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The Role of Chromatin Remodeler BAF in Neuronal Activity-Induced Transcription

A thesis submitted in partial satisfaction of the requirement for the degree of Master of Science

in

Quantitative and Systems Biology

by

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Committee in charge:

Maria Zogbhi, Ph.D. (Chair)
Xuecai Ge, Ph.D.

To God who gave the strength and opportunity to try.
To my husband and babies who gave me encouragement to keep going.

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List of Abbreviations

ASD (autism spectrum disorder)
ATP (adenosine triphosphate)
IEG (Immediate early genes)
dIEG (delayed Immediate early genes)
rIEG (rapid Immediate early genes)
BRD 98 (BAF inhibitor, BRD-K98645985)
KD (knockdown)
RNAi (RNA interference)
PD (MEK inhibitor, PD0325901)
FK (calcineurin inhibitor, FK-506)
MAPK (Mitogen associated protein kinase)
CaN (calcineurin)
SWI/SNF (switch/sucrose non-fermenting)
CDH (chromodomain-helicase-DNA binding)
INO80 (inositol requiring 80)
ISWI (imitation switch)
BAP (brahma Associated Protein)
PBAP (polybrahma Associated Protein)
BAF (bromo-associated factors)
cBAF (canonical rromo-associated factors)
nBAF (neuronal bromo-associated factors)
npBAF (neuroprogenetor Bromo-associated factors)
GBAF (glial bromo-associated factors)
PBAF (polybromo-associated factors)
Pol II (RNA polymerase)
SRF (serum response factor)
SWI/SNF (switch/sucrose non-fermenting ATP-dependent chromatin remodeler)
CDH (chromodomain-helicase-DNA binding ATP-dependent chromatin remodeler)
INO80 (inositol requiring 80 ATP-dependent chromatin remodeler)
ISWI (imitation switch ATP-dependent chromatin remodeler)
mSWI/SNF (mammalian switch/sucrose non-fermenting ATP-dependent chromatin remodeler)
esBAF (embryionic stem cell bromo-associated factors)
Bic (bicuculline)
4AP (4-aminopyridine)
PROTAC (ACBI1; BRG1/BRM targeting BAF protein degrader)
ACBI1 (PROTAC; BRG1/BRM targeting BAF protein degrader)

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Acknowledgements

This project would not have been possible without the support of my advisor and mentor, Ramen Saha, who encouraged me through the difficulties and guided my intellectual growth. Thanks to all the Saha Lab staff for the help during unstable and COVID changes that led to timely data organization. Thanks to my committee members, Maria Zogbhi and Xuecai Ge for the support and guidance. Thank you to UC Merced and NIH for the scholarships and grants that allowed for my studentship along this project. Special thanks to the UC Merced, SNS Graduate Student Coordinator, Jan Zarate for the clear answers to my unending questions and guidance towards making this degree and project a possibility. Finally, a great thanks to my husband, mom, and babies that were so patient, loving, and supportive through the ups and downs of this journey.

Abstract

The BAF complex is an ATP dependent chromatin remodeling complex that is known to facilitate gene transcription. However, underlying mechanisms are not fully understood. We expected neuronal activity-induced gene transcription of delayed IEGs to require BAF complex-dependent chromatin remodeling and to be impaired when the latter functions sub-optimally, while rapid IEGs were expected to not need BAF as they already have accessible chromatin. Pharmacological inhibition or degradation of cBAF, but not the PBAF, and RNAi knockdown (KD) of nBAF subunit 170 and 53b significantly attenuated transcription of neuronal immediate early genes including rapid IEGs. BAF subunit KD didn't alter phosphorylation of MAPK-ERK, a necessary signaling cascade for transcription. Also, pulse-chase with the nBAF inhibitor showed that the BAF complex is required for IEG transcription on a continuous basis. Taken together, using multiple experimental approaches, this is the first study to demonstrate a critical role of nBAF in neuronal activity-induced transcription.

Introduction

Activity-induced gene transcription has been linked to neuronal development and downstream neuronal regulation disorders making it important for memory formation and may have implications on psychiatric disorders involving fear memory[1-4]. For example, there may be connections with neuro-epigenetic mechanisms and autism spectrum disorder, major depression, panic disorder, and even schizophrenia, to name few[4-9]. The intricately orchestrated developmental processes of the central nervous system are designed to specialize cells through differentiation, making both neurons and glial cells. Many factors are considered for this differentiation process, such as chromatin regulation and other various epigenetic changes. A mutation in transcriptional machinery or varying the heterochromatin arrangement will cause a change in transcription regulation and therefore alter protein synthesis. This can then affect the cellular identification and pushes the non-specified cells towards their final fate. It is easy to see how a minor change to the instructions of these cells can lead to a completely catastrophic domino effect. In other words, the system and proteins involved are essential for proper neuronal formation and plasticity.

Activity-Induced Immediate Early Gene Transcription

Single specific mutations to specific genes can be linked to a disease or disorder; however for Autism Spectrum Disorders (ASD), as well as many other intellectual and neurodevelopmental disorders, there is a longer list of genes involved with the phenotypes known for these disorders. Understanding those genes and their regulation can lead to understanding the impact they can have on the disorder. Fortunately, by narrowing the field of study to certain groups of genes, we can start to understand at least some of the processes that may be affected by these disorders. For example, the genes transcribed within minutes after neuronal stimulation are used in the long-term processes of cells. When normal gene transcription was inhibited in rat hippocampal neurons immediately after were stimulated, the long-term potentiation that was previously established was no longer seen. In fact, many of the activity-induced genes have critical periods for cells to respond with late phase long term potentiation[10].

These genes immediately transcribed within minutes of neuronal stimulation are referred to as immediate early genes (IEGs)[1, 11, 12]. Activity-induced IEGs have their own expression patterns with different mechanisms behind them. Under sustained activity, three separate and distinct waves of gene transcription are seen: rapid IEGs (rIEGs), delayed IEGs (dIEGs) and secondary response genes, each group being transcribed in that respective order, from the immediate response of minutes to a few hours, where each group has its own temporal and mechanistic signatures[11]. Listed in Table 1 are the several IEGs observed

throughout the experiments we conducted. Their differences are found in the transcription mechanisms themselves (Figure 1).

Neither the rapid or delayed IEGs need *de novo* translation; meaning, they both have the necessary protein complexes translated for transcription premade, though perhaps not in place along the DNA. In other words, they do not require translation of transcriptional machinery. However, each group seems to rely on separate cell signaling pathways and mechanisms to varied extents. Rapid IEGs, such as *Arc,* require the Mitogen-Activated Protein Kinase (MAPK) pathways and have transcription factors prebound to their enhancer and promoter regions. The necessity of MAPK in the brain is well known as it can be linked to anxiety, addiction, and neuronal development disorders[12]. As part of the RAS-RAF-MEK-ERK pathway, MAPK is a step into cellular transcription which then can involve more protein interactions for regulation[13-15]. These are key factors to these genes which can swiftly transcribe and therefore translate RNA to functional protein.

Table1: List of rapid and delayed IEGS			
Rapid IEGs		Delayed IEGs	
Arc	Gadd45g	Nfil3	Ifing
Nfra3	Cyr61	Bdnf	Egr3
cFos	Btg2	Nurr1	Klf4
Npas4	Ppp1r15a	Fam46a	Trib1
Fbxo33	Erg2	Cartpt	Tiparp
Dusp1	Zif268	Edn1	Vgf
Egr4	Gadd45b	Fosl2	Nurr77
Arf4	Dusp6	Orl670	Maff
Nup98		Rasl11a	Trib1
		Dusp5	Fosl1

Table 1. List of rapid and delayed IEGs used throughout the experiments to be discussed.

Unlike rapid IEGs, delayed IEGs, such as *Bdnf*, require more time or sustained neuronal activity for the genes to be transcribed[16]. This aspect of their time specific transcription isn't fully understood. We do know, however, that for proper transcription, DNA needs to be accessible. The enhancer/promoter region must be accessible and near each other for transcription factors to bind, such as RNA polymerase (Pol II) or SRF[16, 17]. Without such accessibility, transcription will be attenuated. Perhaps the delayed IEGs require the use of ATP-dependent chromatin remodelers, such as neuronal BAF, for the essential accessibility to then recruit the transcription factors into the promoter and enhancer regions.

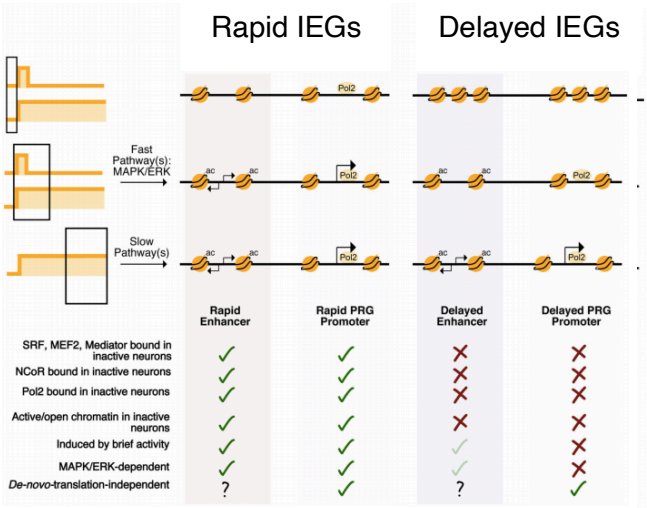

Figure 1. Description of the separate waves of immediate early gene (IEG) transcription*. Rapid IEGs have transcription machinery available and depend on MAPK for optimal transcription. (modified from Tyssowski, 2018)*

Chromatin remodelers

There are various kinds of mechanisms responsible for chromatin accessibility during transcription. The main remodeling purpose is to alter the way histone octamers interact with the chromatin itself. They may include sliding of nucleosomes, changing the composition of octamers, or changing conformation of the nucleosomal DNA[18, 19]. Remodelers may either cause post-translational modifications to a histone, methylation for example, or remodelers will interrupt the contact between the inaccessible DNA and the histones involved through ATP hydrolysis[20]. ATP hydrolysis restructures the nucleosome configuration to essentially move along or off the DNA making space for cellular mechanisms to initiate transcription as a transcription regulation system. There are four kinds of ATP-dependent chromatin remodeling complexes which include SWI/SNF (switch/sucrose non-fermenting), CDH (chromodomain-helicase-DNA binding), INO80 (inositol requiring 80), and ISWI (imitation switch)[18, 21, 22].

The SWI/SNF complex, first described based on two separate functions in yeast, is a well conserved ATP-dependent chromatin remodeler across many species[23, 14]. *Drosophila* contain BAP (Brahma Associated Protein) and PBAP (polybrahma Associated Protein), while mammals contain the equivalent BAF (Bromo-associated factors) and PBAF (polybromo-associated factors) complexes. These multi-subunit complexes have been significantly studied as a transcription regulators[18, 24, 25]. It may very well have significant implications on IEG regulation even in neurons, as it has been linked to developmental defects in the brain in several studies[26-28].

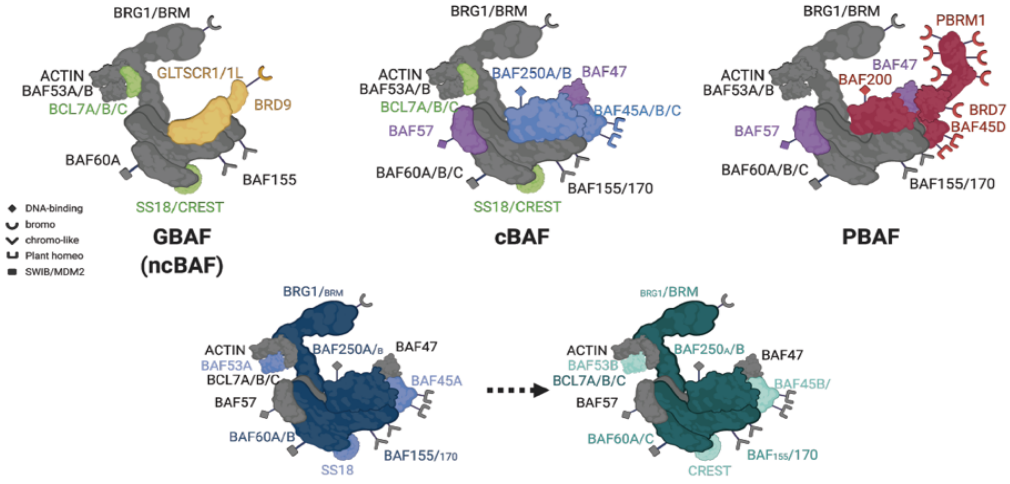

Figure 2. Distinct structures of BAF remodeling complexes. *Color refers to subunits shared between all three complexes (grey), specific to GBAF (ncBAF) (yellow), BAF (blue), or PBAF (red), or shared between GBAF and BAF (green), or BAF and PBAF (purple). cBAF undergoes substantial subunit rearrangement during differentiation from neural progenitors to neurons. Subunits in light blue/green undergo complete switching, while subunits in dark blue/green display alterations in relative stoichiometry. (from Dr. Emily Dykhuizen, Purdue University)*

The BAF complex

The BAF complex, otherwise known as mSWI/SNF or mammalian SWI/SNF, contains at least 15 subunits with specialized functions. As it is an ATP-dependent chromatin remodeler, it includes ATPase subunits, BRM and BRG1, to aid with the ATP hydrolysis necessary for altering chromatin accessibility. The neuronal specificity of BAF depends on the type of cell it becomes as the cells differentiate. As neuronal development advances and cell differentiation begins to happen, the cells go from embryonic stem cells to neural progenitor cells, and eventually become proper neurons as they end their mitotic cycle. With these cellular changes come internal changes to the canonical BAF composition as well. For example, some of the changes to canonical subunits of esBAF (embryonic stem BAF) are that BRG1 and 250a can now be interchanged to the ATPase BRM and 250b in the npBAF (neural progenitor BAF). In addition to that, the dimer between 155:155 can now also be 155:170 in npBAF[29](Table 2, Figure 2).

When neural progenitor cells exit mitosis and become mature neurons, they also go through changes that alter the subunit composition. BAF53a, BAF45a and d, and CREST replace their respective counterparts, BAF53b, BAF45b and c, and SS18. Not only that, but the proportion of BAF170 to BAF155 is increased as well. When these changes to BAF are inhibited, the cells cannot progress developmentally to the next stages normally[29-32](Table 2, Figure 2).

Mutations to specific genes that alter this complex have been linked to specific developmental, intellectual, or general pathological problems. For example, a single point mutation for BAF250a domain V1068G in mice will inhibit proper BAF binding that results in embryonic death[34]. In other cases, loss of other subunits not directly tasked with the function in question will have a cascade effect almost as drastic as if targeting the main components of the complex itself[29, 35]. It seems as if proper function of many subunits must be in harmony for the complex to optimally operate. Most of the literature seems to point to specific subunits in association to cancers or developmental disorders. Interestingly, subunits that have seemingly similar functions such as BRG1 and BRM as ATPases, have been linked to cancer and neurodevelopment disorders. Similarly, BAF250a has been mostly studied as a link to cancer, BAF250b has been mostly seen involved in neurodevelopmental [29]. The involvement of these subunits must not be merely coincidental despite fulfilling similar roles in the BAF complex.

Does this mean that each subunit functions at least slightly different in the complex, perhaps affecting chromatin accessibility and therefore affecting the overall protein synthesis and cellular function at a larger scale? More work must be done to understand the specific function each individual subunit. Whether it is a difference in domains between subunit equivalents, understanding what the links between proper function between BAF and transcription are will create the baseline to compare the malfunctioning or pathological circumstances. Only then

may we shed some light on more problems or perhaps even solutions to the epigenetic chaos that can lead to cases of cancer, autism, and even schizophrenia. For this reason, we targeted separate subunits of the BAF complex to understand the orchestrated function of the entire complex.

Table 2: List of known BAF subunits				
Name	***Alias***	***Function***	***Location***	***Neuro-Pathological Involvement***
BAF 250a	ARID1a		esBAF, npBAF, nBAF	Coffin-Siris syndrome
BAF250b	ARID1b		npBAF, nBAF	Autism, schizophrenia, Nicolaides-Baraitser syndrome, intellectual disability
BAF180	PBRM1		esBAF, npBAF, nBAF	Coffin-Siris syndrome
BAF200	ARID2		esBAF, npBAF, nBAF	Coffin-Siris syndrome
BRM	SMARCA2	ATPase	npBAF, nBAF	Coffin-Siris syndrome, schizophrenia
BRG1	SMARCA4	ATPase	esBAF, npBAF, nBAF	Coffin-Siris syndrome, autism
SNF5, INI1	SMARCB1			
BAF155	SMARCC1		esBAF, npBAF, nBAF	Autism
BAF170	SMARCC2		npBAF, nBAF	Autism
BAF60 a	SMARCD1		esBAF, npBAF, nBAF	
BAF60 b	SMARCD2		esBAF,	
BAF60 c	SMARCD3		npBAF, nBAF	
BAF57	SMARCE1		esBAF, npBAF, nBAF	Coffin-Siris syndrome
BAF53a	ACTL A		esBAF, npBAF	
BAF53b	ACTL B		nBAF	
BAF45a	PHF10		esBAF, npBAF	
BAF45b/c	DPF1/2		nBAF	
BAF45d	DPF3		esBAF, npBAF	
ß Actin	ACT B		esBAF, npBAF, nBAF	
BRD9			esBAF, npBAF, nBAF	
SS18			esBAF, npBAF	
BAF47			esBAF, npBAF, nBAF	Coffin-Siris syndrome, Kleefstra syndrome
CREST			nBAF	Amyotrophic lateral sclerosis
BCL11a/b			esBAF, npBAF, nBAF	Autism, schizophrenia
BCL7a/b/c			esBAF, npBAF, nBAF	

Table 2. List of known BAF subunits, aliases, location in BAF developmental stages, function, and pathological associations[25, 29] .

Materials and Methods

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 37°C with 5% CO_2, replacing half of media every 4 days.

Stimulation: Neurons were used routinely between 10–14DIV. To induce synaptic stimulation, we triggered neuronal activity by co-treating neurons with 50μM Bicuculline (Sigma-Aldrich) and 75μM 4-Aminopyridine (Acros Organics) (or a DMSO control). Neurons were collected at various time points. 2μM PD184352 (Tocris) was added with bicuculline and 4-Aminopyridine. 5μM FK506 (Tocris) was added with bicuculline and 4-Aminopyridine. 5μM BRD7i (a kind gift from Dr. Emily Dykhuizen, Purdue University) was added 3 hours prior to treatment with bicuculline and 4-Aminopyridine. 20μM BRD98 (a kind gift from Dr. Emily Dykhuizen, Purdue University) was added 3 hours prior to treatment with bicuculline and 4-Aminopyridine, 30 minutes prior as part of pretreatment, or simultaneously with bicuculline and 4-Aminopyridine. 1-2.5μM ACBI1 and cis-ACBI1 (gift from opnMe) were added 3 hours prior to treatment with bicuculline and 4-Aminopyridine.

Plasmids and lentiviral preparation

Several web-based software programs were used to design hypervariant-specific shRNA for RNAi. The shRNA were inserted into pLKO.1-puro (designed by RNAi consortium or TRC; obtained from Addgene) following the protocol on the Addgene website. Self-inactivating HIV lentivirus particles were produced by transfecting 293T cells with the shRNA vector, envelope (pMD2.G; Addgene), and packaging plasmids (psPAX2; Addgene). These constructs were packaged into lentiviruses as mentioned in our publication[36].

Short-Hairpin RNA-dependent Knockdown

Neurons between 10-14DIV were infected via engineered lentiviruses (MOI: 2-5) with scrambled RNA, BAF170 knockout RNA or BAF53b knockout RNA for a 5 days total at 37°C with 5% CO_2, replacing half of media every 4 days. Target knockdown was verified by western blots.

Western Blotting

Neurons were lysed in ice-cold 1X RIPA buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nadeoxycholate, 0.1% SDS, 0.1% NP-40) supplemented with 1:100 protease/phosphatase inhibitor cocktail (Thermo, #78442). Lysates were sheared by sonication (low setting; three cycles on Bioruptor), cell debris pelleted at 15,000 rpm for 1 minutes at 4°C, and clarified supernatant transferred to pre-chilled 1.5 mL microcentrifuge tube. The various cell extracts were denatured at 95°C, for 5 minutes, using either 4X-Laemmli sample buffer (BIO-RAD #1610747). Denatured protein samples were resolved on 4-20%- (BIO-RAD, #4568095) or 4- 15%- (BIO-RAD #456-1083) Mini PROTEAN® gels in Tris/Glycine/SDS (BIO-RAD #1610772). Resolved proteins were transferred onto LF PVDF membrane, using the BIO-RAD TBT RTA kit and protocol using either 10-20%MeOH or 20%EtOH-containing transfer buffer (#1704272). PVDF membranes were incubated at 4°C overnight with appropriate primary antibodies in 1X TBS-T with 3% BSA. Primary antibodies included the following antibodies: b-Actin (Thermo #AM4302, RRID: AB_2536382), BAF 170 (Cell Signaling #12760), and BAF53b (Neuromab #75-311). Next day, membranes were washed three times in 1X-TBST for 5 min each, probed with either goat-anti-Mouse-647 (RRID: AB_2535808) or goat-anti-Rabbit-546 Alexa Fluor (RRID: AB_2534093) secondary antibodies (Life Technologies) for 45 minutes at room temperature, washed three times with 1X TBS-T for 5 min each, and imaged using BIORAD Multiplex ChemiDocTM Imaging System.

RNA extraction and qPCR

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). Primer sequences listen in Table3 below. 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.

Table 3. Pre-mRNA IEG primer sequences			
		Forward	Reverse
Delayed IEGs	Vgf	TTGCGAGCGTTCTCTGACCATTTG	CCCGAAGAGAAACGTTTAGCAGAG
	Trib1	TAAACCGGTGACTTTCTCTCCGCA	AGGATCACTTCCACGATGCCAGTA
	Orl670	GTT GCC CTT CTG TGG ACC CAA TTT	TAG ACC CAC TGT TGG CAA AGA CCA
	Nurr1	CGACATTTCTGCCTTCTCCTGCAT	TGTGATTAGGGAAAGCCATGACCG
	Nur77	ACCAACTCTTCTGGCTTCCCTTAC	GGCTGGTTGCTGGTGTTCCATATT
	Nfil3	AGTCGAAAGAACAGCTTCGTGGGT	AAGCCAACTGAACTTGCCTGACTC
	Maff	ATCCCTTATCCAGCAAAGCCCTGA	GGGCTTCCAGTTAGGAAAGGTGGAAA
	Ifing	TGTTTCCCAAGGACGGTAACACGA	TGCTGATGGCCTGGTTGTCTTTCA
	Fosl1	CCTGTGAGCAGGTAAGGAACAGAGAT	ATTCCCTCATAGCAAGATCCAGCC
	Tiparp	CACCTTTCTTCCTTTCCTGGTAGG	CGGTGGTTTCCATGTCCATGATGT
	Rasl11a	ACCAGTGATGTGAAGGGAGTGGTT	ATGCCAATGAATGTGCTTCCTCCC
	Klf4	GTGTGCTTCACCCAGCAAGTCAAT	AGTCGCTTCATGTGAGAGAGTTCC
	Fosl2	TGCCTTCGGATTGTGGTATGTCCT	TGACCAGTTCACAGACAGCAGACA
	Fam46a	TCGTGCTGGATTGCCTGTTAGACT	AAGCCCATCAGGTGGCTTTACCTT
	Egr3	TGTGAGTTCTGTGGGCGCAAGTTT	AAAGAAGGGATCTGAGAGGCGGAT
	Edn1	TGTCTACTTCTGCCACCTGGACAT	ATGGCTAGGACTACAATGCCCTCT
	Dusp5	GACCAGCTTATGACCAGGTATGTG	AATGGGATGTGAGGAAGGAGCAGA
	Bdnf	TGTCTCTGCTTCCTTCCCACAGTT	TGGACGTTTGCTTCTTTCATGGGC
	Cartpt	TGCAGGAAGTCCTGAAGAAGCTCA	TCCTGCCAAAGTAAGGGAAGAGGA
Rapid IEGs	Zif268	TTCGGCTCTCATCGTCCAGTGATT	AACCGGGTAGTTTGGCTGGGATAA
	Pppp1r15a	ACAATGACTCAGTGCTGTGACCTG	AGAAAGAGTGGGCTTCCTTCCAGT
	Nup98	GGGCTTTGGTACAACGTCAACA	GCTACACTGAGTTAAGCCAGCTAC
	Npas4	GTTGCATCAACTCCAGAGCCAAGT	ACATTTGGGCTGGACCTACCTTCA
	Gadd45g	ACTCACGGCGCTTGTTCTTTCACA	ATTCAGGACTTTGGCGGACTCGTA
	Gadd45b	TCCCTCTGACACTCCTTCTTTCCT	GCAGAACGATTGGATCAGGGTGAA
	Egr4	TCCTTTGGCAGGCGACTTCTTGA	CCAGGAAGCAGGAGTCTGTTAAGT
	Egr2	TCCACGTGCCTGTATTCTCATCGT	AGCTACTCGGATATGGGAGATCCA
	Dusp6	TCCTGTGCCTCTCACAAGCTGAAA	AACTTACTGAAGCCACCTGCCAGA
	Dusp1	CTCTACGACCAGGTTAGTAGGAGT	ACAGCCGCTTTCTCTATTCTCCCT
	Cyr61	ATGTATGAGTTTCAGCGTGTGGCG	GTCTGCCTTCTGACTGAGCTGTAA
	Btg2	CTCTCTCTCTTGTTTCCTCCACAG	TGTGGTTGATGCGGATACAGCGAT
	Arf4	CACAGTTTGGGATGTTGGTGGTCA	TGTACGGCATTTCTCACCTGGGTA
	Arc	GAATTTGCTATGCCAACTCACGGG	AGTCATGGAGCCGAAGTCTGCTTT
	cFos	ACAGCCTTTCCTACTACCATTCCC	CTGCACAAAGCCAAACTCACCTGT

Study design and statistical analysis

The study was not pre-registered with any journal. Data presented were generated from both hypothesis confirming and exploratory experiments. For rat primary culture experiments, sample size calculation was based on our previous publications[16]. There were no pre-determined exclusion criteria for animal work. Statistical analyses were conducted using GraphPad Prism 7 (RRID: SCR_002798, GraphPad software, San Diego, CA). Error bars represent standard error of the mean throughout the article. No tests for outliers were conducted, therefore all data-points were included. Effects were determined by t-test for generation of specific *P*-values, details are indicated throughout in figure legends. Specific *P*-values are indicated on figures, * indicates *P*-value under 0.05.

Results

In efforts to initially understand the differences between rIEG and dIEG regulation, we investigated the cell signaling pathways required for 20 representative delayed IEGs. We induced and sustained synaptic transmission in E18 rat cortical neurons. Since rapid IEGs need MAPK to optimally be transcribed but isn't known to be entirely necessary for delayed IEGs, we first targeted the MAPK cell signaling pathway. Knowing that the calcineurin (CaN) is also

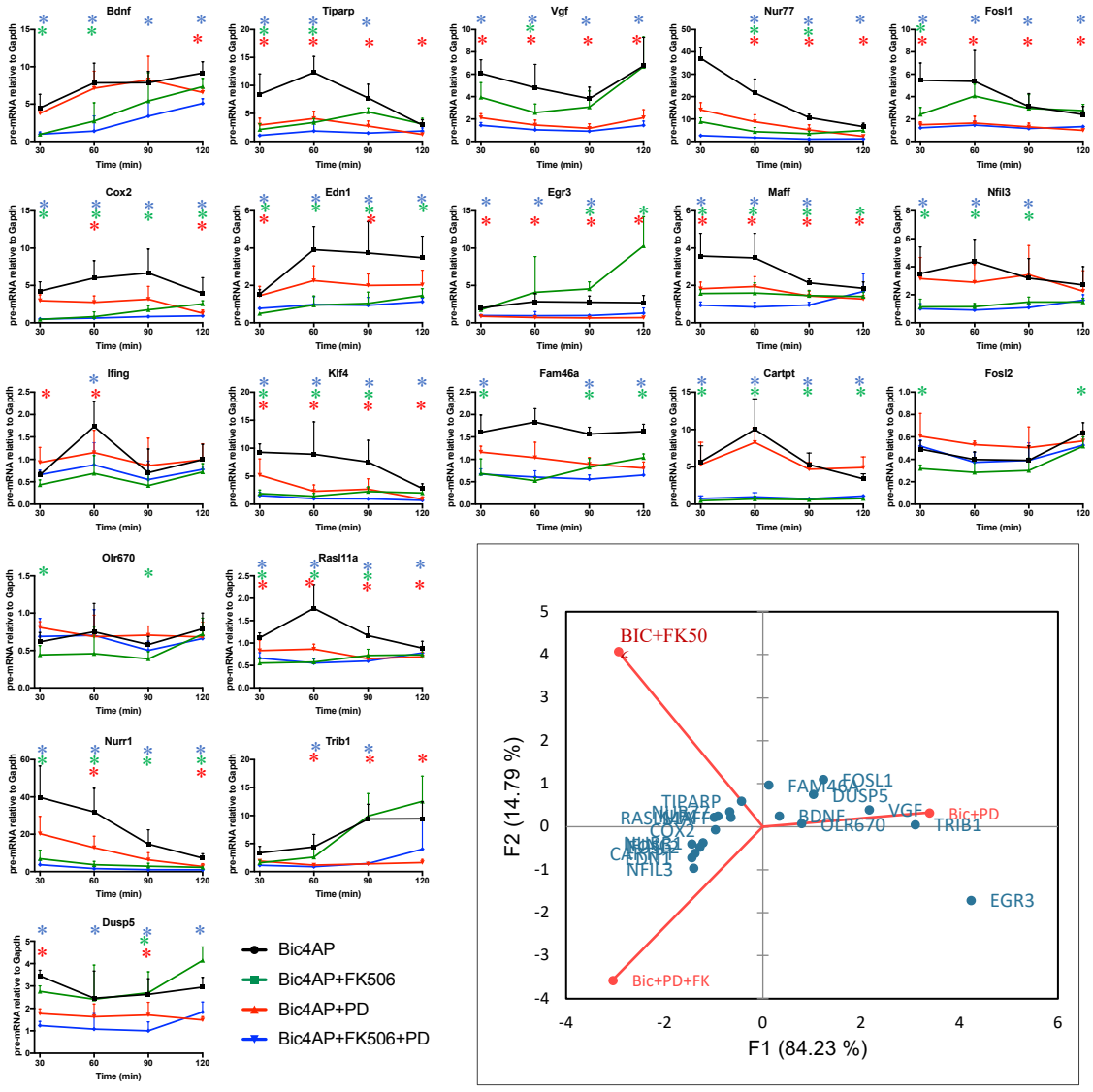

Figure 3. Delayed IEGs employ distinct cell signaling pathways. Left: *Transcription profiles of 20 dIEGs displaying the effects of inhibiting either or both calcineurin or MAPK pathways at 30, 60, 90, and 120 minutes.* **Right:** *PCA analysis of transcription times for each gene; MAPK was the principal component to their categorization. n=4-5 biological replicates.*

necessary for transcription in other instances[37], we also targeted that pathway. Simultaneous to the sustained synaptic transmission via bicuculline (Bic) and 4-aminopyridine (4AP), we either inhibited MAPK pathway, CaN pathway, or both and measuring the pre-mRNA transcribed at 30, 60, 90, and 120 minutes (Figure 3a). We can see that each of the 20 genes expresses separate effects. While some genes are more affected by the inhibition of CaN, like *Bdnf* or *Cartpt*, others, like *Dusp 5* or *Trib1*, are more affected when MAPK was targeted. Others yet, show similar sensitivities to any and all inhibition, such as *Rasl11a* and *Tiparp* (Figure 3, left).

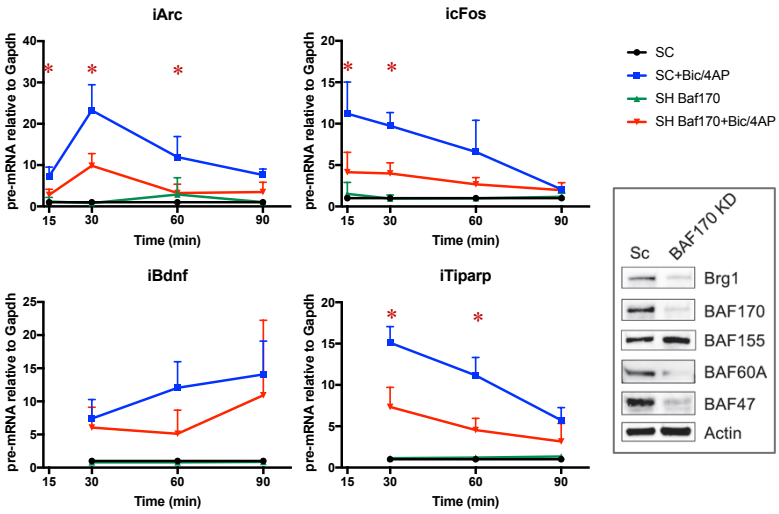

***Figure 4*. BAF is necessary for both rapid and delayed IEG transcription. Left:** *Transcription profiles of 2 rapid IEGs (top) and 2 delayed IEGs (bottom) after BAF 170 depletion. N= 3-4 biological replicates* **Right:** *Western Blot images representing the presence of several BAF subunits after BAF 170 KD.*

With such preliminary results, we went forward with grouping analysis methods to categorize the 20 genes in question by the dominant signaling pathway used. We looked at the peak transcription times for each gene to isolate the variation that can arise from comparing the several gene profiles with varied peak times. Using principal component analysis, it was clear that the most essential signaling mechanism was still the MAPK pathway as it was the principal component with the other two inhibition treatments being of lesser, though still significant importance to the genes themselves with it being the primary component of analysis for the gene's categorization (Figure 3, right).

Since the analysis performed on the dIEGs was not conducive to a clear categorization based on the two signaling mechanisms, we decided to explore the necessity of chromatin remodeling as the possible regulatory mechanism that creates the temporal differences in IEG transcription. From previous research, we know that rapid IEGs already have open chromatin in their inactive state while the same was not shown for the delayed IEGs[16]. Therefore, we looked into the BAF complex as the chromatin remodeler responsible for delayed IEG regulation while not being necessary for rapid IEG transcription. We targeted the known chromatin remodeling complex BAF by starting with a 5-day short-hairpin knockdown (KD) of the canonical BAF170 subunit. Again, inducing synaptic transmission using bicuculline (bic) and 4-aminopyridine (4AP), we observed the transcription levels of two representative dIEGs, Bdnf and Tiparp, along with two rIEGs for positive control, Arc and cFos. When comparing the effects of BAF170 KD to the control groups treated with scramble RNA, we indeed see a lower rate of pre-mRNA transcription in those genes treated with BAF170 KD. In fact, we also saw the same effects on the rIEGs tested (Figure 4, left). Interestingly, while assessing the efficacy of BAF170 knockdown procedures, we also see that several other BAF subunits were affected with the single KD process (Figure 4, right). Together, this suggests that the BAF complex may be involved in general IEG transcription regulation mechanisms.

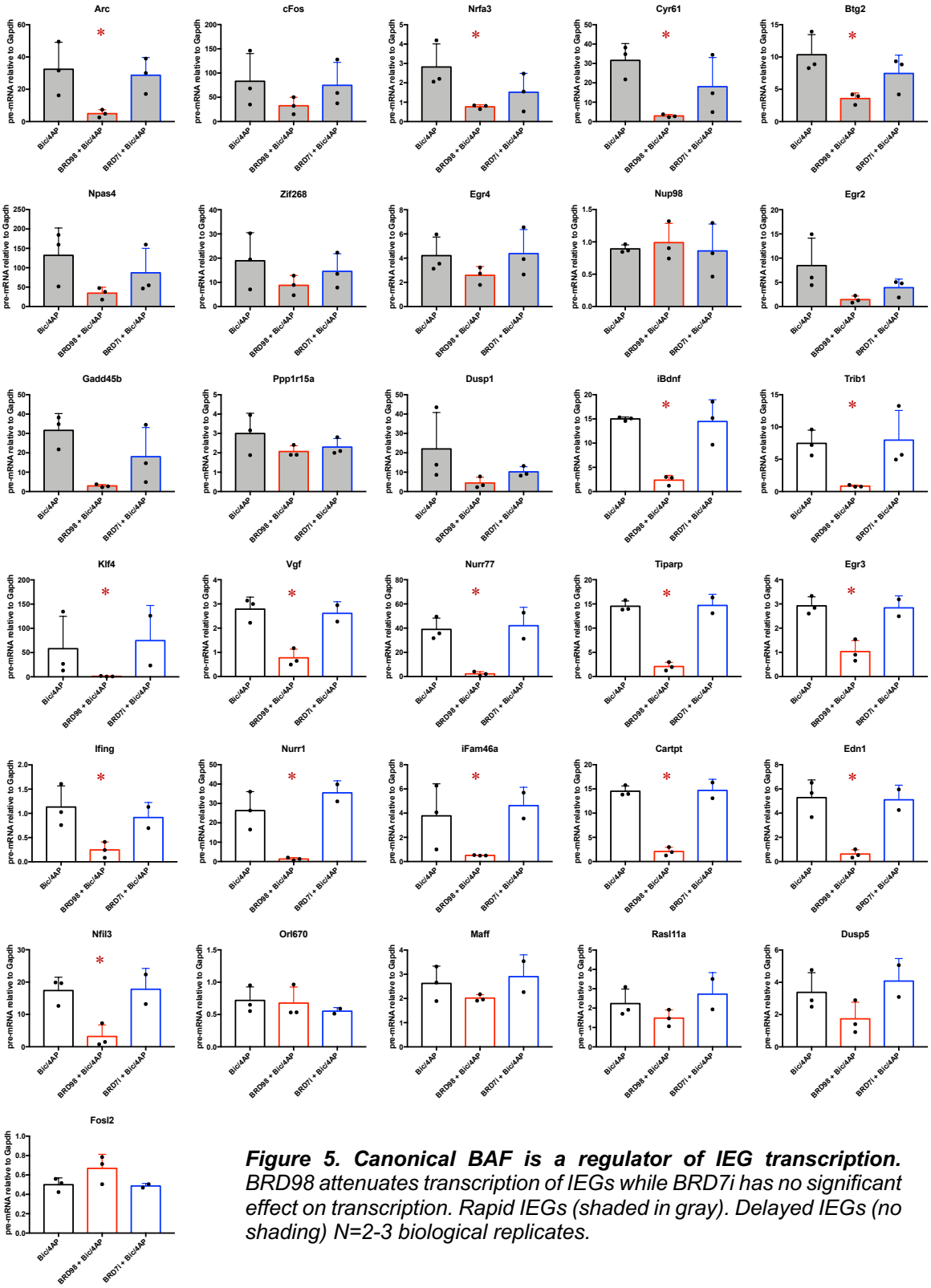

Figure 5. Canonical BAF is a regulator of IEG transcription. *BRD98 attenuates transcription of IEGs while BRD7i has no significant effect on transcription. Rapid IEGs (shaded in gray). Delayed IEGs (no shading) N=2-3 biological replicates.*

To confirm the specificity of BAF complex involved, we looked both in and out of the canonical BAF structure. Inhibitors BRD7i, and BRD98 were selected to target and inhibit the PBAF and cBAF complexes respectively (Figure 5 & 6)[38]. Because altering the specific function of BAF via viral infections for the KD procedures could cause altering to the homeostatic processes of the cells, or adaptive cellular processes taking over, confirming the use of BAF for transcription regulation through alternate means was essential. We collected data from 13 rIEGs and 18 dIEGs after simultaneous inhibition of BAF subunits and synaptic stimulation with bicuculline (Bic) and 4-aminopyridine (4AP). Given that we worked with rat cortical neurons specifically, we see that the effect of pharmaceutical inhibition was only seen in the cBAF subunits, and not the BRD7 subunit from PBAF, as expected (Figure 5). 13 of the 18 dIEGs demonstrated a significant decrease in pre-mRNA transcription when inhibiting with BRD98, while 4 of the 13 rIEGS showed the same effects. None of the genes, however, showed a decrease during BRD7 inhibiton. This significantly suggests that the BAF involved is in fact the cBAF as well as, confirms the findings from Figure 4, which reveal BAF involvement in IEG transcription.

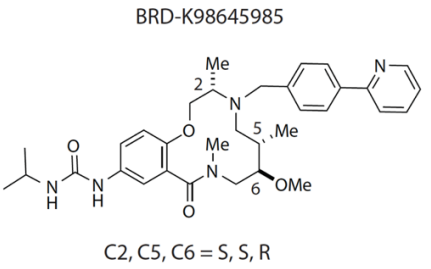

Figure 6. Structure of BAF inhibitor, BRD98. *(from Dr. Emily Dykhuizen, Purdue University)*

Due to the possibility of further interactions between subunits despite the use of pharmaceutical inhibitors, we decided to use a protein degrader, ACBI1, to specifically target the BRG1/BRM subunits, in cBAF. ACBI1, also known as PROTAC has been developed and use for oncology research targeting BAF related cancers and can be applied to other realms of research[39]. By degrading this major ATPase subunits, as opposed to inhibition, we can cause the breakdown of the BAF complex with higher specificity and success than other methods such as knockdowns (Figure 7). In doing so, we can get a better idea of the function as it will have little possibility to interact with any other substance. With similar cellular induction, we recorded the pre-mRNA transcription levels of dIEG and rIEGs previously observed after bicuculline (Bic) and 4-aminopyridine (4AP) stimulation on its own, with ACBI1, or with cis-ACBI1 as our positive control as it has a different configuration to the standard ACBI1 which should not bind or degrade BAF (Figure 7). 16 of the 34 IEGs demonstrated a significant decrease of transcription when treated with ACBI, again showing a large portion of IEGs relying on BAF for transcription (Figure 8). This method isn't perfect and may have nonspecific targets, however, combined with the several previous methods used, we can be certain of our conclusions.

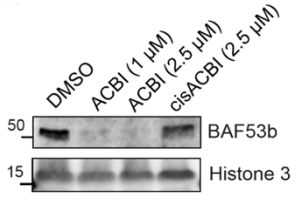

Figure 7. ACBI efficacy on BAF53b degradation. *A representative western blot image of the effects of ACBI on BAF53b. (from Poston, R et. al. 2018)*

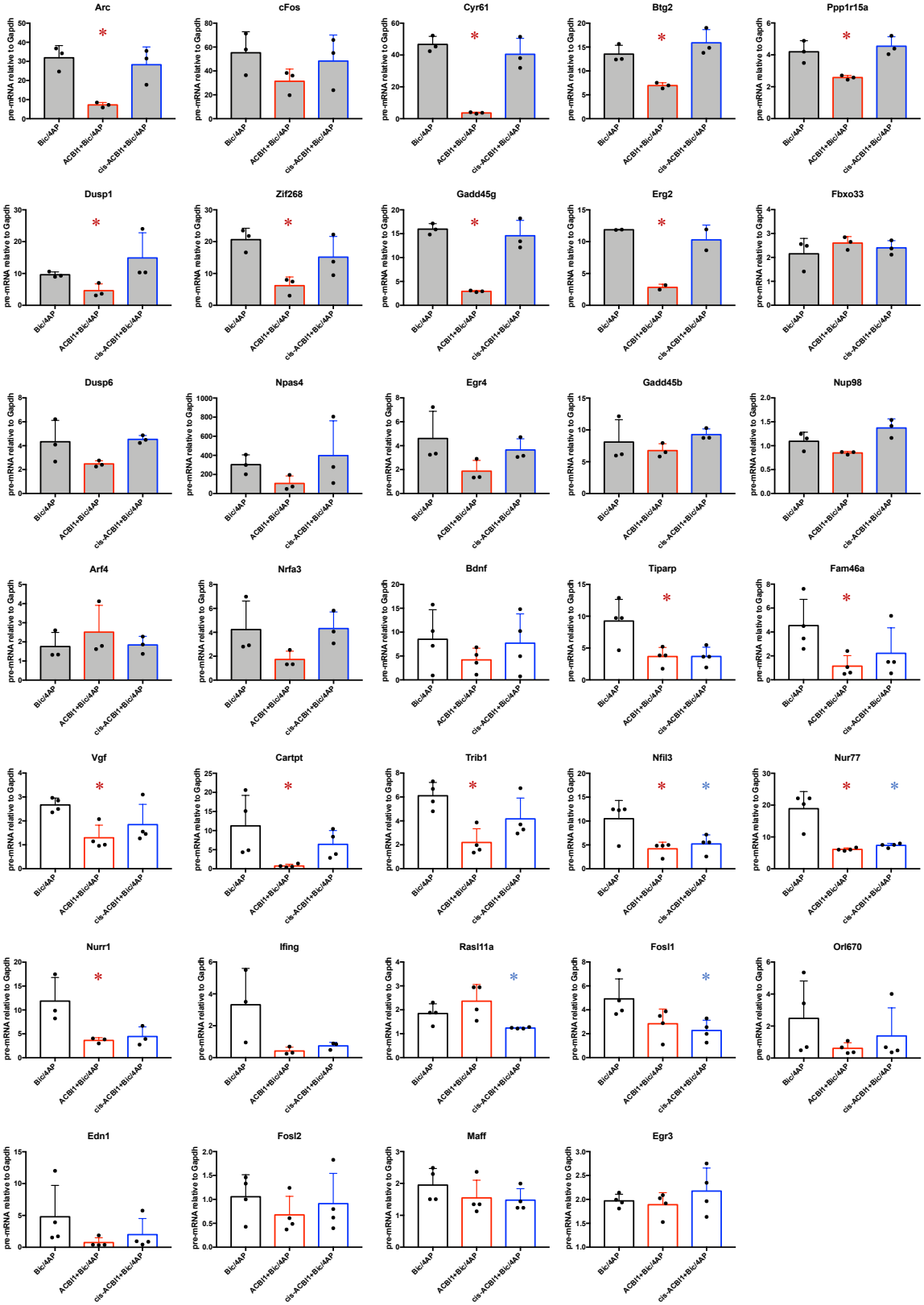

Figure 8. BAF degrader, ACBI1 confirms the necessity of BAF for several IEGs when compared to the cis arrangement of ACBI1 as well as baseline transcription. *Rapid IEGs (shaded in gray). Delayed IEGs (no shading) N=4 biological replicates.*

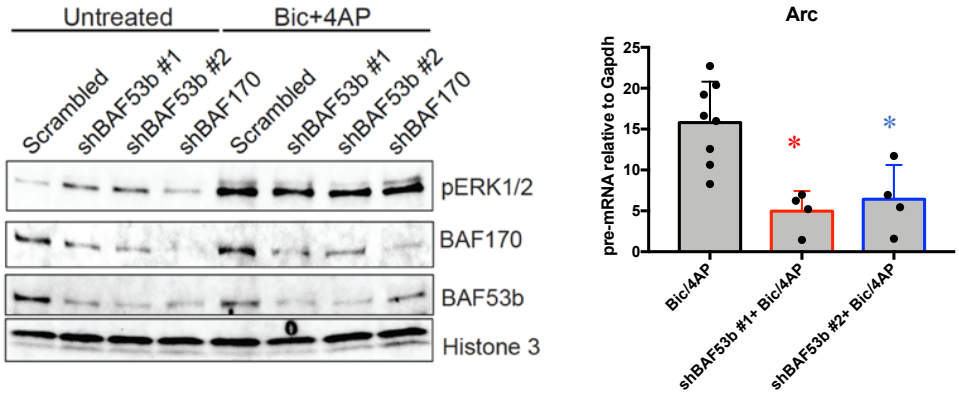

Figure 9. Synaptic function is uninterrupted with BAF 170 and 53b knockdowns. Left: *Western blot images depicting pERK, for neuronal function check, and BAF subunits 53b and 170 after BAF170 and 53b knockdowns.* ***Right:*** *Baf53b knockdown shows slight decrease in Arc pre-mRNA transcription. N=4-6 biological replicates.*

To verify the proper synaptic connection and cellular function, we checked for pERK levels after both BAF170 and 53b knockdowns. All the knockdown treatments showed no decreased just as expected when compared to the scrambled RNAi treatment (Figure 9, left). This means that our neuronal function can be assumed normal. Interestingly, when we specifically targeted neuron specific BAF53b using the same KD processes as before, we also saw a significant decrease in transcription in Arc (Figure 9, right). A more expansive look at a larger set of genes will grant more clarity in this subunit's effects. This only makes the mechanism by which this complex works the more intriguing.

Confirming that a majority of the IEGs observed require BAF for proper transcription, we can begin to question the mechanism by which this role is carried out. To begin, we revisited the use of the inhibitor BRD98 at two separate time points. We created treatments groups that were either subjected to a simultaneous inhibition using BRD98 and bicuculline (Bic)/4-aminopyridine (4AP) stimulation, as well as a 30-minute pretreatment with BRD98 before cellular stimulation. As seen previously, BRD98 decreased transcription in a majority of the genes observed. We found that 12 out of the 13 rIEGS were drastically affected by both simultaneous and pre-inhibition. In fact, there was little difference between the effects of pretreatment and simultaneous inhibition when compared to the uninhibited stimulation treatments (Figure 10). This means that the time BAF is necessary is ongoing and not just needed to initiate the transcription in some way. This may suggest that BAF isn't only a regulator to initiate transcription. BAF may be necessary for ongoing transcription to occur.

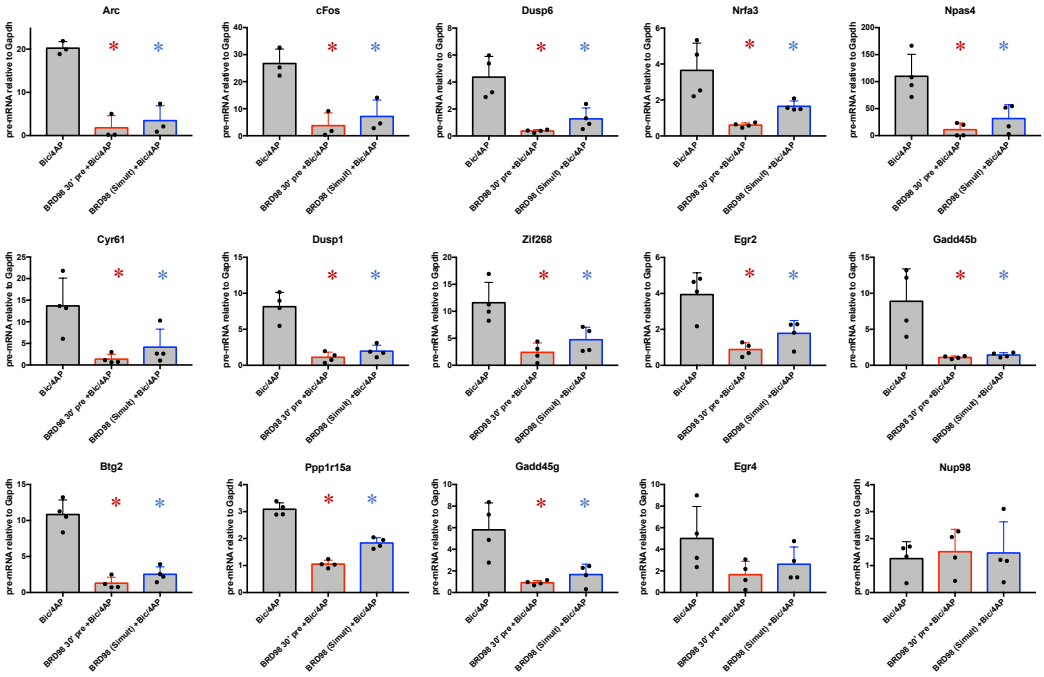

Figure 10. Rapid IEGs need BAF for continuous transcription*. BRD89 inhibition 30 minutes prior to cellular stimulation, as well as simultaneous inhibition with stimulation show attenuated transcription. N=4 biological replicates.*

Discussion

Activity induced transcription has a long way to go before being fully understood. IEGs specifically, have come a long way and yet the distinctions between the several categories of genes are not clear. Unfortunately, testing whether there is reliance on MAPK, CaN, or both did not give us a clear distinction of the groups rather reminded us that they are indeed a single general group of genes prior to being separated into categories by the more general reliance on MAPK by most genes, as well as the further explored necessity of BAF for their transcription.

While the BAF complex is known to play a role in many neurobiological disorders, the specific role and mechanisms it employs in activity induced transcription are not known. What we know is that without proper BAF function, as seen by BAF170 knockdown, transcription is attenuated in both rapid and delayed IEGs. While we expected delayed IEGs to be affected by the lack of available chromatin remodeling by BAF, this finding opened up many more unexpected questions over BAF's role because rapid IEGs already have open chromatin, yet they also seem to require BAF. In fact, it was the pivotal point that propelled the rest of the study by confirming these results through several different methods.

In trying to understand the extent of this pattern of function among the IEGs, we extended the list of IEGs views as well as explored other avenues of inhibition. In collaboration with Emily Dykhuizen at Purdue University, we were able to effectively inhibit the canonical BAF complex using BRD 98 in order compare it to inhibition of BRD7, a known PBAF subunit. The isolation of pharmaceutical inhibition allowed for more precise identification of the specific BAF complex structure we were investigating while showing the necessity of the complex on over half the genes observed. BRD7i inhibition did not have any effect on IEG transcription while BRD98 caused a significant decrease in the transcription of 17 of 31 genes.

Because pharmacological drugs can have non-specific artifacts, we additionally targeted BAF by degrading the structure using a degrader (ACBI1, also known as PROTAC). ACBI1 targets the main ATPase subunits of BAF, BRM and BRG1essentially, rendering the entire complex unable to function. Again, we saw a large portion of IEG transcription attenuated when degrading the major subunits. Interestingly, there were some IEGs continuously unaffected by the inhibition or degradation of BAF. Nup98 for example, didn't show any decrease in transcription when BAF was inhibited with BRD89 nor when degraded with ACBI1. This can set a whole new standard or pattern of genes that may not require BAF as the rest of the genes observed. Meaning, there may be a subset of IEGs that do not follow the standard BAF regulation set by the BAF experiments in this study. Following up on these particular genes can be a new avenue for understanding BAF by understanding the cases in which it isn't a regulator.

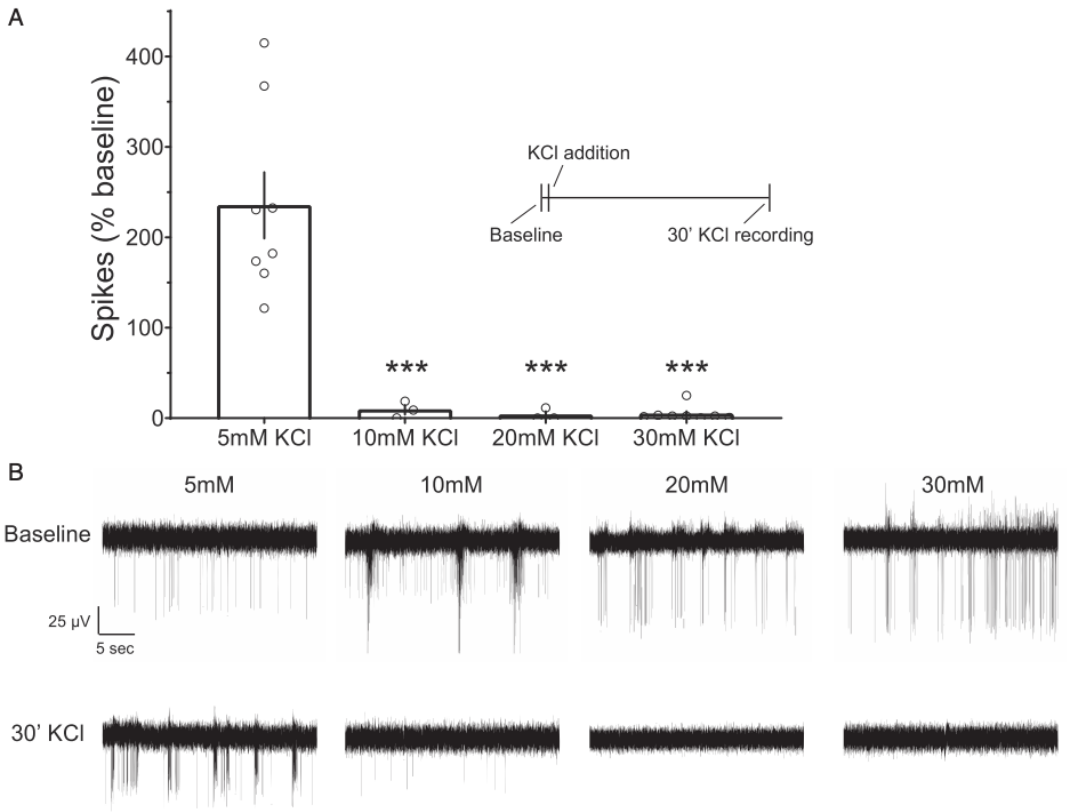

Figure 11. Neuronal Activity Under Elevated Extracellular KCl In Vitro. A: Spikes under KCl treatment as a % of baseline activity before treatment. Spiking is observed under 5mM KCl treatment, which matches [KCl] in standard media conditions, but disappears as [KCl]o rises. Spike data were compared using ANOVA. *** $P < 0.001$. B: Example recordings during baseline measurements (top) and KCl treatments (bottom). (From Rienecker, Kira D A et al., 2020)

Finally, to begin understanding the neuronal purpose of BAF, we did a simple test where we conducted a pretreatment of BAF inhibition before cellular stimulation and compared it happening simultaneously. In doing so, we are looking at the events before, during, and after stimulation with regards to the function of BAF as a possible transcription regulator for IEGs. As the results show, not only is needed for transcription at a pretreatment level, but also when simultaneously used it attenuated the transcription, thus suggesting that BAF may be involved continuously as opposed to solely being essential at the initial point of transcription.

Based on this, we've come closer to understanding BAF by finding that cBAF, is necessary for much of the activity-induced IEG transcription. However, this role of BAF is not generally agreed upon. When we began our exploration of the BAF complex's role in 2018 with the data presented in this piece to support the claim that BAF is a positive transcription regulator. However, in 2020, Wenderski *et. al.* from the Gerald Crabtree laboratory at Stanford University presented this same subunit as a repressor linked to ASD[40]. We believe the method of treatment and experimental design itself is conducive to cellular adaptations that may affect

the outcome observed. Keeping this particular possibility in mind, we kept the experiments to a short timeframe, attempting to minimize cell homeostatic adaptations or interactions to occur. The repetitive confirmation from BRD89 and ACBI1 only strengthens the suggested necessity of BAF for proper neuronal transcription function.

We also precisely chose our neuronal induction method to be done using bicuculline (Bic) and 4-aminopyridine (4AP) because we can be sure that synaptic transmission is allowed. As with the previous claim that BAF53b may be a repressor[40], the researchers used a KCl induction treatment for their experiments, which has been shown to depolarize the membrane but render it electrically inert[41]. As seen in Figure 11, from recordings in multiple electrode arrays (60MEA200/30-Ti, MultiChannel Systems (MCS), Reutlingen, Germany), the baseline cellular activity detected in the top panel is completely muted after 30-minute addition of different concentrations of KCl (bottom panel)[40]. Further studies using the methods presented by the Crabtree and our labs, with more selective stimulation and experimental methods, can help clarify the circumstances for different conclusions.

Further work to specifically find the role of BAF in transcription regulation has to begin with assessing where the complex is found during the transcription process. While the involvement of nBAF can be confirmed, the mechanism it employs is unknown. For that, ChIP may be the simplest method to begin to narrow the interactions BAF can have at the transcription sites and how its dysfunction affects the processes. As it is a chromatin remodeler, BAF can be affecting the direct accessibility of genes in a very specific way among neuronal activity induced IEG transcription. Perhaps the involvement it has with other transcription factors, RNA polymerase (Pol II) primarily. This idea isn't far-fetched as there has been research demonstrating the involvement of BAF in Pol II elongation in yeast. They showed that the yeast equivalent of BAF, SWI/SNF is required for the histone eviction at the elongation sites specifically[42].

BAF, though known as a chromatin remodeler involved with many disorders, isn't known specifically down to the subunit functions. Therefore, the key to understanding the links BAF has with these disorders, and to find potential therapeutic interventions to some of them, is lying in the understanding of the specific mechanisms and location of relevant subunits that control this chromatin remodeler and its functions.

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