ m diameter, NA 0.48) were implanted bilaterally at the injection sites (150 μ m below pial surface). Mice were allowed to recover for at least 10 days prior to recording.

Fiber photometry recordings of bulk calcium activity from V1

For photometry recordings, we delivered blue light via an LED (Plexon LED Driver PLexBright LD-1, 20 μ W output, calibrated prior to each recording session) and patch cable (Doric). Recordings demonstrated very similar visual responses from each hemisphere, so a data from a single hemisphere was used per mouse.

Experimental paradigm during GCaMP6 recordings

We used the following visual stimulation paradigm during recordings. Singly-housed mice at the end of their 12-hr dark cycle (~7 am) were fitted with a patch cable for photometry recordings and moved, together with their home cage, to a light- and sound-isolated cabinet. The cabinet was initially fully dark, other than IR illumination (light source: HTX-F5-48-23), used for concurrent collection of videography to track mouse locomotion using a IR-sensitive camera (Flea3 1.3 MP Mono USB3 Vision camera, FL3-U3-13Y3M-C; Lens: H2Z0414C-MP).

Recordings were collected in darkness for one hour prior to bright light illumination of the home cage (two GE White 18" Fluorescent Light Fixtures, part # UCF18P and F15T8, 15W/60Hz) placed on either side of the mouse home cage. For the subsequent 3 hours, the cage was illuminated with the visual stimulation paradigm described above. Black heat-shrink tubing was used to prevent room light from affecting photometry signals. We confirmed that

contamination of photometry signals by illumination of the cage was negligible, by recording photometry signals in the absence of delivery of blue light via the patch cable at the end of each recording session.

All photometry signals and timestamps from stimuli delivery and videography were acquired on a standard PC and data acquisition board (National Instruments).

Histology

In a subset of experiments (4/8), fiber localization was confirmed histologically to be in area V1 and among strongly GCaMP6-expressing cell bodies. Mice were given an overdose of tribromoethanol, perfused with 10% formalin, and brains were cut in 40- μ m coronal sections and stained with 4'-6-diamidino-2-phenylindole (DAPI) to visualize nuclei. Sections were then imaged on a digital slide scanner (Olympus VS120).

Western blotting

Mouse cortical neurons

To detect protein expression in mouse cortical neurons, neurons were collected in cold lysis buffer (for pERK and ARC western blots - 1% Triton X-100, 50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, and freshly added protease and phosphatase inhibitors from Roche Applied Science Cat. # 05056489001 and 04906837001, for pElk-1 western blots - RIPA buffer (10mM tris pH 7.4, 1% NP-40, 150mM NaCl, 0.1% SDS, 1mM EDTA, 1mM Na(3)VO(4), 0.1% Sodium Deoxycholate) with protease and phosphatase inhibitors). Lysed neurons were treated with 4X sample buffer (40% glycerol, 8% SDS, 0.25M Tris-HCL, pH 6.8, 10% 2-mercaptoethanol) and boiled for 5 minutes. Samples were centrifuged at full speed for 3 minutes before loading on NuPage 4-12% Bis-Tris Gels (Invitrogen). Gels were run at 140V for 55 minutes. We transferred onto nitrocellulose membranes using the BioRad transfer system at

114V for 1h and 7min. Membranes were blocked in 5% milk-TBST for 1 hour. They were treated with primary antibody in 5% milk-TBST for at least one hour at room temperature or overnight at 4C. To visualize protein, blots were incubated with secondary antibody in TBST in the dark for 45 minutes. Blots were imaged using a LiCor Odyssey and quantified using ImageJ. Primary antibodies used were: rabbit anti-phosphoERK1/2 (Cell Signaling Technology 4370, 1:1000), mouse anti-GAPDH (Pierce, GA1R, 1:10000), rabbit anti-ARC (Synaptic Systems, 156-003, 1:1000), mouse anti-pElk-1 (Santa Cruz, sc-8406X, clone B4, 1:1000), rabbit anti-GAPDH (Cell Signaling D16H11, 1:1000). Secondary antibodies used were: IDR dye 680 goat anti-rabbit (LiCor, 1:10000), IDR dye 800 goat anti-mouse (LiCor, 1:10000).

Rat cortical neurons

To detect protein expression in rat cortical neurons, neurons were disrupted by brief sonication (three cycles of 30 sec in low setting in Bioruptor at 4C) and then cleared of debris by high-speed centrifugation (14500 RPM for 1 minute). The supernatant was collected in separate tubes and resolved by gel electrophoresis on 4-20% pre-cast gels (Life technology) and transferred to a nitrocellulose membrane using the iBlot gel transfer apparatus (Life technology). Immunoblots were incubated with primary antibody overnight. Blots were visualized with a LiCor Odyssey infrared scanner after immunolabeling primary antibodies with Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 680 (ThermoFisher). Images were processed using the Odyssey 2.1 software. Primary antibodies used were: rabbit anti-phosphoERK1/2 (Cell Signaling Technology 4370), H4 (Cell Signaling Technology 2935), Actin (Millipore, AM4302).

Nuclear Isolation

Nuclear lysate was prepared from treated neurons by first liberating the nuclei in a non-ionic detergent buffer (10mM HEPES (pH 7.9), 10mM KCl, 2mM MgCl₂, 0.5mM dithiothreitol,

0.1% NP-40) for precisely 30 seconds and subsequently lysing them in NETN buffer (0.5% NP-40, 1mM EDTA, 50mM Tris, 120mM NaCl, pH 7.5) freshly supplemented with 0.5% protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktails (Sigma). Nuclear liberation was confirmed under the microscope before the released nuclei was scraped and dissolved in the NETN buffer.

Immunocytochemistry

To detect nuclear phospho-CaMKIV levels, after stimulation, neurons were fixed in 4% PFA for 15 minutes. Neurons were then washed twice in PBS and blocked and permeabilized for 30 minutes using 1% BSA in PBS + 0.25% Triton-X100 (BSA-PBST). Neurons were then incubated overnight at 4C in BSA-PBST and phospho-CaMKIV antibody (1:500, Santa Cruz sc-28443-R). They were then washed 3 times with PBS and incubated for 1 hour at room temperature in secondary antibody (1:1000 ThermoFisher, R37117). They were then washed once with PBS, incubated for 10 min with DAPI (Roche, 10236276001) in PBS, and washed again with PBS. Neurons were imaged with a Leica inverted microscope. Images were taken with LAS software and quantified using ImageJ.

Chromatin immunoprecipitation (ChIP)

Media on the neurons was removed and neurons were fixed in crosslinking buffer (10 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% formaldehyde) for ten minutes at room temperature, and this reaction was quenched using 125mM glycine for 5 minutes. For H3K27ac ChIP, 250,000 neurons were used per ChIP sample. For Pol2 ChIP, 2 million neurons were used per sample. Neurons were then washed with cold PBS and then collected in PBS with 0.25% BSA and pelleted by centrifuging at 700 x g for 15 minutes. Cell pellets were stored at -80C. Neurons were sonicated using a Covaris E3 sonicator in lysis buffer (10 mM Tris pH 8.0,

1mM EDTA, 1 mM EGTA, 1X Roche complete EDTA-free protease inhibitors, 0.15% SDS).

Sonication was done for 8 minutes per samples with 200 cycles/burst, a 2% duty cycle at power level 3. This reliably produced fragments between 100 and 700bp in length. Samples were then supplemented with ChIP Buffer to make SDS-ChIP buffer (10 mM Tris pH 8.0, 0.1% SDS, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.3 mM EGTA, 1X Roche complete EDTA-free protease inhibitors). For H3K27ac ChIP, Protein A beads (Dynabeads) were washed with 1% BSA/TBST and added to the fragmented DNA for a pre-clear and rotated at 4C for one hour. A different set of protein A beads was pre-treated with 0.48ug of antibody (Abcam, ab4729)/sample for H3K27ac ChIP. The same procedure was followed for Pol2 ChIP, but with Protein G Dynabeads and 4ug antibody (Abcam, ab817) per crosslinked input. Following the pre-clear, pre-clear beads were removed, an aliquot of fragmented DNA was set aside as the input, and antibody-treated beads were incubated with the fragmented DNA overnight at 4C. were washed twice with cold low salt wash buffer (0.1% SDS, 20 mM Tris pH 8.0, 1% Triton X-100, 150 mM NaCl, 2 mM EDTA), twice with cold high salt wash buffer (0.1% SDS, 20 mM Tris pH 8.0, 1% Triton X-100, 500 mM NaCl, 2 mM EDTA), twice with cold LiCl wash buffer (1% NaDOC, 10 mM Tris pH 8.0, 1% NP40, 250 mM LiCl, 1 mM EDTA), and once with room temperature TE. Crosslinks were reversed by incubating samples in TE+1%SDS at 65C overnight. Samples were then treated with RNase A (Ambion) and Proteinase K (New England Biolabs), and DNA was eluted using MinElute Columns (Qiagen) according to the instructions of the manufacturer.

Chromatin immunoprecipitation sequencing (ChIP-seq)

H3K27ac ChIP-seq

For H3K27ac ChIP-seq, libraries were prepared using 5ug of immunoprecipitated DNA or input DNA with the NuGen Ultralow V2 1-96 library prep kit. Libraries were sequenced on an Illumina NextSeq500 to a depth of at least 30 million reads per library. Reads were aligned to

mouse genome mm9 using bowtie2 (Langmead and Salzberg, 2012). The resulting SAM files were made into BED files using SAMtools and BEDtools, with reads extended to 300 base pairs (Li et al., 2009; Quinlan and Hall, 2010) and then into bigWig files using UCSC-tools (Kuhn et al., 2013). Reads were assigned to individual enhancers or promoters using bedtools map and data was analyzed using R.

For downstream analysis, H3K27ac ChIP-seq data was input-normalized and then normalized by dividing by the geometric mean of control enhancers identified based on their location near the same control genes used for ARG-seq (control enhancer selection described in Capture RNA sequencing section). The data used for plotting (Supplementary Table A2.7) included the mean input-normalized and control-normalized signal from the same regions targeted by eRNA-seq of each enhancer for two biological replicates, averaging each enhancer across replicates prior to plotting, and including only enhancers captured in eRNA-seq. Plots in Supplementary Figures A2.4 and A2.8 were made as describe in the “Published ChIP-seq data” section (see below).

Pol2 ChIP-seq

For Pol2 ChIP-seq, reads were aligned to mouse genome mm9 using the STAR aligner (Dobin et al., 2013). The resulting SAM files were made into read-extended (200 bases per fragment) BED files using SAMtools and BEDtools (Li et al., 2009; Quinlan and Hall, 2010) and then into bigWig files using UCSC-tools (Kuhn et al., 2013). For analysis, the metaseq (Dale et al., 2014), numpy (Van Der Walt et al., 2011), and matplotlib (Hunter, 2007) python packages were used to process aligned bam files, extend reads to 200 bases, and to produce read-depth- and input- normalized data. TSS positions were obtained from UCSC gene annotations and refseq gene databases (see Supplementary Table A2.8). For two genes (*Amigo3*, *Dusp5*), we used Refseq TSSs that are now deprecated. The mean Pol2 density at each TSS was measured using 600bp

windows centered (-300bp to +300bp) on the TSS. ARG gene lists were filtered for a single TSS per gene, using the TSS with greatest average Pol2 density of all samples within single biological replicate. Additional analysis was performed in R. Given across-sample variability in read-depth- and input-normalized data, the samples were further normalized to Pol2 ChIP-seq density measured at constitutively active, non-activity-regulated control gene promoters—similar to the across-sample ChIP-seq normalization methods adopted by others for quantitative analysis of peaks (Shao et al., 2012). Specifically, data from each sample was normalized to the median value of a distribution of Pol2 density values occurring at ~800 constitutively active TSSs (-300 to +300bp) with unchanging mRNA levels under KCl as measured by RNAseq (Kim, et al. 2011).

Published ChIP-seq Data

For analysis of published data, data from Kim et al. 2010 was used as aligned and processed by the authors and downloaded from GEO as bigwig files. Data from Telese et al. 2014 was downloaded from GEO as fastq files, re-aligned to mm9 using bowtie2, and processed like the H3K27ac data in this study. Data from ENCODE was downloaded as processed by the authors. Signal was binned across TSSs and enhancers and input-normalized using the Python package metaseq (Dale et al., 2014). Plots were made using R, smoothing with the lowess function.

Luciferase assays

The sequences for enhancer e5 was amplified using PCR from genomic DNA extracted from wildtype (C57BL/6J) mice, utilizing primers that included flanking KpnI and XhoI sites (ATACGGTACCCGAGACTACGTCA, ATGTCTCGAGATTAAAAAGGCC). These amplified sequences were cloned into pTAN02, an ITR-containing AAV screening vector containing minimal human pFos upstream of the Firefly luciferase gene (Nguyen et al., 2016) with the KpnI

and XhoI sites. Additionally, pTAN02 without an enhancer insert was included as a “no enhancer” control. Primary cortical neuron cultures (see above) were transfected using PEI (4:1 PEI:DNA mass ratio) on DIV5. These cultures were co-transfected with an internal control Renilla luciferase construct, pTK-RN, at a fixed mass ratio of 9:1, Firefly construct:Renilla construct. Each experiment was run in triplicate. 30 minutes prior to depolarization, 10uM U0126 in DMSO or a DMSO vehicle was added to the culture media. Cultures were depolarized for 12 hours. A non-depolarized control received a media change with no additional KCl. Cultures were collected on the night of DIV7 and prepared using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. The lysate was assayed over a 10 second period using the GloMax 20/20 Single Tube Luminometer (Promega), and the luciferase activity was calculated as a ratio of the Firefly to Renilla output values.

QUANTIFICATION AND STATISTICAL ANALYSIS

We have included most statistical details in our Figure legends, including p-values, statistical tests used, ‘n’s for each experiment, and a description of to what ‘n’ refers. Biological replicates refer to biological material from different mice (all experiments), with biological replicate samples also collected on a different day (in vitro experiments only).

Gene Classification

In vitro

In experiments in mouse cortical neurons, our gene lists consisted of genes that showed significant induction ($FDR < 0.05$) of at least 1.5 fold at any time point in ARG-seq experiments, as determined by edgeR (173/251 captured ARGs). We classified genes as PRGs if they showed less than a 2-fold reduction in expression in 6h-KCl-treated neurons in the presence of cycloheximide. SRGs showed a greater than 2-fold reduction in the presence of cycloheximide

(FDR<0.05 by edgeR). We classified PRGs as rapid if they had higher induction at 1h compared to 6h and delayed if they had higher induction at 6h compared to 1h. All rapid PRGs showed >2-fold pre-mRNA induction by 20min of stimulation. We eliminated four PRGs from our analysis due to ambiguity in our classification scheme, which exclusively relied upon kinetics of induction to distinguish rapid from delayed PRGs. We eliminated two genes (*Vgf* and *Homer1*) because their expression peaked at 6 hours of KCl stimulus, but they showed robust and significant pre-mRNA induction at 20 minutes. We also eliminated two genes (*Gadd45b* and *Nfkbid*) because while their mRNA induction peaked at 1h, they did not show a trend towards pre-mRNA or mRNA induction at 20 minutes of KCl. For significance testing in the classification, we used edgeR's glmFit and glmTreat functions (Robinson et al., 2009). PCA was performed using the prcomp function in R using normalized mRNA expression values. Specifically, to better assess expression kinetics, each gene was normalized such that its lowest expression value was set at 1 and its highest at 10.

In vivo

For in vivo data in Figure 2.4, gene classification was based on in vitro mouse data. However, we eliminated delayed PRGs with higher induction at 30 minutes compared to 150 minutes of visual stimulus.

Functional Annotation

Functional annotation was performed using PANTHER version 13.1 (Mi et al., 2017) (Supplementary Table A2.5). Text of the table reflects output from the program with duplicate entries deleted. Colors in table represent manual classification. Genes were identified as directly regulating transcription if they were annotated as transcription factors/cofactors or as binding to DNA. Genes were identified as indirectly regulating transcription if they were annotated as part

of a signaling pathway likely to regulate transcription. Genes were also identified as indirectly regulating transcription if they are not channels, receptors, or secreted proteins that were annotated as regulating transcription but not as transcription factors or binding to DNA.

Nearest-neighbor classifier

Our first classifier for post-hoc determination of in vitro activity pattern based on in vitro gene expression used the maximum expression at any time point for each gene, such that the kinetics of gene induction did not contribute to the classifier. It compared each replicate in a testing set to all replicates in a training set using Euclidean distance and classified based on the minimum distance. It was run with both separate testing and training sets (6 biological replicates each, randomly sorted) and leave-one-out cross validation. This classifier was run using all genes targeted by ARG-seq, only induced ARGs, and only control (non-induced) genes.

Our second classifier tested in vivo activity pattern and was trained using in vitro gene expression. We used 60-minute time points for both training and testing sets to enable detection of both rPRGs and dPRGs. The 11 ARGs used were *Egr1*, *Fos*, *Bdnf*, *Npas4*, *Cdkn1a*, *Crem*, *Grasp*, *Maml3*, *Scg2*, *Pcsk1*, and *Egr2*. To compare expression without influence of the absolute magnitude of expression, which differs between in vivo and in vitro experiments, data for each experiment (i.e., in vitro or in vivo), was quantile normalized between genes. The classifier then compared each replicate in the in vivo testing set to all replicates in the in vitro training set using Euclidean distance and classified based on the minimum distance.

scRNA-seq analysis

Data

We used raw scRNA-seq (inDrops method) expression values from neurons in the visual cortex that had been exposed to 0, 1 or 4 hours of sustained visual stimulation (Hrvatin et al., 2017). We limited our analysis to only neurons classified with high confidence as excitatory neurons by Hrvatin et al. Our analysis was done on data from n=4 individual visual cortices for each time point pooled together.

Activity History Inference

Briefly, to infer activity history, each gene in each neuron at 1h was first called as ON or OFF based on the distribution of expression of that gene in excitatory neurons from the unstimulated visual cortex. Next, the numbers of rPRGs and dPRGs that were ON or OFF in each cell were summed. The number of genes ON in each gene class was used to determine whether that class as a whole was ON or OFF, based on thresholds set using data from unstimulated neurons (see below). The rPRG and dPRG states were then used to infer activity history as inactive (or unchanged from unstimulated), BRIEF, or SUSTAINED in response to visual stimulation.

A detailed description: For this analysis we used read-depth normalized data. We started by determining whether each rPRG or dPRG was induced in each neuron. A gene was defined as induced in a neuron from the stimulated cortex if its expression in that neuron was greater than a threshold set based on the expression of that gene in neurons from unstimulated cortex. This threshold was set at the 95th percentile of expression values for that gene in all the excitatory neurons in unstimulated cortex.

We then used these classifications of individual genes to determine whether neurons induced our gene classes (i.e., rPRGs or dPRGs) as a whole. We counted the number of rPRGs and dPRGs induced in each neuron. We set a threshold for the number of genes in each class that needed to be induced for that class to be considered ON in the neuron. We determined this

threshold separately for rPRGs and dPRGs. To determine this threshold, we compared distributions of rPRG or dPRG metagenes between the stimulated and unstimulated samples. rPRG and dPRG metagenes were summed expression of all rPRGs or dPRGs, respectively, in each cell. We specifically compared metagene distributions between stimulated OFF neurons (i.e., neurons in the stimulated cortex for which the class is OFF) and unstimulated neurons (i.e. neurons from the unstimulated cortex), as our goal was for the stimulated OFF neurons to be similar to the unstimulated neurons to ensure that the class is actually OFF in stimulated OFF neurons. The threshold was therefore set as the maximum number of genes induced in the class for which the distribution of metagene expression for the stimulated OFF neurons was the same as or slightly left-shifted (i.e., less expressed) compared to the unstimulated neurons. More specifically, the threshold was set at the number of genes induced in the class that produced the minimum distance between distributions where $p > 0.1$ by the Kolmogorov–Smirnov test and the stimulated OFF distribution was left-shifted from the unstimulated distribution.

We defined BRIEF neurons as having rPRGs ON and dPRGs OFF, SUSTAINED neurons as having dPRGs ON, and inactive neurons as having rPRGs and dPRGs OFF. For most classification of BRIEF and SUSTAINED neurons, we used the lists of rapid PRGs and delayed PRGs defined in Figure 2.1 of this paper. We also defined dPRGs among significantly induced genes in the in vivo data: dPRGs showed significant induction at 4h (FDR<0.05, 2-fold induction, unpaired, two-sided rank-sum test on bulk neurons) similar (<1.4 fold different) expression at 1h and 4h following stimulus. In this analysis, we defined genes for each layer individually.

For the analyses to determine whether the population of BRIEF neurons was significant, we asked whether BRIEF neurons were responding to the visual stimulus or reflective of an expected proportion of rapid-PRG-expressing cells among unstimulated neurons. We compared neurons from the stimulated cortex classified as having dPRGs OFF to unstimulated neurons. We

used a Fisher's exact test to assess enrichment for rPRG-ON cells among d-PRG-OFF cells compared to unstimulated cells, expecting an odds ratio not equal to 1 if there was a difference in the proportion of BRIEF neurons between dPRG-OFF neurons and unstimulated neurons. We performed this analysis on all excitatory neurons together as well as for each layer individually.

Differential gene expression analysis

Differential gene expression analysis was performed using an unpaired, two-sided Wilcoxon rank-sum test comparing all BRIEF neurons to all SUSTAINED neurons. We confirmed that the package Monocle2 (Trapnell et al., 2014) gave us identical results. We also performed DE analysis using DECENT (Ye et al., 2017) and used it to generate imputed read counts. DECENT had greater power to detect differentially expressed genes, but revealed similar trends (i.e., differential expression of deep layer markers in BREIF neurons).

RNA-seq

Expression analysis

We quantified pre-mRNA transcription using intron reads from total RNA-seq data (Gaidatzis et al., 2015; Gray et al., 2014).

For ARG-seq and total RNA-seq figures, we plotted a mean of the control-normalized expression levels for each gene from several biological replicates. All p-values reported in the figure legends for comparisons between two groups of genes are from an unpaired non-parametric two-tailed Wilcoxon rank-sum test (unless otherwise noted). A paired test was used when comparing between the same set of genes in two conditions. We confirmed significance using a two-tailed Student's T-test (log-normalized if comparing fold-inductions). We also confirmed that the differences observed via analysis of the mean expression levels were replicated in each biological replicate individually ($p < 0.05$, rank-sum test).

For ARG-seq and eRNA-seq, we confirmed using the Tukey HSD test in conjunction with ANOVA that expression from control genes or control enhancers in read-depth-normalized samples and spike-in-normalized samples is not affected by membrane depolarization, visual stimulation, or addition of U0126/SL327 (adjusted $p > 0.8$).

Comparison to other gene lists

Comparison between the genes induced in our study in vitro and the genes induced in three in vivo brain studies was performed with lists generated in a previous study (Cho et al., 2016). Comparison between the genes induced in our study in vitro and the genes induced in mouse macrophages and human cancer cell lines was performed using gene lists of induced genes generated by the authors of the previous studies (Escoubet-Lozach et al., 2011; Tullai et al., 2007). Human cancer cell line genes were converted to their mouse orthologs using the Mouse Genome Database (Blake et al., 2017) prior to analysis.

Analysis of photometry signals

All data analysis of GCaMP6 photometry signals was performed in MATLAB (Mathworks). For estimating the time course of changes in V1 calcium activity during each presentation of a 60-s lights-on stimulus onset (and during the first hour of recording in the dark, during 'pseudo-trials' in which the light was not actually switched on), we first estimated the mean GCaMP6 fluorescence in the 10-s period prior to stimulus onset, F_0 . We then calculated the fractional change in fluorescence at each time point from -20 s to 80 s relative to stimulus onset, as $(F(t) - F_0)/F_0$.

ChIP-seq

H3K27ac

We confirmed using the Tukey HSD test in conjunction with ANOVA that read-depth-normalized signal at control enhancers was not affected by stimulation or by addition of U0126 (adjusted $p > 0.8$). We also performed one replicate using *Drosophila* spike-in chromatin (Active Motif #61686, #53083) according to the instructions of the manufacturer and observed that U0126 treatment did not result in global H3K27ac changes. The plots shown only include enhancers that should and increase in H3K27ac with neuronal activity: 248 of the 940 putative enhancers reproducibly gain H3K27ac within the first hour of stimulation in two biological replicates (> 1.3 -fold change). All p -values reported are from the two-tailed non-parametric Wilcoxon rank-sum tests, but we confirmed significance using the Student's t -test. Unpaired tests were used if comparing between two groups of enhancers, and paired tests were used if comparing between the same group of enhancers in two conditions. We also performed a Student's t -test comparing the mean signal across all enhancers from each replicate for each gene class without U0126 to the mean signal across enhancers from each gene class with U0126 and found no significant difference ($p > 0.6$). We also compared each enhancer individually, and again found no significant change in H3K27ac signal at any enhancer with U0126 ($p > 0.9$, Bonferroni corrected).

Pol2

Additional analysis was performed in R. Given across-sample variability in read-depth- and input-normalized data, the samples were further normalized to Pol2 ChIP-seq density measured at constitutively active, non-activity-regulated control gene promoters—similar to the across-sample ChIP-seq normalization methods adopted by others for quantitative analysis of peaks (Shao et al., 2012). Specifically, data from each sample was normalized to the median value of a distribution of Pol2 density values occurring at ~800 constitutively active TSSs (-300 to +300bp) with unchanging mRNA levels under KCl as measured by RNA-seq (Kim, et al. 2011). As

a separate analysis, rPRG and dPRG TSS lists were filtered for TSS's with mean Pol2 ChIP-seq density greater than a threshold condition defined as two standard deviations above the mean value of un-expressed (Kim, et al. 2011) negative control TSS. For fold change analysis, fold-change was calculated at each TSS using the average unstimulated Pol2 density value obtained from two DMSO- and two U0126- treated samples.

Published ChIP-seq Data

For the enhancer data, in addition to the data shown in the figures, we also compared only those rapid and delayed enhancers near delayed PRGs. In unstimulated neurons, for SRF, CREB, MEF2, MED23, MED1 and NCoR we compared binding -6kb to +6kb from the centers of rapid enhancers compared to delayed enhancers and as reported in the main text found greater binding at rapid enhancers ($p < 0.009$, rank-sum test, including only enhancers within 100 kb of delayed PRGs). Active histone marks H3K27ac, H3K4me2, H3K4me1, and H4K16ac were also higher in a comparison of the same rapid compared to delayed enhancers in unstimulated neurons ($p < 0.01$, rank-sum test, only enhancers within 100 kb of delayed PRGs).

DATA AVAILABILITY

The RNA-seq and ChIP-seq have been deposited in GEO under ID code GSE111899.

APPENDIX 2.

SUPPLEMENTAL MATERIALS RELATED TO CHAPTER II

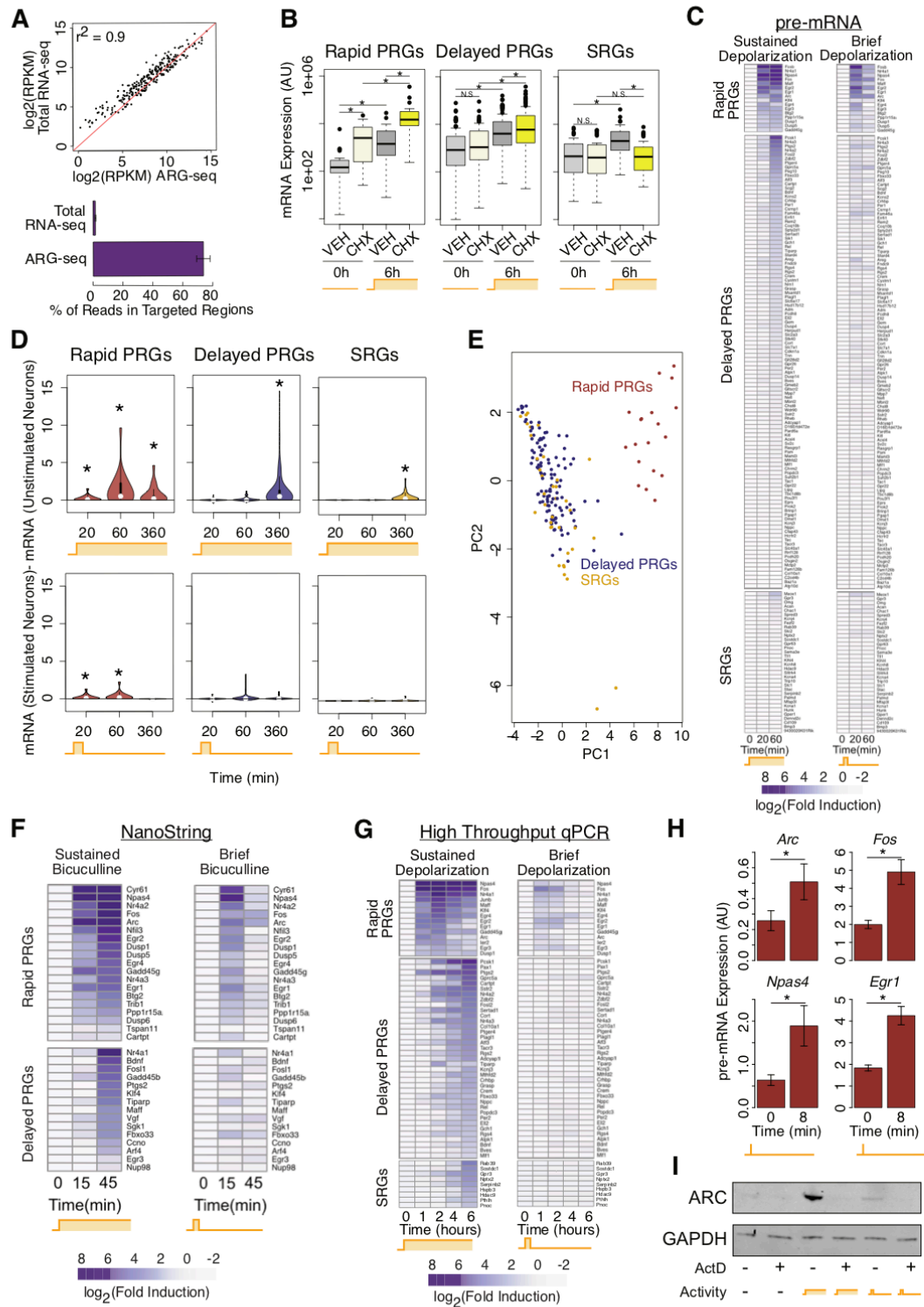
This appendix includes the supplementary figures and tables related to the results in Chapter II. This Appendix was originally published in: Tyssowski KM, DeStefino NR, Cho JH, Dunn CJ, Poston RG, Carty CE, Jones RD, Chang SM, Romeo P, Wurzelmann MK, Ward JM, Andermann ML, Saha, RN, Dudek SM, Gray, JM.: Different Neuronal Activity Patterns Induce Different Gene Expression Programs. Neuron 2018, 98:530–546.e11.

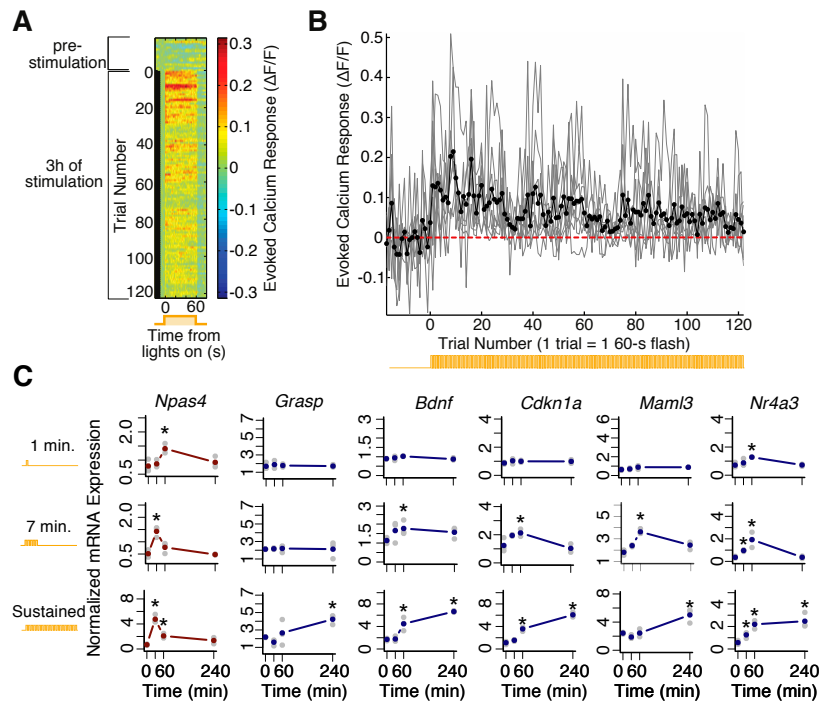
Author contributions same as in Chapter II.

SUPPLEMENTARY FIGURE A2.1. BRIEF NEURONAL ACTIVATION INDUCES ONLY THE FIRST OF THREE WAVES OF GENE INDUCTION

A. Top: Gene induction from total RNA-seq and ARG-seq are highly correlated (r^2 by Pearson correlation). Shown is one representative comparison of one biological sample prepared with total RNA-seq and ARG-seq. Bottom: ARG-seq provides ~76-fold enrichment of reads in targeted regions. Error bars are $\pm$ SEM from $n=12$ libraries. **B.** Summary of primary response gene (PRG) and secondary response gene (SRG) sensitivity to the translational inhibitor cycloheximide (CHX), after distinguishing SRGs from PRGs based on >50% reduction in expression in the presence of CHX at 6 hours of stimulus and $FDR < 0.05$ by edgeR (* $p < 0.01$, paired rank-sum test, means for each gene from $n=2$ biological replicates). **C.** There is little pre-mRNA induction of delayed PRGs (dPRGs) with brief neuronal activation, based on total RNA-seq comparison of pre-mRNA induction using reads that align to annotated introns. Shown are means from $n=2$ biological replicates. No dPRG or SRG pre-mRNAs are significantly induced in response to brief activity ($FDR > 0.05$). Genes induced by brief membrane depolarization are enriched for rapid PRGs (rPRGs) at the pre-mRNA level ($p < 10^{-8}$, Fisher's exact test). Gene classes as in Figure 2.1. **D.** The greater response of rPRGs to brief stimulation is unlikely to be an artifact of their greater induction overall, as shown in violin plots. Note in particular the 360-minute time point for dPRGs at top (sustained) and bottom (brief) and compare to the 60-minute time point for rPRGs at top and bottom. Same data as in Figure 2.1B, with biological replicates averaged prior to plotting. **E.** Principal component analysis of the kinetics of gene induction distinguishes rPRGs from dPRGs and SRGs. Based on data shown in Figure 2.1B, see methods for details. **F.** Bicuculline/4AP treatment in rat neurons recapitulates rPRG and dPRG differences observed in mouse neurons with KCl-depolarization, based on NanoString comparison of gene induction upon sustained or brief neuronal activation using bicuculline/4AP treatment in rat cortical neurons. Shown are means from $n=3$ biological replicates. Only genes induced >2 fold at any time point are shown. Genes induced by brief membrane depolarization are enriched for rPRGs ($p=0.001$, Fisher's exact test). rPRGs were defined based on reaching 50% of their maximum induction by 15 minutes or having greater than 4-fold induction by 15 minutes. Delayed genes are mostly dPRGs, based on their classification in mouse, although we did not confirm this via translational inhibition in rat neurons. **G.** Validation of RNA-seq and ARG-seq results using a high-throughput qPCR (Fluidigm)-based comparison of gene induction in response to sustained or brief neuronal activation with KCl-mediated depolarization. Shown are means from $n=4$ biological replicates. Genes induced by brief membrane depolarization are enriched for rPRGs ($p < 10^{-7}$, Fisher's exact test). Gene classes as in Figure 2.1, and this data is the same as that shown in Figure 2.1C. **H.** 10-second treatment with bicuculline/4AP followed by TTX induces rPRG pre-mRNA. Plotted is means from $n=5$ biological replicates. Error bars are $\pm$ SEM * $p < 0.05$, paired student's t-test. **I.** ARC protein is induced by brief neuronal activation in a transcription-dependent manner. Representative western blot of ARC protein expression two hours after sustained or brief KCl-depolarization in the presence or absence of the transcriptional inhibitor Actinomycin D (ActD) (10 μ g/mL). $n=1$ of 3 biological replicates.

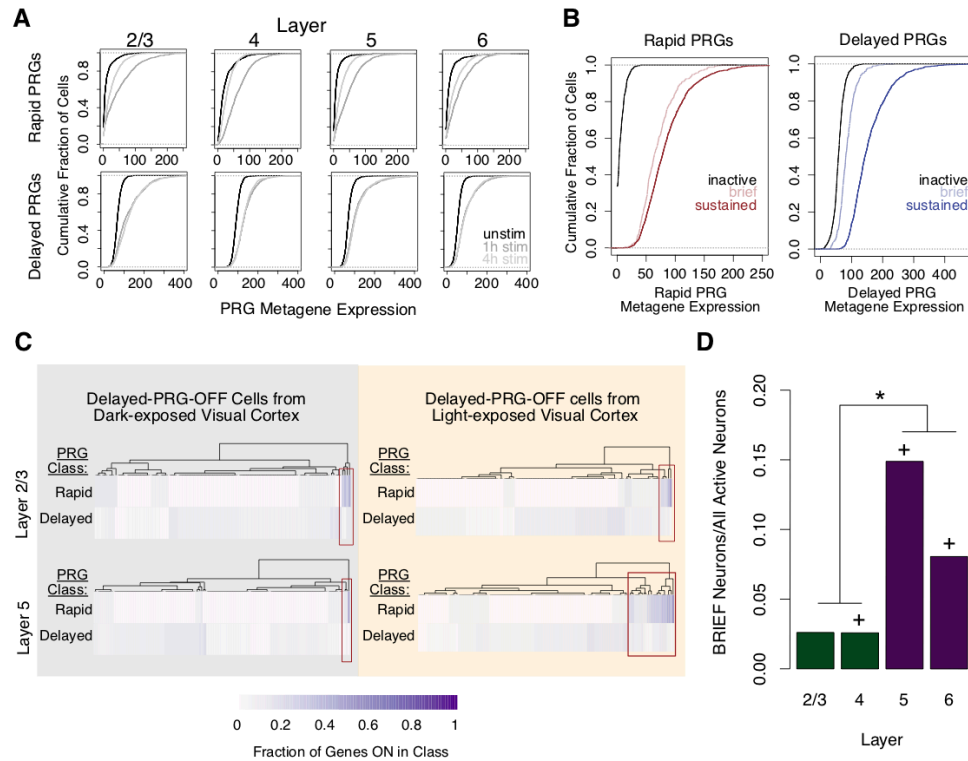
Supplementary Figure A2.1 (continued)





SUPPLEMENTARY FIGURE A2.2. BRIEF VISUAL STIMULATION INDUCES ONLY THE FIRST OF THREE WAVES OF GENE INDUCTION IN VIVO

A. In vivo fiber photometry recording of light-evoked changes in bulk neuronal calcium activity in primary visual cortex (V1). Mice were exposed to up to 3 hours of a repeated visual stimulus of lights-on for 60s followed by lights-off for 20s. Each row represents a time course of activity (fractional change in fluorescence; $\Delta F/F$; positive signals indicate net increase in calcium activity in V1 neurons) during presentation of a single lights-on stimulus, averaged across 8 sessions (8 mice, 1 session/mouse). There was a significant, ~13% increase in activity for the first trial ($p = 0.04$) compared to the pre-stimulus no-light trials (trials lacking a black bar at right and numbered less than 0 in the plot). Visual stimulation in the first hour of stimulation evoked a 9.3% average increase in activity ($p = 0.0005$). Visual stimulation in the second hour evoked a 6.1% average increase in activity ($p = 0.0005$). Visual stimulation in the third hour evoked a 4.7% average increase in activity ($p = 0.00007$). p-values: one-sided Student's t-test ($n = 8$ mice). **B.** Same data as in (A). Gray lines indicate mean $\Delta F/F$ for each trial (from 0-60 s post stimulus onset compared to the 10 s prior to stimulus onset) for each of the eight individual mice. Black line is the mean evoked response across mice, with each dot representing the mean evoked response for a single trial. **C.** Additional genes beyond those shown in Fig. 1E, showing ARG induction in the visual cortex following visual stimulation, as measured by qPCR. Colored points are mean of 3 biological replicates. Gray points are values from individual biological replicates. Gene categories defined as in Figure 2.1. *significant induction compared to 0h time point, $p < 0.05$ Student's t-test, fold induction > 1.5 .



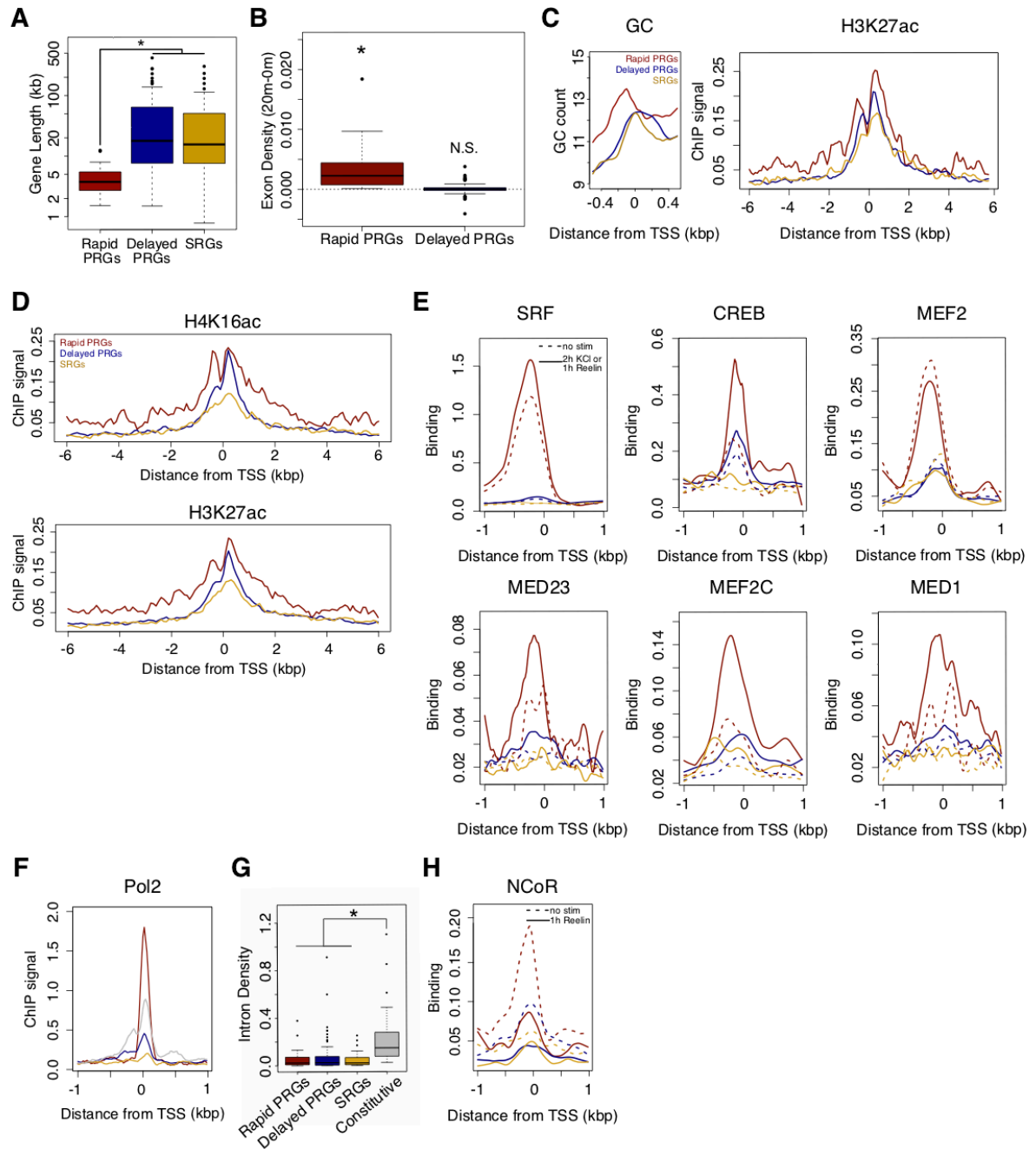
SUPPLEMENTARY FIGURE A2.3. NEURONAL ACTIVITY PATTERN CAN BE INFERRED FROM ARG EXPRESSION

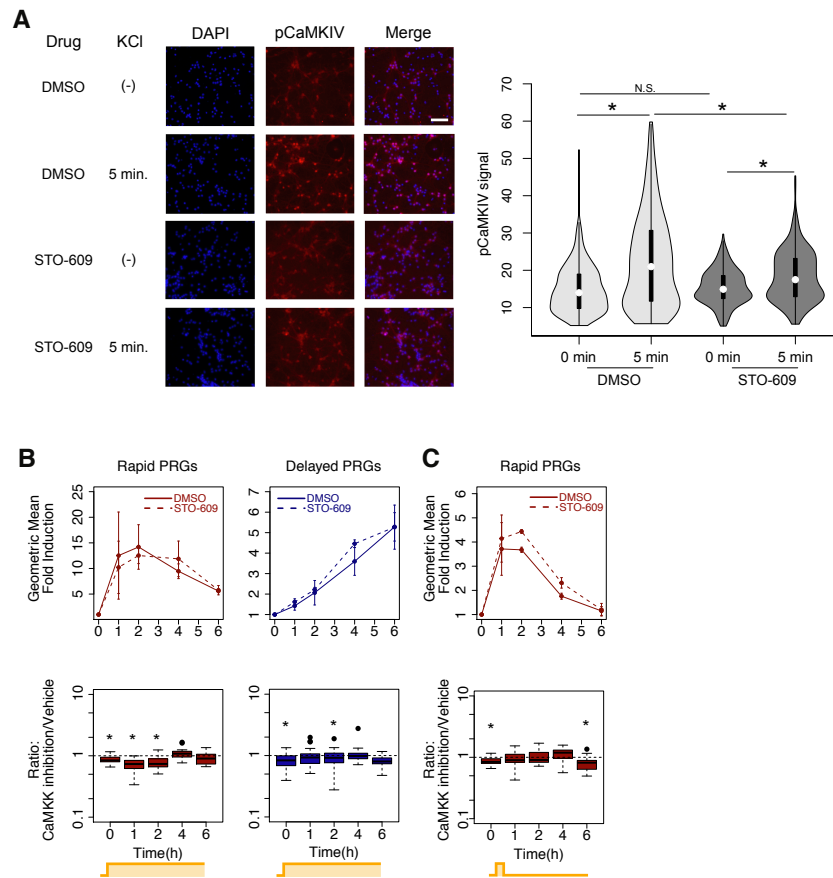
A. Both rPRGs and dPRGs are induced by 1h of visual stimulation in all cortical layers ($p < 10^{-7}$, rank-sum test). Metagene expression is average expression of all rPRGs (19) or dPRGs (116) from read-depth-normalized data for each timepoint in each layer. scRNA-seq data (Hrvatin et al., 2017) is from the visual cortex of mice exposed to sustained visual stimulus. Evidence of induction of dPRGs in all layers suggests that our gene list is not biased toward any layer. The consistent dPRG induction across layers does not invalidate our finding of more putative BRIEF neurons in deeper layers, since the BRIEF neurons we found are a relatively small minority population. **B.** Expression of rPRG and dPRG metagenes in BRIEF, SUSTAINED, and inactive neurons, as classified in Figure 2.2. Metagene expression was computed as in (A), except that they were made from cell populations defined based on activity state instead of layer. **C.** Layer 5 has more putative BRIEF (rPRG-expressing dPRG OFF neurons) in the light than in the dark ($p = 0.002$, OR = 0.47, Fisher exact test), suggesting that rPRG expression in BRIEF neurons in the light is due to visual cortex stimulation (see methods). The number of putative BRIEF neurons in the light and dark is similar in layer 2/3 ($p = 0.4$, OR=1.6, Fisher exact test). Each box in the heatmap represents a neuron. The color of the box represents the fraction of either rPRGs (top) or dPRGs (bottom) that are induced in that neuron (out of all rPRGs or dPRGs induced in that layer). The dendrogram represents hierarchical clustering using Euclidean distance. Red boxes indicate clusters of neurons that have large number of rPRGs induced. **D.** Deep compared to upper layers are enriched for BRIEF neurons ($*p < 10^{-15}$, Fisher exact test). Similar to Figure 2.2G, but BRIEF and SUSTAINED neurons defined using dPRG lists made specifically for each layer. +significant population of BRIEF neurons; $p < 0.001$, Fisher exact test comparing the number of rPRG ON neurons among dPRG OFF neurons in the stimulated cortex to the number of rPRG ON neurons among unstimulated neurons.

SUPPLEMENTARY FIGURE A2.4. RAPID PRGs HAVE OPEN CHROMATIN AND PRE-BOUND TRANSCRIPTION FACTORS

A. rPRGs are shorter than dPRGs and SRGs ($p < 10^{-6}$, rank-sum test). Genes classified as in Figure 2.1. **B.** Reads from total RNA-seq that map to first exons of genes in each category shown on the y-axis as the increase from 0 to 20 minutes of KCl-depolarization, normalized by the length of the exon (read density) (show is one of $n=2$ biological replicates), $*p < 0.01$, rank-sum test for both biological replicates, difference from zero. **C.** Chromatin state at rPRGs, dPRGs, and SRGs, shown as metaplots of the geometric mean by gene category. ChIP-seq data is from unstimulated mouse neurons. GC content and H4K27ac are significantly different (sum of the region shown) between rPRGs and dPRGs or SRGs ($p < 0.01$, rank sum test). **D.** rPRGs have a more active chromatin state than dPRGs or SRGs in vivo. Chromatin state at rPRGs, dPRGs, and SRGs is shown as metaplots as in (C). ChIP-seq data is from mouse hippocampus without specific hippocampal activation. H4K16ac and H3K27ac are significantly different between rPRGs and dPRGs (or SRGs, $p < 10^{-5}$, rank sum test on area under the curve). ChIP-seq data from Telese et al., 2015. **E.** Transcription factor binding in unstimulated and stimulated mouse neurons from ChIP-seq, shown as metaplots as in (C). MED23 and MED1 binding is greater at rPRGs than dPRGs in unstimulated neurons ($p < 0.009$, rank sum test, for significance of SRF, MEF2, and CREB in unstimulated neurons see figure 2.3B). For SRF and CREB stimulation was with 2h of KCl-mediated depolarization (Kim et al., 2010). For MEF2, MEF2C, MED1, and MED23 stimulation was with 1h of reelin (Telese et al., 2015). SRF, CREB, MEF2, MEF2C, MED1, and MED23 binding is greater at rPRGs than dPRGs or SRGs in stimulated neurons ($p < 0.0014$, rank-sum test). Stimulated signal shown for comparison. **F.** rPRGs and constitutively expressed control genes have more Pol2 binding in unstimulated neurons from ChIP-seq than dPRGs or SRGs ($p < 0.04$, rank-sum test for area under the curve shown). Data from Kim et al., 2010. **G.** There is no difference in transcription of rPRG, dPRG, and SRG classes in unstimulated neurons based on pre-mRNA expression levels from RNA-seq ($p > 0.5$, rank sum test). $* = p < 10^{-5}$, rank-sum test. **H.** NCoR is selectively bound to rPRGs in unstimulated neurons, and its binding is reduced with stimulation ($p < 0.009$, rank sum test). Stimulation was with 1h of reelin (Telese et al., 2015).

Supplementary Figure A2.4 (continued)





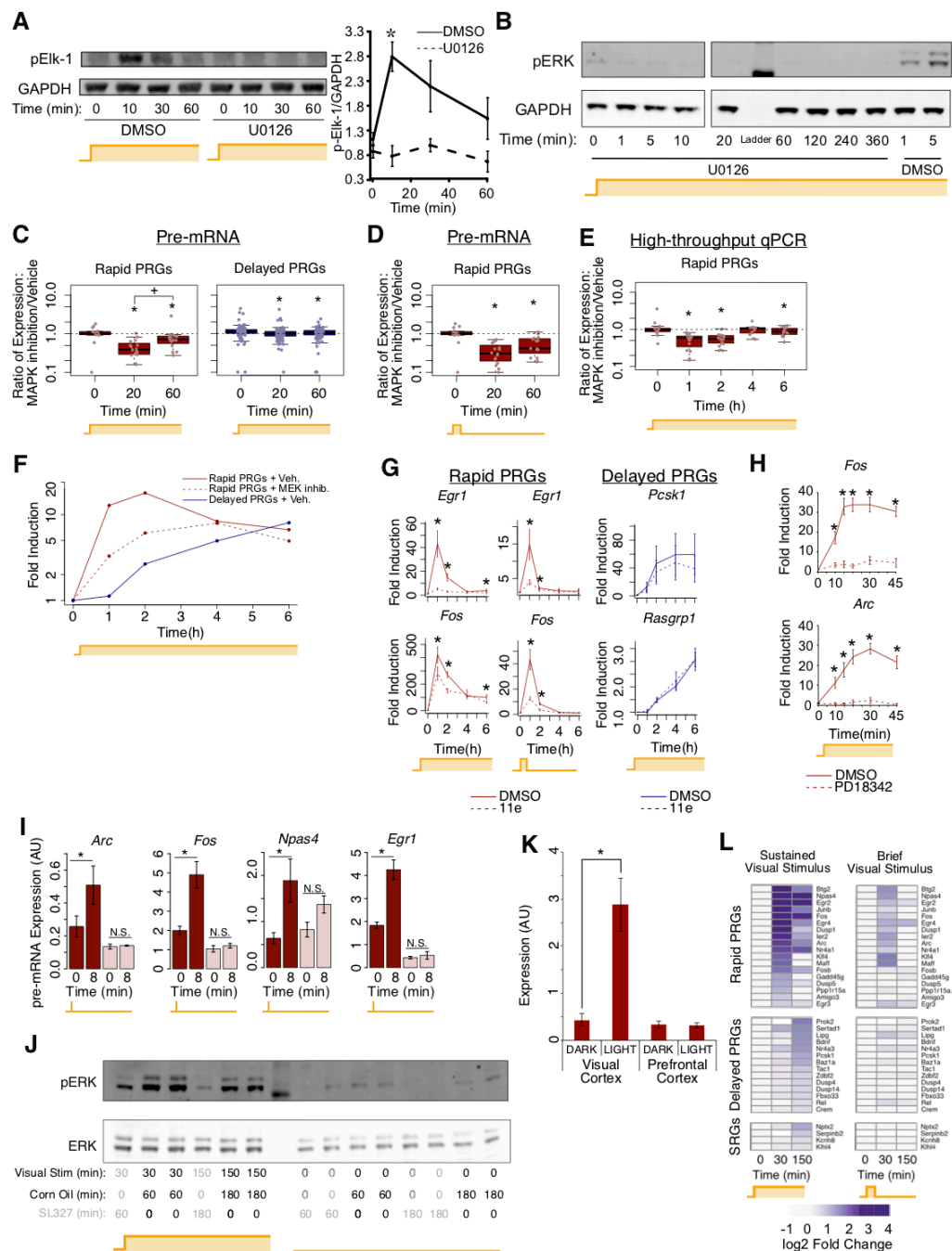
SUPPLEMENTARY FIGURE A2.5. THE CAMKK/CAMKIV PATHWAY IS NOT REQUIRED FOR PRG INDUCTION

A. Left: immunocytochemistry for phospho-CaMKIV reveals pathway activation by 5 minutes of KCl-mediated membrane depolarization, seen by an increase in nuclear staining. 3 μ M of the CaMKK inhibitor STO-609 blocks nuclear accumulation of phospho-CaMKIV. Scale bar = 50 μ m, same for all images. Right: Quantification of nuclear phospho-CaMKIV signal (i.e., signal the overlaps with DAPI). Shown is a representative example of n=2 biological replicates. *p<0.00001, unpaired, two-tailed, t-test. **B.** High-throughput qPCR-based gene expression for 15 rPRGs (left) and 35 dPRGs (right) induced by sustained KCl treatment in the presence or absence of 3 μ M STO-609 (*significantly different from 1, p<0.05, rank-sum test). Metagene averages of fold induction (top) and boxplots showing expression of all tested genes (bottom). Error bars are +/- S.E.M. from each of n=2 biological replicates of geometric means of all genes in the category. **C.** Same data as in (B) but for brief (1-min.) KCl stimulation. Error bars are +/- S.E.M. from each of n=2 biological replicates of geometric means of all genes in the category.

SUPPLEMENTARY FIGURE A2.6. MAPK/ERK IS REQUIRED FOR THE FIRST (RAPID PRG) BUT NOT SUBSEQUENT WAVES OF GENE INDUCTION

A. Elk-1 phosphorylation is rapid and MAPK/ERK-dependent. Representative western blot using an antibody recognizing phosphorylated Elk-1 (pElk-1). On the right, quantification of western blotting from $n=2-3$ biological replicates. *pElk-1 blocked by MEK inhibition, $p<0.05$, unpaired Student's t-test. Error bars are $\pm$ S.E.M. **B.** The U0126 inhibitor blocks MAPK/ERK pathway activation throughout a time course of neuronal stimulation via KCl-mediated depolarization. Representative western blot using an antibody recognizing phosphorylated ERK (pERK). Upper and lower bands are the phosphorylated p44 and p42 ERK paralogs (ERK1 and ERK2), respectively. Mouse cortical neurons were activated with continuous 55mM KCl-mediated depolarization for the indicated amount of time in the presence (or absence) of MEK inhibitor U0126 (10 μ M added 30 minutes before stimulation). Samples were run on two blots that were run, transferred, and treated with antibody together. **C-D.** Total RNA-seq intron-aligned reads to measure pre-mRNA expression for all rPRGs or dPRGs in the presence or absence of the U0126 MEK inhibitor upon KCl-depolarization. (C) Sustained activation (D) Brief activation. (*significantly different from 1, $p<0.003$, rank-sum test, $^+p<0.05$, rank-sum test). Expression of rPRGs is less affected by MEK inhibition than expression of dPRGs ($p = 0.003$; rank-sum test, 16 rPRGs vs. 109 dPRGs using mean for each gene from $n=2$ biological replicates at the time point of maximum induction). **E.** High-throughput qPCR (Fluidigm) to measure mRNA expression for all PRGs in the presence or absence of the U0126 MEK inhibitor upon neuronal activation with KCl. Expression of rPRGs is less affected by MEK inhibition than expression of dPRGs ($p = 0.003$; rank-sum test, 15 rPRGs vs. 37 dPRGs using mean for each gene from $n=3-4$ biological replicates at the time point with maximal induction). *significantly different from 1, $p<0.004$, rank-sum test. **F.** MEK inhibition blunts and delays the first wave (rPRGs), making it more similar to the second wave (dPRGs), based on median expression of rPRGs and dPRGs from high-throughput qPCR (Fluidigm) upon KCl-depolarization. **G.** Expression of representative rPRG and dPRG mRNA measured by qPCR in the presence or absence of the ERK inhibitor 11e (625nM) upon sustained or brief neuronal activation with KCl. * $p<0.01$, paired, two-sided, t-test. Error bars represent $\pm$ S.E.M. **H.** Expression of representative rPRG pre-mRNA measured by qPCR in the presence or absence of the MEK inhibitor PD184352 (2 μ M) with sustained bicuculline/4AP treatment. * $p<0.01$, student's T-test. Error bars represent $\pm$ S.E.M. **I.** rPRG pre-mRNA induction in response to a 10s bicuculline treatment requires MAPK/ERK signaling. MEK inhibited by 10 μ M U0126. Plotted is means from $n=3-5$ biological replicates. Error bars represent $\pm$ S.E.M. * $p<0.05$, paired student's t-test. Vehicle-treated data same as Supplementary Figure A2.1H. **J.** SL327 blunts MAPK/ERK pathway activation in vivo. Representative western blot of visual cortices from dark-housed mice treated with an intraperitoneal injection of corn oil or the MEK inhibitor SL327 (100mg/kg) and then exposed to visual stimulation (or left in the dark). Blotting was performed with an antibody recognizing phosphorylated ERK (pERK). Upper and lower bands are the phosphorylated p44 and p42 ERK paralogs (ERK1 and ERK2), respectively. **K.** Visual stimulation selectively induces gene expression in the visual cortex but not prefrontal cortex. qPCR of *Fos* mRNA expression in the visual and pre-frontal cortex of mice exposed to visual stimulation. Mice were dark-housed for three days and exposed to light for thirty minutes. * $p<0.01$, $n=4$ mice. Error bars represent $\pm$ S.E.M. **L.** Profiling of gene expression in visual cortex before and after room-light visual stimulation, using ARG-seq. Only genes induced >1.4 fold in any condition in vitro were included (see methods). Data are means from $n=2-4$ mice. rPRG, dPRG, and SRG gene categories were defined from in vitro data as in Figure 2.1. Genes induced by brief visual stimulus are enriched for rPRGs ($p=0.03$, Fisher's exact test).

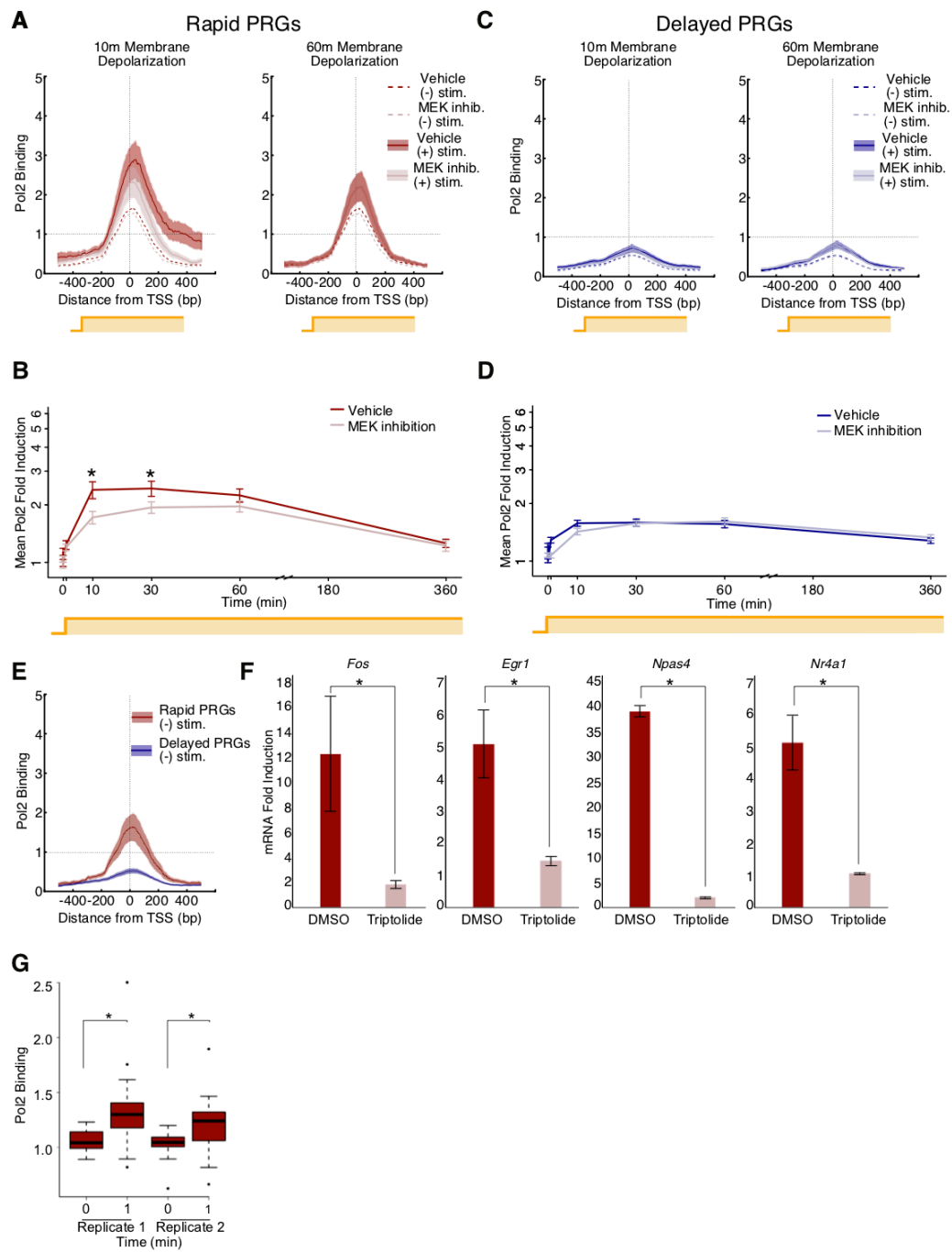
Supplementary Figure A2.6 (continued)



SUPPLEMENTARY FIGURE A2.7. MAPK/ERK MEDIATES FAST RECRUITMENT OF POL2 TO RAPID PRG PROMOTERS

A. RNA Polymerase 2 (Pol2) binding (ChIP-seq) at the promoters of rPRGs, 10 and 60 minutes after KCl-mediated neuronal activation in the presence or absence of MEK inhibitor U0126 (10 μ M). Solid lines represent the mean and shading the S.E.M. across loci. Data shown are from the second of two biological replicates (see Figure 2.5 for the first replicate). The KCl-dependent fold-increase in mean Pol2 density (-300bp to +300bp) is significant under both vehicle and U0126 treatments (FDR<0.001 in each of two biological replicates, paired rank sum test). **B.** ChIP-seq-based time course of fold-change in Pol2 occupancy at rPRG promoters (-300bp to +300bp), with or without MEK inhibition. Shown are mean fold-change values, with +/- S.E.M error bars. *FDR <0.01 in each of two replicates (see also Figure 2.5B), paired rank-sum test on fold-change values with adjustment for multiple comparisons. **C.** RNA Pol2 binding (ChIP-seq) at the promoters of dPRGs, as in (A). (KCl-dependent increase, FDR <0.001 in each of two biological replicates). **D.** ChIP-seq-based time course of Pol2 occupancy at dPRG promoters. As in (B). The KCl-dependent fold-increase in mean Pol2 density (-300bp to +300bp) is significant under both vehicle and U0126 treatments (*FDR<0.001 in each of two biological replicates paired rank sum test). **E.** Promoter-binding of Pol2 in unstimulated neurons at rPRG and dPRG promoters (significantly different, $p<0.001$, rank sum test on area from -300 to +300). **F.** rPRG mRNA fold-change qPCR measurements following 30-minutes of depolarizing KCl, in the presence of vehicle or the transcription initiation blocker triptolide (10 μ M, 5-minute pre-treatment). * $p<0.05$ one-tail Student's t-test on log-normalized fold-change values. Error bars represent +/- S.E.M. **G.** Average unstimulated and one-minute rPRG promoter-bound Pol2 KCl fold-change values in samples treated with the vehicle, shown for two biological replicates. *FDR <0.01.

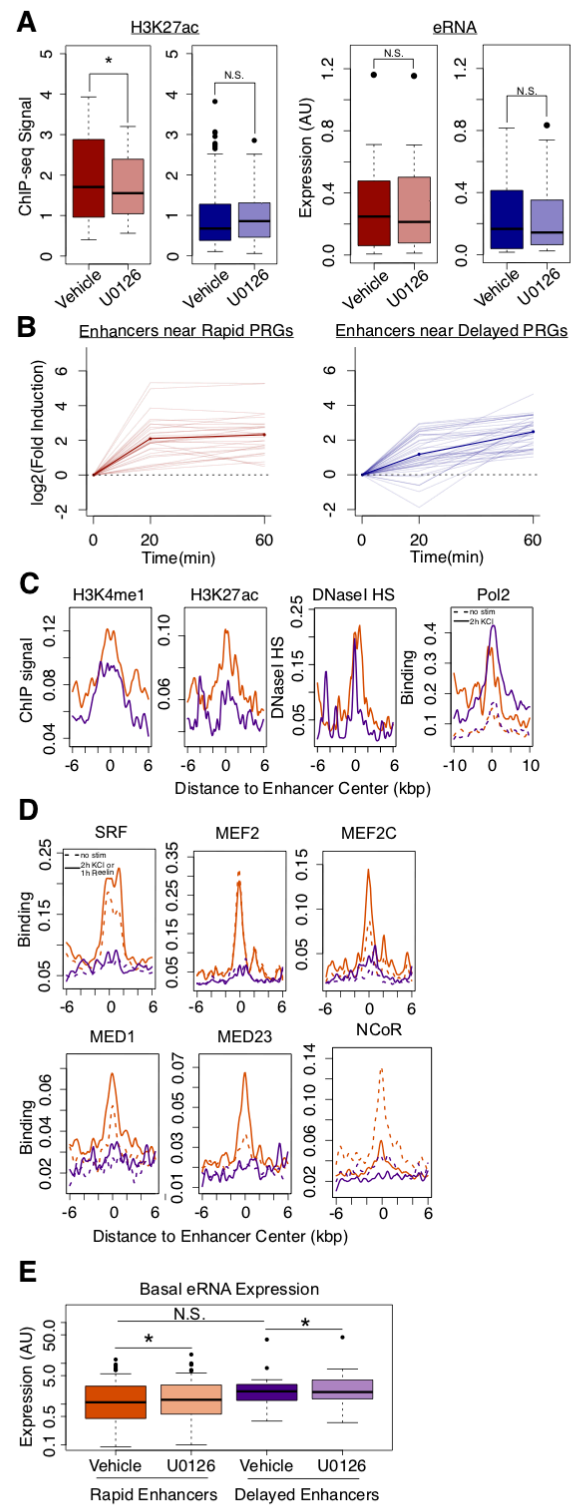
Supplementary Figure A2.7 (continued)



SUPPLEMENTARY FIGURE A2.8. MAPK/ERK IS REQUIRED FOR RAPID ERNA INDUCTION BUT NOT H3K27 ACETYLATION AT ENHANCERS

A. Effect of MEK inhibition on H3K27ac and eRNA expression in unstimulated neurons. There is a slight effect of MEK inhibition on H3K27ac in unstimulated neurons (* $p < 0.02$, rank-sum test). H3K27ac from ChIP-seq and eRNA from total RNA-seq. **B.** Enhancers near rPRGs exhibit more rapid induction than enhancers near dPRGs, based on eRNA-seq (as done in Figure 2.7C with total RNA-seq data). Rapid enhancers have greater induction at 20 minutes than delayed enhancers ($p = 0.01$, rank-sum test). The dark line is the geometric mean of all enhancers shown and light lines represent individual enhancers. Enhancer expression kinetics roughly mirror that of nearby promoters. **C.** DNaseI HS and H3K27ac and H3K4me1 occupancy prior to stimulation is greater at rapid than delayed enhancers (based on area under the curves, $p < 0.01$, rank sum test). Pol2 binding is not significantly different between rapid and delayed enhancers ($p > 0.1$). Shown as metaplots of the geometric mean of the signal all enhancers in the category. H3K4me1 and Pol2 ChIP-seq data from Kim et al., 2010. DNaseI HS data from ENCODE. **D.** Transcription regulator binding in unstimulated and stimulated mouse neurons from ChIP-seq also shown as metaplots as in (C). MEF2C and MED1 binding is greater at rapid enhancers than delayed enhancers in unstimulated neurons ($p < 0.01$, rank-sum test, for significance of other transcription factors in unstimulated neurons see figure 2.7G). For SRF, stimulation was with 2h of KCl-mediated depolarization (Kim et al., 2010). For MEF2, MEF2C, MED1, NCoR and MED23 stimulation was with 1h of reelin (Telese et al., 2015). SRF, MEF2, MEF2C, MED1, and MED23 binding is greater at rapid enhancers than delayed enhancers in stimulated neurons ($p < 0.011$, rank-sum test). NCoR binding is lost with stimulation. Stimulation shown for comparison. **E.** eRNA expression at rapid and delayed enhancers in unstimulated neurons in the presence or absence of U0126. There is no statistically significant difference between rapid and delayed enhancers under vehicle treatment ($p > 0.01$, rank-sum test). There is a very minor yet significant effect of U0126 treatment on both classes of enhancers (* $p < 0.01$, rank-sum test). Data from eRNA-seq.

Supplementary Figure A2.8 (continued)



SUPPLEMENTARY TABLE A2.1. TARGETED CAPTURE OLIGONUCLEOTIDES.

Oligonucleotides used to make biotinylated probes for ARG-seq and eRNA-seq. Sequences include in-vitro-transcription promoters and amplification primer sites. *Electronic attachment.*

SUPPLEMENTARY TABLE A2.2. ARG-SEQ AND ERNA-SEQ DATA.

Data from each replicate of each experiment normalized for read depth and by controls, as in methods. *Electronic attachment.*

SUPPLEMENTARY TABLE A2.3. FLUIDIGM HIGH-THROUGHPUT QPCR DATA AND PROBES.

Data from high-throughput qPCR from neurons stimulated with brief and sustained KCl-depolarization. Neurons treated with a DMSO vehicle, 10 μ M U0126 or 3 μ M STO-609. *Electronic attachment.*

SUPPLEMENTARY TABLE A2.4. NANOSTRING DATA.

Data from NanoString from bicuculline-treated neurons. *Electronic attachment.*

SUPPLEMENTARY TABLE A2.5. FUNCTIONAL ANNOTATION OF ARGs BY CLASS.

Functional annotations of each ARG available using PANTHER (Mi et al., 2017). Genes were identified as directly regulating transcription if they were annotated as transcription factors/cofactors or as binding to DNA. Genes were identified as indirectly regulating transcription if they were annotated as part of a signaling pathway likely to regulate transcription. Genes were also identified as indirectly regulating transcription if they are not channels, receptors, or secreted proteins that were annotated as regulating transcription but not as transcription factors or binding to DNA. *Electronic attachment.*

SUPPLEMENTARY TABLE A2.6. QPCR PRIMERS, RELATED TO STAR METHODS.

DNA primer name	Sequence	Species	RNA
Fos Fw	GGCTCTCCTGTCAACACACA	Mouse	mRNA
Fos Rv	TGTCACCGTGGGGATAAAGT	Mouse	mRNA
Egr1 Fw	GGGATAACTCGTCTCCACCA	Mouse	mRNA
Egr1 Rv	CCTATGAGCACCTGACCACA	Mouse	mRNA
Pcsk1 Fw	TGCAGGTGAAATTGCCATGC	Mouse	mRNA
Pcsk1 Rv	GGCCAGGGTTGAATCCAATTG	Mouse	mRNA
Rasgrp1 Fw	TGACAACTGTGCTGGCTTTC	Mouse	mRNA
Rasgrp1 Rv	TGCACTGTTTGTGGCAGTTC	Mouse	mRNA
Egr2 Fw	TTGACCAGATGAACGGAGTG	Mouse	mRNA
Egr2 Rv	TGCCCATGTAAGTGAAGGTC	Mouse	mRNA
Scg2 Fw	CAGAGAGGAGCATGCTTGGA	Mouse	mRNA
Scg2 Rv	TCGGCTCCAGAGATGAGGAA	Mouse	mRNA
Crem Fw	GCAAAAGCCCAACATGGCTG	Mouse	mRNA
Crem Rv	CATGTGACTTGGGGCAAGGT	Mouse	mRNA
Cdkn1a Fw	CGGTGTCAGAGTCTAGGGGA	Mouse	mRNA
Cdkn1a Rv	AGGATTGGACATGGTGCCTG	Mouse	mRNA
Grasp Fw	CAGCACTGGAGGACTATCACC	Mouse	mRNA
Grasp Rv	TCTTCCAACGGAATCCTGAGC	Mouse	mRNA
Maml3 Fw	AGCACCCGAGTAAAACCCAG	Mouse	mRNA
Maml3 Rv	CCATTCTGCTGGTCCCCATT	Mouse	mRNA
Nr4a3 Fw	CTCTAAAGACGGAACCGCCA	Mouse	mRNA
Nr4a3 Rv	GTCGGGATAGGCGAAGCAG	Mouse	mRNA
Npas4 Fw	GGGTGTCTTCTTTGCTGGAG	Mouse	mRNA
Npas4 Rv	CTGCCACAATGTCTTCAAGC	Mouse	mRNA
Bdnf Fw	TCCACCAGGTGAGAGTG	Mouse	mRNA
Bdnf Rv	GCCTTCATGCAACCGAAGTA	Mouse	mRNA
Gapdh Fw	CGTCCCGTAGACAAAATGGT	Mouse	mRNA
Gapdh Rv	TCGTTGATGGCAACAATCTC	Mouse	mRNA
Arc Fw	GAATTTGCTATGCCAACTCACGGG	Rat	pre-mRNA
Arc Rv	AGTCATGGAGCCGAAGTCTGCTTT	Rat	pre-mRNA
Fos Fw	ACAGCCTTTCCTACTACCATTTCC	Rat	pre-mRNA
Fos Rv	CTGCACAAAGCCAACTCACCTGT	Rat	pre-mRNA
Npas4 Fw	GTTGCATCAACTCCAGAGCCAAGT	Rat	pre-mRNA
Npas4 Rv	ACATTTGGGCTGGACCTACCTTCA	Rat	pre-mRNA
Egr1 Fw	TTCGGCTCTCATCGTCCAGTGATT	Rat	pre-mRNA
Egr1 Rv	AACCGGGTAGTTTGGCTGGGATAA	Rat	pre-mRNA
Gapdh Fw	AACATGCACAGGGTACTTCGAGGA	Rat	pre-mRNA
Gapdh Rv	ACGACATACTCAGCACCAGCATCA	Rat	pre-mRNA

SUPPLEMENTARY TABLE A2.7. POL2 CHIP-SEQ, NORMALIZED DATA.

Electronic attachment.

SUPPLEMENTARY TABLE A2.8. H3K27AC CHIP-SEQ, NORMALIZED DATA.

Electronic attachment.

APPENDIX 3.

PRELIMINARY DATA RELATED TO CHAPTER II

This chapter includes preliminary data related to the results in Chapter II. I performed all experiments, analyzed data, and wrote this section. Jesse Gray advised.

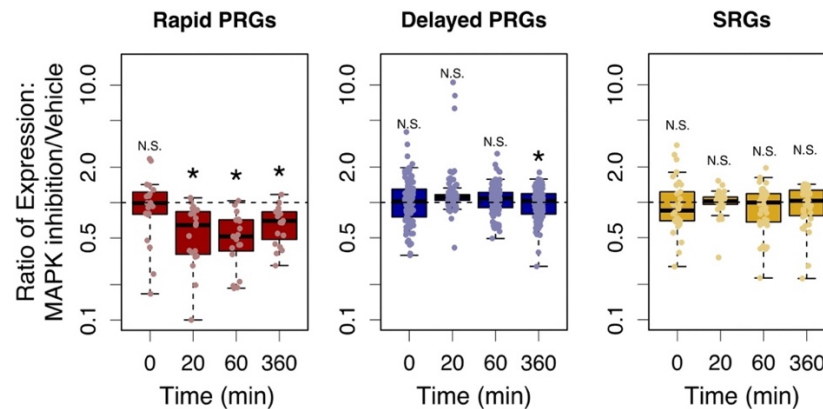
EFFECT OF MAPK/ERK INHIBITION ON SRG INDUCTION

MAPK/ERK signaling is required for rapid induction of rPRGs, and many rPRGs are thought to regulate transcription of SRGs. Therefore, SRGs could be regulated by MAPK/ERK signaling indirectly via rPRG protein products. To assess this possibility, I analyzed the ARG-seq data described in Chapter II to determine the effect of U0126 (a MAPK/ERK inhibitor) treatment on gene SRG induction. This analysis was left out of the paper because it is hard to tell if effects are direct or indirect and the goal of the paper was to find mechanistic differences between rPRGs and dPRGs.

Surprisingly, I found that MAPK/ERK pathway inhibition had no effect on SRG induction at any time point (Supplementary Figure A3.1). (Note that when I did this with Fluidigm high-throughput qPCR, it seemed that SRGs might turn on slightly slower in the presence of U0126, but this could be due to the selection of the few SRGs tested not being representative. However, it could also be due to inclusion of the 2h and 4h time points. ARG-seq with more time points could answer this question).

It is somewhat surprising that MAPK/ERK pathway inhibition has little effect on SRG induction, given its large effect on rPRG induction. However, since rPRGs are eventually induced in response to sustained stimulation in the presence of MAPK/ERK inhibition, it is possible that this delayed induction is sufficient to drive SRG induction. However, the finding that MAPK/ERK signaling is not required for SRG induction suggests that rapid induction of rPRG transcription factors is not required for SRG induction. This raises the question: why are rPRGs induced so quickly? Is there any function of the rapidity of their induction? Perhaps the rapidity is more important for the effector genes like *Arc*. It is also possible, as mentioned above, that SRGs are

induced slightly slower in the presence of MAPK/ERK inhibition but that we do not have the right time points to detect this.



SUPPLEMENTARY FIGURE A3.1. MAPK/ERK INHIBITION DOES NOT AFFECT SRG INDUCTION

Induction of rPRGs, but not dPRGs or SRGs is greatly reduced by MAPK/ERK pathway inhibition. SRG induction is unaffected at each time point. Data from ARG-seq as shown in Figure 2.3.

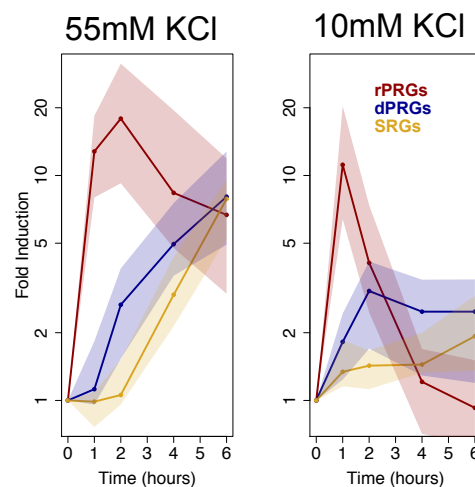
*significantly different from 1, $p < 0.01$, rank-sum test.

THE EFFECT OF STIMULUS STRENGTH ON ARG INDUCTION

Observations by Matt Friesse suggested that weaker KCl stimulation (10mM instead of 55mM) resulted in better induction of *Nr4a* genes compared to *Egr* genes. Therefore, in my early experiments, I tested the effect of stimulus strength on ARG induction. We decided not to include this data in the final publication because it is difficult to determine what a weaker KCl stimulus means – i.e., what it corresponds to *in vivo*.

First, to test the effect of weaker KCl stimulation on ARG induction, I stimulated with 10mM KCl instead of 55mM KCl (stimulation and culture otherwise same as in appendix 1). I assessed gene induction using high-throughput Fluidigm qPCR. I found that in response to 10mM KCl, all 3 classes of genes were induced, but rPRGs and dPRGs turn off more quickly with 10mM compared to 55mM KCl, and SRGs do not turn on as strongly (Supplementary Figure

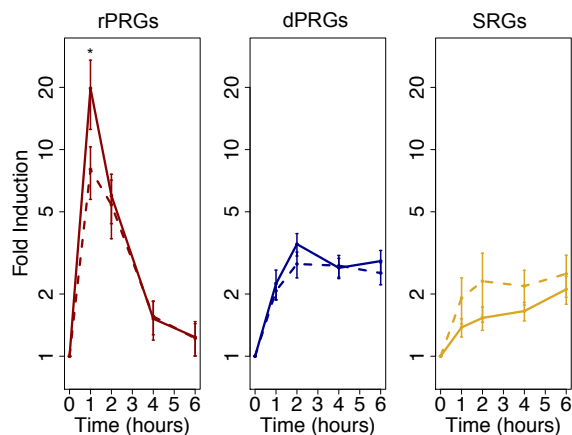
A3.2). The finding that SRGs do not turn on as strongly is consistent with them acting as integral sensors.



SUPPLEMENTARY FIGURE A3.2. 55MM VS 10MM KCL TREATMENT

Data from high-throughput qPCR, as described in Appendix I. Solid lines represent the expression of the median gene (as determined in the mean of n=3-6 biological replicates). Shading represents the upper and lower quartiles.

I next asked how MAPK/ERK inhibition affected gene induction in response to 10mM KCl stimulation. I inhibited neurons with U0126 and assessed gene induction in response to 10mM KCl stimulation with Fluidigm high-throughput qPCR. As with 55mM KCl stimulation, I found that only rPRGs are affected by MAPK/ERK inhibition in response to weak stimulation, but the effect of MAPK/ERK inhibition seems to be less than observed with 55mM stimulation (Figure 2.3): following 1h of stimulation, I still observe induction of rPRGs. Like with 55mM KCl stimulation and visual cortex stimulation, dPRG and SRG induction do not appear to be affected by MAPK/ERK inhibition in response to 10mM stimulation (Supplementary Figure A3.3). These findings suggest that MAPK/ERK signaling has less of an effect on gene induction in response to weak compared to strong stimulation. One possible explanation of this is that MAPK/ERK may respond better to large, fast changes than to weaker changes.

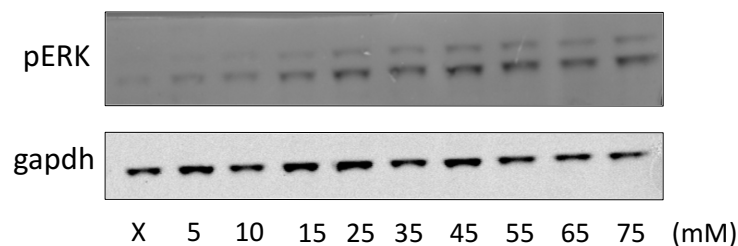


SUPPLEMENTARY FIGURE A3.3. THE EFFECT OF MAPK/ERK INHIBITION ON GENE INDUCTION IN RESPONSE TO WEAK STIMULATION

From neurons stimulated with 10mM KCl. Gene expression from Fluidigm qPCR. Lines are means of all genes from each class. Solid lines are DMSO controls, dotted lines are U0126. Error bars represent +/- S.E.M. * $p=0.00006$, rank-sum test

Because I observed that MAPK/ERK inhibition had less of an effect on gene induction in response to weak stimulation, I thought it possible that MAPK/ERK pathway activation was less robust in response to weak compared to strong stimulation. Therefore, I performed western blotting for phospho-ERK in response to several concentrations of KCl. I only performed this experiment once. I found that ERK phosphorylation increases monotonically with KCl concentration (Supplementary Figure A3.4).

While the differences between 10mM KCl and 55mM KCl stimulation are interesting, the results are difficult to interpret. It is likely that 10mM KCl results in less calcium influx into the neuron than 55mM KCl, suggesting that MAPK/ERK signaling could be measuring calcium influx. However, calcium measurements in response to different KCl concentrations have not been done. It could be interesting for future experiments to assess the role of MAPK/ERK stimulation on ARG induction in response to high vs. low frequency prolonged stimulation, which should also affect calcium influx.



SUPPLEMENTARY FIGURE A3.4: pERK INCREASES WITH INCREASING CONCENTRATIONS OF KCl

Western blot for pERK (two bands are p44 and p42 paralogs). 5 minutes of treatment with different concentrations of KCl. Western blotting done as explained in Appendix 1.

THE ROLE OF OTHER PATHWAYS IN ARG INDUCTION

Neurons activate many signaling pathways in response to elevations in activity, including the MAPK/ERK pathway, the CaMKII pathway, the CaMKK/CaMKIV pathway, and calcineurin (Flavell and Greenberg, 2008). I was therefore interested in whether other signaling pathways regulated induction of specific ARG subsets, like the MAPK/ERK pathway regulates induction of rPRGs. To test this possibility, I stimulated neurons with brief and sustained 55mM KCl stimulation (as in Appendix I) in the presence of several signaling pathway inhibitors (Supplementary Table A3.1).

SUPPLEMENTARY TABLE A3.1. INHIBITORS AND CONCENTRATIONS USED TO TEST THE ROLE OF CELL-SIGNALING PATHWAYS IN ARG INDUCTION.

Drug	Concentration Used	Pathway Inhibited
SB203580	15uM	p38 MAPK
CsA	1uM	calcineurin (CaN)
Myr-AIP	5uM	CaMKII
CAPE	35uM	NF κ B

As in the other plots in this Appendix, I analyzed the genes for which we have Fluidigm probes (Supplementary Table A2.3) according to their kinetic gene class as defined in Chapter II.

It should be noted that I did not verify the efficacy of any of these inhibitors. I found the following (Supplementary Figure A3.5):

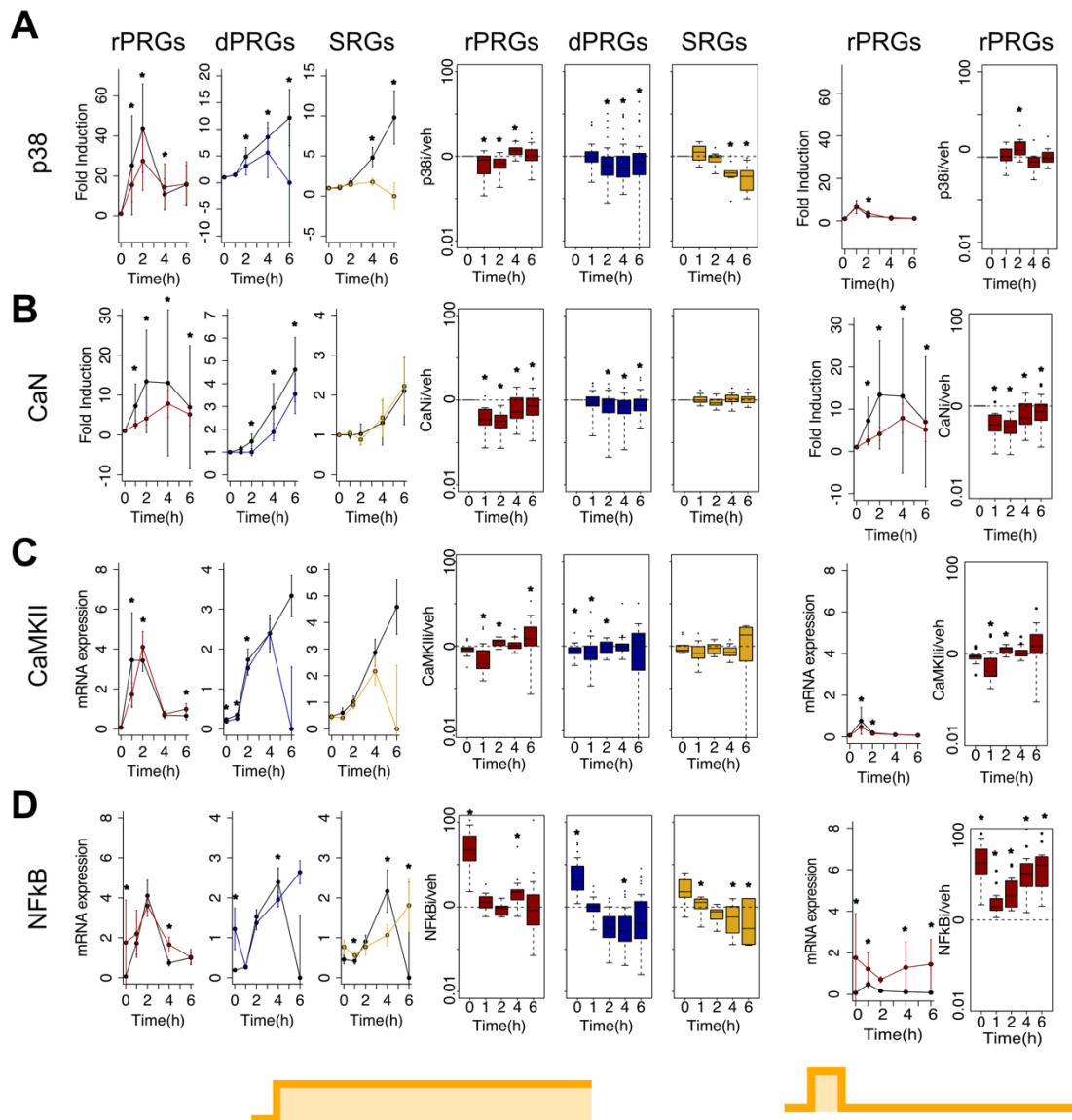
p38 (SB203580): The p38 MAPK pathway appears to be required for SRG induction (although there is the caveat that there are very few SRGs measured by Fluidigm). p38 pathway inhibition appears to completely block SRG induction but have a lesser effect of dPRG and rPRG induction (Supplementary Figure A3.5A).

Calcineurin (CsA): Calcineurin appears to regulate rPRGs. Like MAPK/ERK pathway inhibition, inhibition of calcineurin with CsA blocks rPRG induction in response to brief and sustained activity but has only a subtle effect on dPRG induction and no effect on SRG induction (Figure A3.5B).

CaMKII (Myr-AIP): CaMKII inhibition with the specific peptide inhibitor Myr-AIP has little effect on gene induction. While there are “significant” effects, they are not consistent across time points and are small in magnitude (Supplementary Figure A3.5C). However, it is possible that this inhibitor was not effective.

NF κ B (CAPE): NF κ B inhibition with CAPE appears to dramatically increase baseline gene expression (i.e., ARG expression in unstimulated neurons). [Note that the 0h time point in these experiments is treated with the drug for 6.5h.] It is therefore difficult to assess its effect on ARG induction in response to stimulation (Supplementary Figure A3.5D).

Of these findings, it would be most interesting to follow up on the finding that p38 specifically seems to regulate SRGs. To confirm this finding, it would be important to (a) confirm that the p38 inhibitor is indeed blocking p38 and (b) determine the effect of p38 inhibition on more SRGs using RNA-seq or ARG-seq. If p38 is indeed required for SRG induction, it could be because p38 is required for the translation of the rPRG protein products (such as FOS) that



SUPPLEMENTARY FIGURE A3.5: EFFECT OF P38, CALCINEURIN, CAMKII, AND NFκB INHIBITORS ON ARG INDUCTION.

A. SRG expression is most affected by p38 inhibition. Fluidigm qPCR gene expression. Black line = DMSO, colored line = inhibitor. Boxplots are the same data as the line graphs but show the ratio of induction in the presence of the inhibitor to the presence of the vehicle. * = $p < 0.05$ rank-sum test. Left = sustained depolarization, right = brief depolarization. **B.** Like (A) except with a CaN inhibitor. **C.** Like (A) except with a CaMKII inhibitor and showing mRNA expression instead of fold induction due to drug-effect on the baseline of some genes. **D.** Like (C) but with a NFκB inhibitor.

regulate SRG induction. Alternatively, it is possible that p38 acts more directly to regulate SRG induction, for example, by activating AP1 post-translationally or by remodeling chromatin, both observed roles of p38 in other systems (Fowler et al., 2011; Shaulian and Karin, 2002).

APPENDIX 4.

BLUE LIGHT INDUCES NEURONAL-ACTIVITY-REGULATED GENE EXPRESSION IN THE ABSENCE OF OPTOGENETIC PROTEINS

This chapter is from a paper published on bioRxiv. Tyssowski, K.M., Gray, J.M., 2019. Blue light induces neuronal-activity-regulated gene expression in the absence of optogenetic proteins. bioRxiv 572370. <https://doi.org/10.1101/572370>. It may be published elsewhere in the future.

Author contributions: *I designed the study, performed experiments, and wrote the manuscript. JMG advised on all aspects.*

INTRODUCTION

With the development of optogenetic technologies over the past decade (Beyer et al., 2015; Boyden et al., 2005), it has become increasingly common to expose biological samples to high-powered light. Optogenetics enables light-based control over diverse cellular functions--including neuronal firing (Lin, 2011), transcription (Nihongaki et al., 2015; Polstein and Gersbach, 2015), and cell signaling (Beyer et al., 2015)--via exogenous proteins that are activated by specific wavelengths of light. Results of such experiments can be difficult to interpret if light by itself, in the absence of optogenetic proteins, affects cellular processes. Therefore, it is important to characterize how light exposure affects biological samples.

Sustained light exposure can form free radicals that affect cellular processes. In cell culture experiments, hours-long light exposure lowers cell viability via toxic oxidation and free radical formation in the media (Blum, 1932; Dixit and Cyr, 2003; Richardson, 1893; Stoien and Wang, 1974; Waldchen et al., 2015). Furthermore, *D. melanogaster* larvae and *C. elegans* are sensitive to free radicals that accumulate internally when the animals are exposed to visible light for hours to days (Bhatla and Horvitz, 2015; De Magalhaes Filho et al., 2018; Guntur et al., 2017). In both cell culture and animal studies, shorter (i.e., blue-er) wavelengths of light have greater negative effects than longer wavelengths. Despite the potential for side effects with blue light, the majority of optogenetic proteins, including channelrhodopsin (ChR2) and cryptochrome-2 (CRY-2) (Beyer et al., 2015; Boyden et al., 2005), are activated by blue light in

the 450-500 nm range, although other optogenetic proteins are responsive to longer wavelengths of light (Klapoetke et al., 2014; Lin, 2011; Lin et al., 2013). In neuroscience, blue light stimulation has been used with exogenous channelrhodopsins to control neuronal firing for hours to days (Fong et al., 2015; Goold and Nicoll, 2010; Grubb and Burrone, 2010; Park et al., 2015), time scales likely to result in light-induced toxicity. Indeed, exposing cultured neurons to 20 hours of blue light flashing at 1Hz reduces viability (Stockley et al., 2017). However, less severe cellular changes that may occur separately from, or in early stages of, cell death have not been characterized.

Here we focused on assessing the role of light exposure on transcription. We were particularly interested in characterizing the effects of light on transcription in neurons because optogenetically-driven neuronal activity induces expression of activity-regulated genes, such as *Fos* (Schoenenberger et al., 2009). Therefore, optogenetics could be a useful tool to precisely control neuronal activation to study the resulting activity-regulated gene expression. Furthermore, optogenetics can be used to directly control transcription (Nihongaki et al., 2015; Polstein and Gersbach, 2015) and to control signaling pathways that regulate transcription (Beyer et al., 2015), suggesting that experimenters may wish to measure transcriptional outputs of optogenetic experiments in many contexts.

We therefore asked whether neuronal transcription is affected by one to six hours of blue, red, or green light exposure. We chose light wavelengths that activate published channelrhodopsin variants (Klapoetke et al., 2014; Lin, 2011; Lin et al., 2013) and timepoints relevant to activity-regulated gene induction (Tyssowski et al., 2018; West and Greenberg, 2011). We found that cultured neurons without channelrhodopsin induced the activity-regulated genes *Fos*, *Npas4*, and *Bdnf* when exposed to one or six hours of blue light, but not when exposed to red or green light. Our findings suggest light by itself, in the absence of optogenetic proteins, can induce transcription. Therefore, experiments that measure transcription following

long-term optogenetic stimulation should take precautions, such as using longer-wavelength light, to avoid experimental confounds from this light-induced transcription.

MATERIALS AND METHODS

Cell Culture

Cortices were dissected from embryonic day 16 (E16) CD1 or C57/Bl6 mouse embryos of mixed sex. They were dissociated with papain (Worthington, (L)(S)003126). 250,000 dissociated cells/well were plated on 48-well Lumos OptiClear plates (Axion), which have opaque well walls and had been coated overnight with poly-ornithine (30mg/mL, Sigma) and laminin (5ug/mL) in water and then washed once with PBS. Cultures were maintained at 37C at 5% CO₂ in BrainPhys media (StemCell Technologies) without phenol red supplemented with SM1 (StemCell Technologies) and penicillin/streptomycin (ThermoFisher).

Light Stimulation

Light stimulation was done using the Lumos system programmed with AxIS software with power set at 100% (Axion Biosystems). According to the manufacturer, 100\% power corresponds to 3.9mW/mm² for blue light, 1.9mW/mm² for green light, and 2.2mW/mm² for red-orange light. These irradiance measurements were taken from the bottom of a well with no media (personal correspondence with Axion Biosystems). The wavelengths used were 475nm (blue), 530nm (green), and 612nm (red-orange). The temperature was maintained by putting the plate on a 37C warming plate (Bel-Art). The CO₂ was maintained at 5% throughout the duration of the recording using the base provided with the Axion Lumos system. Neurons were silenced with APV (100uM, Tocris) and NBQX (10uM, Tocris) at least 8h before stimulation to replicate

conditions that would be used in optogenetic experiments. Light-exposed wells and wells left in the dark were on the same plate.

Temperature Measurement

We measured temperature using a thermocouple (Omega, 5TC-TT-K-30-36) inserted into a well that was exposed to light stimulation. The temperature on the thermometer attached to the thermocouple was monitored at the start of the stimulation and at least once an hour for the duration of the light exposure (up to 6h).

RNA extraction and qPCR

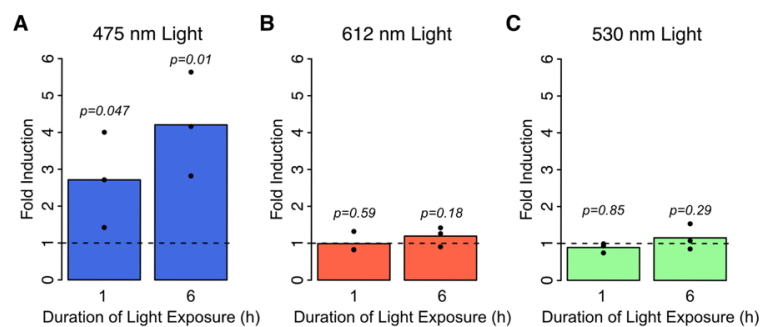
Immediately following stimulation, samples were collected in Trizol (Invitrogen), and total RNA was extracted using the RNeasy mini kit (QIAGEN) with in-column DNase treatment (QIAGEN) according to the instructions of the manufacturer. The RNA was then converted to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). For quantitative PCR (qPCR), we used SsoFast Evagreen supermix (BioRad) with primers for *Fos*, *Npas4*, *Bdnf*, and *Gapdh* in Supplementary Table A2.6. For qPCR analysis, we used a standard curve for each primer to convert Ct values into relative amounts of expression. We normalized our neuronal-activity-regulated gene values by values for the housekeeping gene *Gapdh* in order to control for any differences in the amount of cDNA in each reaction. *Gapdh* values were not significantly different between conditions ($p > 0.1$, two-sided t-test). Furthermore, *Gapdh* mRNA is highly expressed and highly stable, making it less likely to be altered by small changes in transcription. Each biological replicate was from a different dissection on a different day. T-tests testing fold induction were performed on log fold change values from biological replicates testing the difference from a fold change of 1.

Neuronal Activity Measurement

Neuronal activity was measured using neurons plated on Axion MEAs in 48-well optical plates. MEA plates were coated as above. Neurons from post-natal day 0 or 1 (P0-1) mice were dissociated and cultured as above. Recording was done using the Axion Maestro with the Axion software.

RESULTS

To determine the effect of light exposure on neurons, we exposed cultured cortical neurons that did not express channelrhodopsin to a pattern of blue (475 nm) light consisting of 2-ms pulses at a frequency of 10 Hz. We used a light intensity of around 3.9 mW/mm² (see Methods), which is similar to, or less than, the light intensity recommended for optogenetic activation of channelrhodopsin and similar molecules (Klapoetke et al., 2014; Lin, 2011; Lin et al., 2013). After light exposure, we assessed the mRNA expression of the neuronal-activity-regulated gene *Fos* using qPCR. We found neurons exposed to just one hour of 10 Hz flashing blue light have 2.7-fold higher *Fos* mRNA expression than neurons left in the dark (Supplementary Figure A4.1A). Following six hours of light exposure, we observed a four-fold induction of *Fos* mRNA, suggesting that blue light exposure--in the absence of optogenetic proteins--induces *Fos* mRNA expression.



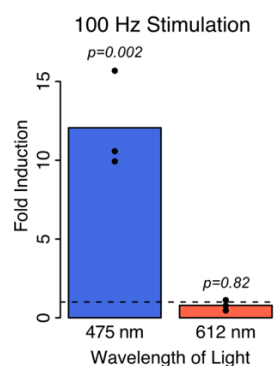
SUPPLEMENTARY FIGURE A4.1. BLUE, BUT NOT RED OR GREEN LIGHT INDUCES *FOS* EXPRESSION

continued overleaf

Supplementary Figure A4.1 (Continued) Cultured cortical neurons without channelrhodopsin were exposed to a pattern of 10Hz, 2-ms pulses of (A) 475nm (blue), (B) 612 nm (red), or (C) 530nm (green) light for 1 or 6 hours. Induction of the activity regulated gene *Fos* was measured using RT-qPCR. Values plotted are the fold induction in mRNA expression at 6h compared to neurons not exposed to light. Bars represent the average of three biological replicates and dots are the values from each replicate. p-values from a one-sided Student's t-test.

We next asked whether *Fos* is induced by red (612 nm) or green (530 nm) light. We exposed neurons to six hours of the same 10-Hz pattern and found that neither red nor green light exposure induces *Fos* expression (Supplementary Figure A4.1B-C).

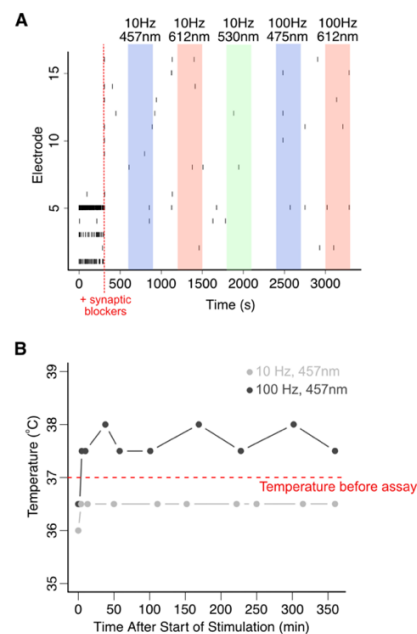
We then asked whether increasing the amount of blue light exposure increases gene induction. When we changed the pattern of blue-light stimulation to a frequency of 100 Hz, we found that neurons showed a 12-fold increase in *Fos* expression after six hours of light exposure. This was more than the four-fold increase we saw after six hours of exposure 10 Hz blue light ($p=0.009$, t-test), indicating that more light exposure results in more gene induction (Supplementary Figure A4.2). However, we found that for red light, even the 100 Hz stimulation pattern failed to induce *Fos*, indicating that light-induced gene expression is specific to shorter (blue-er) wavelengths.



SUPPLEMENTARY FIGURE A4.2. 100HZ BLUE, BUT NOT RED, LIGHT INDUCES *FOS* INDUCTION

Cultured cortical neurons without channelrhodopsin were exposed to a pattern of 100Hz, 1-ms pulses of 475nm (blue) or 612 nm (red) light for 6 hours. Induction of the activity regulated gene *Fos* was measured using RT-qPCR. Values plotted are the fold induction in mRNA expression at 6h compared to neurons not exposed to light. Bars represent the average of three biological replicates and dots are the values from each replicate. p-values from a one-sided Student's t-test.

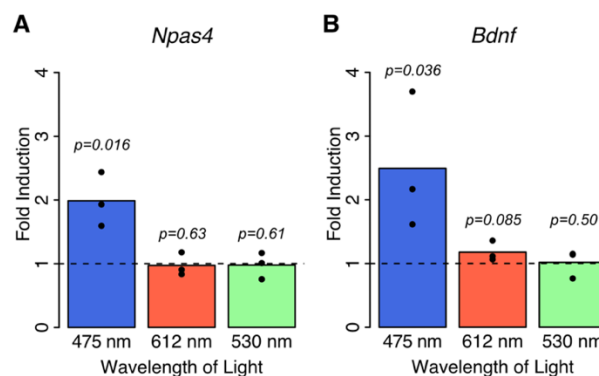
We next investigated whether *Fos* induction might be due to one of several possible secondary effects of blue light exposure. *Fos* is usually induced in neurons as a result of the membrane depolarization that occurs during an action potential. However, neurons without channelrhodopsin grown on multi-electrode arrays did not fire more action potentials when exposed to light (Supplementary Figure A4.3A), ruling out membrane depolarization as a cause of *Fos* induction. We also confirmed that the sustained blue light exposure did not substantially alter the temperature of the media. Even with 100Hz stimulation, the media remained at 37 $\pm$ 1 $^{\circ}$ C for the duration of the experiment (Supplementary Figure A4.3B). These findings suggest that the blue-light-induced gene expression is likely a direct rather than secondary effect of light exposure.



SUPPLEMENTARY FIGURE A4.3. BLUE LIGHT STIMULATION DOES NOT INDUCE ACTION POTENTIALS OR CHANGES IN TEMPERATURE

A. Cultured cortical neurons without channelrhodopsin plated on multi-electrode arrays were exposed to the indicated light conditions. As in all experiments, neurons were silenced before light exposure with synaptic blockers APV and NBQX. Each line represents an action potential. Light is ON at the highlighted times. Representative example from one experiment. **B.** Temperature measurements were taken from a well exposed to blue light at several time points during the course of a 6-hour experiment. The red line represents the temperature at which the neurons were cultured before starting the assay. Representative example from one experiment.

Finally, we asked whether others of the hundreds of neuronal-activity-regulated genes are induced by light exposure. Specifically, we assessed expression of *Bdnf* and *Npas4* mRNA using qPCR. We hypothesized that since *Bdnf* is regulated differently from *Fos* (Tyssowski et al., 2018; West and Greenberg, 2011), it may not be induced by the *Fos*-regulating signaling pathways activated by blue light exposure. However, we found that *Bdnf* mRNA expression is induced by a six-hour exposure to blue, but not red or green, light (Supplementary Figure A4.4A). Induction of *Npas4* mRNA, unlike *Fos* (Fowler et al., 2011), is relatively specific to activated neurons (Lin et al., 2008). We thus reasoned that if the gene induction in response to blue light stimulation were activated as part of a response to oxidation and cell death (Blum, 1932; Richardson, 1893; Stoen and Wang, 1974), a neuronal-activation-specific gene might not be induced. Surprisingly, we found that a six-hour exposure to blue, but not red or green, light also resulted in induction of *Npas4* mRNA (Supplementary Figure A4.4B). We therefore suspect that many neuronal-activity-regulated genes are induced by blue light exposure.



SUPPLEMENTARY FIGURE A4.4. BLUE, BUT NOT RED OR GREEN, LIGHT STIMULATION INDUCES NPAS4 AND BDNF

Cultured cortical neurons without channelrhodopsin were exposed to a pattern of 10Hz, 2-ms pulses of 475nm (blue), 612 nm (red), or 530nm (green) light for 6 hours. Induction of the activity regulated genes (A) *Bdnf* and (B) *Npas4* were measured using RT-qPCR. Values plotted are the fold induction in mRNA expression at 6h compared to neurons not exposed to light. Bars represent the average of three biological replicates and dots are the values from each replicate. p-values from a one-sided Student's t-test.

DISCUSSION

We show in cultured cortical neurons without channelrhodopsin, that extended exposure to blue light induces neuronal-activity-regulated genes. This gene induction does not occur in response to exposure to red or green light. Our findings indicate that blue light is ill-suited to optogenetic experiments that use long-term light exposure and those that assess changes in transcription in response to optogenetic stimulation. This work also emphasizes the importance of including experimental controls (Allen et al., 2015) in optogenetic experiments to determine the effects of light on cells in the absence of light-activated proteins.

Our finding that blue, but not red or green, light induces spurious transcription is consistent with other work demonstrating detrimental effects of short-wavelength light (Cheng et al., 2016; Godley et al., 2005; Stockley et al., 2017; Stoien and Wang, 1974). Several studies that have compared the effects of blue light to other wavelengths of light both in vitro and in *C. elegans* have found that blue light has greater effects on cell viability (Waldchen et al., 2015), *C. elegans* behavior (Bhatla and Horvitz, 2015), and *C. elegans* survival (De Magalhaes Filho et al., 2018). These data suggest that using optogenetic proteins that are activated by longer wavelengths of light (Hochbaum et al., 2014) might allow experimenters to avoid side effects of light exposure.

We speculate that neurons induce transcription in response to blue light due to the oxidation that occurs in biological liquids in response to extended light exposure (Blum, 1932; Dixit and Cyr, 2003; Richardson, 1893; Stockley et al., 2017; Stoien and Wang, 1974). Oxidative stress can induce transcription of primary response genes, including *Fos*, in a variety of cell types via activation of cell-signaling pathways, including the MAPK and NF κ B pathways (Allen and Tresini, 2000). Because oxidative stress activates similar pathways as neuronal activity (West and Greenberg, 2011), we might expect oxidative stress to activate many neuronal-activity regulated

genes. Indeed, we observed that blue light exposure induces all three of the neuronal-activity-regulated genes that we tested.

In neuronal cell culture systems, blue light exposure likely induces oxidation due to the presence of compounds such as riboflavin, tryptophan, and HEPES in cell culture media (Edwards et al., 1994; Godley et al., 2005; Lepe-Zuniga et al., 1987; Spierenburg et al., 1984). BrainPhys, the media used in this study, contains both riboflavin and HEPES (Gage and Bardy, 2014), as does the common neuronal culture media, Neurobasal (manufacturer's pamphlet). Therefore, supplementing neuronal culture media with antioxidants (Dixit and Cyr, 2003; Grubb and Burrone, 2010) or altering it to exclude compounds that cause oxidation (Stockley et al., 2017) may mitigate the detrimental effects of blue light in culture systems. Alternatively, sensitive channelrhodopsins (Schoenenberger et al., 2009) can be used to minimize the duration of light exposure and thus its negative effects. Notably, we suspect that blue light exposure induces transcription in non-neural cultures, as the common cell culture media DMEM also contains riboflavin and HEPES (manufacturer's pamphlet). Thus, spurious blue-light-induced gene expression may be a concern in any experiment that measures transcription in response to an optogenetic stimulus, including those that use optogenetics to directly induce transcription in non-neural cells (Nihongaki et al., 2015; Polstein and Gersbach, 2015).

The toxic oxidation that occurs in culture media suggests that in vitro experiments may be particularly sensitive to blue light exposure. However, oxidation-prone compounds exist within cells and in interstitial fluids, suggesting that light exposure could also affect cells in vivo. Consistent with this idea, exposing *C. elegans* to blue light likely produces free radicals within the worm (Bhatla and Horvitz, 2015), and *C. elegans*, *Planaria*, and *D. melanogaster* have free-radical-detecting cells that respond to light exposure in the absence of cell culture media (Bhatla and Horvitz, 2015; Birkholz and Beane, 2017; Guntur et al., 2017). Alternatively, it is possible that endogenous opsins or cytochromes, which are expressed in our cultures (Tyssowski et al., 2018)

and in the brain (Peirson et al., 2009), play a role in the observed gene induction, in which case we would expect to observe similar activity-regulated gene induction in vivo. Indeed, there is some evidence that blue light stimulation in the absence of channelrhodopsin may induce *Fos* expression in the rat brain (Villaruel et al., 2018), although it is not clear whether this is due to light stimulation or other factors, such as the trauma from implanting the optical fiber. Therefore, it will be important for future work to assess the impact of blue light exposure on neuronal transcription in vivo.

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