Genome-Wide Identification and Characterization of Stimulus-Responsive Enhancers in the Nervous System

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Genome-wide identification and characterization of stimulus-responsive enhancers in the nervous system

A dissertation presented
by
Athar Naveed Malik

to
The Division of Medical Sciences

in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the subject of Neurobiology

Harvard University Cambridge, Massachusetts

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Genome-wide identification and characterization of stimulus-responsive enhancers in the nervous system

Abstract

During development, intrinsic genetic programs give rise to distinct cellular lineages through the establishment of cell type specific chromatin states. These distinct chromatin states instruct gene expression primarily through the genome-wide demarcation of enhancers. In addition to maintaining cellular identity, the chromatin state of a cell provides a platform for transcriptional responses to environmental signals. However, relatively little is known about the influence of extracellular stimuli on chromatin state at enhancers, and it is not clear which enhancers among the tens of thousands that have been recently identified function to drive stimulus-responsive transcription.

In the nervous system, the chromatin state of terminally differentiated neurons not only maintains neuronal identity but also provides a platform for sensory experience-dependent gene expression, which plays a critical role in the development and refinement of neural circuits and in long-lasting changes in neuronal function that
underlie learning, memory, and behavior. Using chromatin-immunoprecipitation followed by high throughput sequencing (ChIP-Seq), we determined the effects of neuronal stimuli on the active chromatin landscape of mouse cortical neurons. We discover that stimulation with neuronal activity and brain derived neurotrophic factor (BDNF) cause rapid, widespread, and distinct changes in the acetylation of histone H3 lysine 27 (H3K27Ac) at thousands of enhancers throughout the neuronal genome. We find that functional stimulus-responsive enhancers can be identified by stimulus-inducible H3K27Ac, and we use this dynamic chromatin signature to discover neuronal enhancers that respond to neuronal activity, BDNF, or both stimuli. Finally, we investigate the transcriptional mechanisms underlying the function of stimulus-responsive enhancers. We show that a subset of stimulus-responsive enhancers in the nervous system require the coordinated action of the stimulus-general transcription factor activator protein 1 (AP1) with additional stimulus-specific factors.

Our studies reveal the genome-wide basis for transcriptional specificity in response to distinct neuronal stimuli. Furthermore, the comprehensive identification of neuronal activity and BDNF-dependent enhancers in cortical neurons provides a critical resource for elucidating the role of stimulus-responsive transcription in synaptic plasticity, learning and memory, behavior, and disease. Finally, the epigenetic signature of stimulus-inducible H3K27Ac may aid in the identification and study of stimulus-regulated enhancers in other tissues.
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DEDICATION

In the name of Allah, the Gracious, the Merciful.

The Holy Quran [1:1]

In the creation of the heavens and the earth and in the alternation of the night and the day there are indeed Signs for men of understanding; Those who remember Allah while standing, sitting, and lying on their sides, and ponder over the creation of the heavens and the earth; and say: “Our Lord, Thou hast not created this in vain; nay, Holy art Thou.”

The Holy Quran [3:191-192]

Blessed is He ... Who has created seven heavens in harmony. No incongruity canst thou see in the creation of the Gracious God. Then look again. Seest thou any flaw? Aye, look again, and yet again, thy sight will only return unto thee confused and fatigued, having seen no incongruity.

The Holy Quran [67:2-5]

Say, ‘My Prayer and my sacrifice and my life and my death are all for Allah, the Lord of the worlds.’

The Holy Quran [6:163]

-----

I dedicate this thesis, everything that has led to it, and any good that may come from it, to Allah, the Lord of the worlds.
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Over the course of my doctoral training, I have had the opportunity to work with and learn from many extraordinary scientists. Foremost among these individuals is my advisor Michael Greenberg. Mike accepted me into his laboratory at a time when I had little experience in molecular biology, biochemistry, genomics, or neuroscience, fields that my work in his laboratory would eventually require. Mike jokes that when I first joined the laboratory, I barely knew how to pipette. While I wouldn’t go that far, I certainly realize that I had a lot to learn. Due to the opportunity that Mike gave me to train in his laboratory, I am now not only a pipetting expert, but I have also learned a great many of the diverse techniques and experimental methods used in his laboratory. But all this is technical. What I am most indebted to Mike for is shaping me as a scientist. Mike is not only a scientist of the highest caliber, he is also an extraordinary scientific mentor. One of the things that I noticed early on about Mike was that he has a clear vision of what it takes to be a successful scientist and how to train someone to get there. Mike has developed within me the intellectual qualities of independent judgment, scientific rigor, depth of thought, concentration, and hard work. Furthermore, Mike has provided me with all of the resources I could imagine to pursue my research. I always felt that if an experiment were scientifically justified, I would have the opportunity to perform it in Mike’s lab. This support enabled me to pursue scientific questions in a manner limited only by my creativity and the hours of the day. This has been a great joy and privilege, and I thank Mike for providing me the opportunity to experience scientific research in such a pure form.
One of Mike’s great skills as a scientist and as a mentor is to assemble individuals from diverse backgrounds into his laboratory to work together to answer scientific problems. I have had the good fortune of working with and learning a great many extraordinary people in Mike’s laboratory. I will start at the beginning of my tenure in the lab, when I had the opportunity to rotate with Yingxi Lin, then a postdoctoral fellow in the lab. Yingxi taught me to be persistent and optimistic, even in the face of failed experiments. She also helped me perform my first ChIP experiments during my rotation. Little did I know that that would serve as a foundation for my later work in the lab! For all this, I thank her. When I first joined the lab, I had the opportunity to work with Steven Flavell, a senior graduate student in the lab. Steve taught me the importance of cultivating good scientific habits, reading scientific literature, and thinking creatively about scientific problems, and I thank him for these important lessons.

After working with Steve, I worked largely independently for a time but then had the opportunity to work with a research assistant, Alex Rubin. Alex has been a pleasure to work with and has been a model research assistant. Alex learns quickly and can function independently. Because of his tremendous efficacy within the lab, I have often gotten overenthusiastic about the experiments that Alex and I might be able to do within a short period of time. Alex has somehow been able to keep up with most of my experimental enthusiasm, but has also kept me grounded in what we might be able to realistically accomplish. For all this, I thank Alex.

One of the challenges in the generation of genomic data is its analysis. As I have generated a substantial amount of genomic data during my doctoral research, analyzing
it has been a considerable challenge. I am indebted to Martin Hemberg, a postdoctoral fellow who has been a longstanding bioinformatics collaborator throughout my doctoral research. Martin has been a great help in performing analyses of genomic data and has taught me a great deal in this regard. I thank Martin for his past and continuing support. In addition to Martin, I would like to thank research scientist David Harmin and postdoctoral fellow Kai-How Farh for their help with various genomic analyses and for helpful discussions. I am also indebted to the course instructors for “Integrated Statistical Analysis of Genome Scale Data,” a course I took at Cold Spring Harbor Laboratory in the summer of 2011. They provided me with a foundation upon which I build the skills necessary to analyze genomic data.

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ATTRIBUTIONS

All work in this thesis was initiated by Athar Malik. Athar Malik conceived and designed all experiments, performed all ChIP-Seq and RNA-Seq experiments, performed the majority of the bioinformatic analyses of ChIP-Seq and RNA-Seq data, assisted with luciferase reporter assays and analysis of luciferase reporter data, and wrote the thesis. Alex Rubin performed the majority of luciferase reporter assays and analysis of luciferase reporter data. Thomas Vierbuchen cloned the AP1 dominant negative A-Fos and assisted with luciferase reporter assays. Martin Hemberg and David Harmin assisted with bioinformatics analyses. Michael Greenberg supervised all research.
1 GENERAL INTRODUCTION

The nervous system enables higher organisms to not only sense and respond to the environment, but also to learn from and remember past experiences. Importantly, these fundamental functions of the nervous system are emergent properties that derive from the ability of individual neurons to sense, respond, and adapt to sensory stimuli. A characteristic component of the neuronal response and adaptation to extracellular stimuli is new gene transcription. This phenomenon, first discovered over 25 years ago, is now known to play a critical role in the development and function of the nervous system, and perturbations in this program of gene expression are thought to contribute to neurological disease. Despite great progress over the past decades in understanding the mechanisms underlying stimulus-dependent programs of gene expression, major questions remain. How is neuronal chromatin affected by extracellular stimuli? What are the enhancers in the genome that drive stimulus-dependent transcription? What is the regulatory logic of these elements? How are specific transcriptional responses generated for different stimuli? What transcription factors play a role in generating these responses? How do multiple transcription factors work together to achieve this purpose? What are the effector molecules regulated by stimulus-responsive transcription factors that mediate adaptive cellular responses to neuronal activity? These are some of the questions that have motivated my thesis work.


1.1 DISCOVERY OF STIMULUS-RESPONSIVE TRANSCRIPTION IN THE NERVOUS SYSTEM

Stimulus-responsive transcription in the nervous system was discovered nearly 30 years ago with the discovery of neuronal activity-dependent transcription of the c-Fos gene. Stimulation of neuronal cells with either the cholinergic agonist nicotine or membrane depolarization with elevated extracellular concentrations of potassium chloride (KCl) was shown induce transcription of c-Fos (Greenberg et al., 1986). In addition to discovering this phenomenon, this first study also showed several key characteristics of neuronal activity-dependent transcription. Activity-dependent transcription was shown to be rapid, occurring with 5 minutes of stimulation. Furthermore, activity-dependent transcription was shown to be transient, lasting approximately 30 minutes after stimulus onset. Finally, activity-dependent transcription was shown to be dependent on extracellular calcium, being abolished when cells were cultured in the presence of the calcium chelator ethylene glycol tetraacetic acid (EGTA). It is interesting to note that this influential study that set the ground for many subsequent discoveries in the nervous system was done in neuronally differentiated PC12 cells. PC12 cells are a cell line derived from a rat pheochromocytoma, which is a neuroendocrine tumor of the medulla of the adrenal gland. Previous work had shown that these cells differentiate into neuronal cells when exposed to nerve growth factor, establishing PC12 cells as a useful culture system to study neuronal cells (Greene and Tischler, 1976). While the initial discovery of neuronal activity-dependent transcription was made in this somewhat artificial cell culture system, neuronal activity-dependent transcription was subsequently observed in many
additional contexts, demonstrating the power of simplified model systems to observe and study biological phenomena.

Soon after the initial discovery of activity-dependent induction of c-Fos in PC12 cells *in vitro*, several groups observed c-Fos induction in response to activity in cultured primary neurons *in vitro* (Hunt et al., 1987; Szekely et al., 1987) and in response to diverse stimuli in the nervous system *in vivo* (Morgan and Curran, 1991). Many of the stimuli that have been shown to induce c-Fos in the nervous system in-vivo are stimuli that increase neuronal activity or activate particular neural circuits. Such stimuli include the convulsant pentylenetetrazol (Morgan et al., 1987; Saffen et al., 1988; Sonnenberg et al., 1989a; Sonnenberg et al., 1989b; Sonnenberg et al., 1989c), the stimulant kainic acid (Le Gal La Salle, 1988; Popovici et al., 1990; Sonnenberg et al., 1989b), electrical stimulation (Daval et al., 1989; Douglas et al., 1988; Dragunow and Robertson, 1987; Hunt et al., 1987; Sagar et al., 1988; Sharp et al., 1989b; Shin et al., 1990; Sonnenberg et al., 1989b; White and Gall, 1987; Winston et al., 1990; Wisden et al., 1990), the glutamate neurotransmitter receptor agonist N-methyl-D-aspartic acid (NMDA) (Cole et al., 1989; Kaczmarek et al., 1988; Sonnenberg et al., 1989b), the γ-aminobutyric acid receptor (GABAR) antagonist picrotoxin (Sonnenberg et al., 1989b), D1-dopamine receptor agonists (Robertson et al., 1989a; Robertson et al., 1989b), β-adrenergic receptor agonists (Gubits et al., 1989), the opiate analgesic morphine (Chang et al., 1988), opiate withdrawal (Hayward et al., 1990), nociceptive and peripheral stimulation (Bullitt, 1989; Draisic and Iadarola, 1989; Menetrey et al., 1989; Presley et al., 1990; Wisden et al., 1990), and light stimulation (Aronin et al., 1990; Rea, 1989; Rusak et al., 1990; Sagar et al., 1988). In addition to the above stimuli that increase
neuronal activity and activate neural circuits, additional stimuli not directly related to
neuronal activity have also been shown to induce c-Fos in the nervous system in vivo,
including surgical lesions and nerve transections (Dragunow and Robertson, 1988;
Sharp et al., 1989a; Sharp et al., 1989c), cerebral ischemia (Jorgensen et al., 1989;
Onodera et al., 1989), heat stress (Dragunow et al., 1989), adrenalectomy (Jacobson et
al., 1990), intracortical NGF injections (Sharp et al., 1989a), and even intracortical
saline injections (Kaczmarek et al., 1988). These data suggest that new gene
transcription is a general hallmark of a stimulated neuron. The nature of the stimulus
does not appear to matter. Whether a neuron is stimulated with a physiological
stimulus, a pathological stimulus, a stimulus related to neuronal activity, or a stimulus
unrelated to neuronal activity, genes such as c-Fos are induced.

It is important to note that c-Fos is not unique in its transcriptional induction by
neuronal activity. Soon after the discovery of the activity-dependent transcription of c-
Fos, other genes were also shown to be induced in response to neuronal activity,
including c-Jun, JunB, and Egr1 (Saffen et al., 1988; Sukhatme et al., 1988). More
recently, investigators have systematically characterized hundred of genes that are
induced in neurons in response to neuronal activity (Altar et al., 2004; Hong et al.,
2004; Li et al., 2004; Nedivi et al., 1993; Park et al., 2006). Genes induced in the
nervous system in response to stimuli can be divided into two waves of induced genes.
The first wave of genes, termed immediate early genes, consists of genes like c-Fos that
are expressed rapidly (within minutes) and transiently in response to stimuli. The
second wave of genes, termed late response genes, consists of genes like brain-derived
neurotrophic factor (BDNF) that are expressed more slowly (over hours) in response to
stimuli. In general, the transcription of immediate early genes does not require new protein synthesis while the transcription of late response genes does require new protein synthesis. Many immediate early genes encode transcription factors that have been proposed to regulate the transcription of late response genes. Late response genes, in turn, are generally thought to encode molecules that effect longer lasting changes within the cell (Sheng and Greenberg, 1990).

Importantly, the stimulus-dependent induction of c-Fos and other genes is not limited to the nervous system. Even before c-Fos was shown to be induced by neuronal activity in neuronal cells, it was shown to be induced by serum and growth factors in fibroblasts (Greenberg and Ziff, 1984; Muller et al., 1984). c-Fos has also been shown to be induced in numerous other diverse circumstances, including cellular differentiation (Mitchell et al., 1985; Muller et al., 1985), wounding of a fibroblast monolayer (Verrier et al., 1986), thyroid hormone stimulation of thyroid cells (Colletta et al., 1986), IL-1 stimulation of lymphocytes (Kovacs et al., 1986), IGF-1 stimulation of skeletal muscle (Ong et al., 1987), growth hormone releasing hormone stimulation of pituitary cells (Billestrup et al., 1987), heat shock stimulation of HeLa cells (Andrews et al., 1987), follicle stimulating hormone stimulation of Sertoli cells (Hall et al., 1988), interferon stimulation of various cells (Wan et al., 1988), and steroid and growth factor stimulation of breast cancer cells (Wilding et al., 1988). The diverse circumstances in which c-Fos is induced suggests that C-FOS may play a critical role in the cellular response to many extracellular signals in many cell types, and that C-FOS may be performing a stimulus-general function that is required for the cellular response to stimulation.
1.2 Function of stimulus-responsive transcription in the nervous system

As soon as c-Fos and other genes were shown to be induced in response to neuronal activity, there was great interest in understanding the function of stimulus-responsive transcription in the nervous system. Neurons were known to exhibit rapid responses to synaptic stimulation that could be explained by the opening of ligand gated ion channels or second-messenger mediated intracellular signaling. However, neurons were also known to exhibit slower, long-term responses to synaptic stimulation that could not be explained by the same mechanisms. At an organismal level, one of the most interesting long-term responses of the nervous system is memory. An appealing hypothesis was that stimulus-responsive genes might underlie memory by facilitating adaptive changes in nervous system structures such as synapses (Morgan and Curran, 1989, 1991; Sheng and Greenberg, 1990). A large body of work has supported roles for stimulus-responsive transcription in memory and synapse development and function (Flavell and Greenberg, 2008; Greer and Greenberg, 2008).

1.2.1 Role in long-term memory

Interestingly, even before the discovery of stimulus-responsive transcription in the nervous system and the speculation that this program of gene expression might contribute to memory, experiments suggested that new protein synthesis was critical for long-term memory. In a classic paper published over twenty years before the discovery
of activity-dependent transcription of c-Fos in the nervous system, investigators demonstrated that intracortical injections of the protein synthesis inhibitor puromycin in mice caused a loss of memory in a behavioral task (Flexner et al., 1963). This suggested that new protein synthesis was an important component of the mechanisms giving rise to memory. Subsequently, a large number of studies provided evidence that extensive inhibition of protein synthesis in the brain through injection of various protein synthesis causes loss of long-term memory, without causing changes in short-term memory or gross changes in behavior (Davis and Squire, 1984).

Further evidence for the role of new gene transcription in memory came from work done in the sea snail Aplysia californica, reviewed in (Kandel, 2001). When the siphon of the snail is touched, the snail exhibits a protective reflex that leads to withdrawal of the gills, similar to how someone might withdraw one’s hand after touching a hot object. The length of time the gills remain retracted serves as an indication of learning by the snail. Normally, the withdrawal reflex leads to retraction of the gills for approximately 10 seconds. However, if the tail of the snail is shocked (indicating to the snail a dangerous environment), the subsequent withdrawal reflex leads to retraction of the gill and siphon for a longer period of time, in a process called sensitization. Furthermore, the animal remembers the shock and exhibits sensitization to subsequent stimuli. One shock will create a short-term memory that will lead to sensitization for a few minutes. Multiple shocks can create a long-term memory that can lead to sensitization for several days. The discovery of the neural circuitry underlying this reflex behavior (Byrne et al., 1978; Hawkins et al., 1981; Kupfermann et al., 1974) enabled the discovery that short-term and long-term sensitization lead to the
strengthening of synaptic connections between sensory and motor neurons, termed heterosynaptic facilitation (Abrams et al., 1984; Brunelli et al., 1976; Castellucci et al., 1970; Frost et al., 1985; Hawkins et al., 1981; Pinsker et al., 1973). These discoveries led to the hypothesis that heterosynaptic facilitation at the cellular level may underlie sensitization seen at the behavioral level. By studying the neural circuit underlying the gill withdrawal reflex, investigators showed that short-term heterosynaptic facilitation does not require new protein synthesis whereas long-term heterosynaptic facilitation does require new protein synthesis (Montarolo et al., 1986). These results paralleled the results seen in mice with intracortical injections of protein synthesis inhibitors and supported a role for new protein synthesis in a cellular correlate of memory. These findings suggested that proteins synthesized during and soon after learning were critical for the establishment of long-term memory. Given that stimulation of neuronal circuits during memory formation gives rise to activity-dependent gene expression, it was hypothesized that this program of gene expression played a role in memory. It wasn’t until later, however, that this hypothesis could be formally tested with studies of the activity-dependent transcription factor CREB (cyclic AMP response element binding protein) in this process.

As investigators began to appreciate the role of new protein synthesis in long-term memory, details began to emerge regarding the underlying molecular mechanisms. Studies of the neural circuit underlying the gill withdrawal reflex showed that the neurotransmitter serotonin mediates long-term heterosynaptic facilitation in Aplysia (Montarolo et al., 1986) and that a second messenger downstream of serotonin, cyclic AMP, was itself capable of producing long-term heterosynaptic facilitation (Schacher et
al., 1988; Scholz and Byrne, 1988). These data suggested that the gene-products important for long-term heterosynaptic facilitation might be inducible by cAMP. It turned out that at this time, separate studies had identified the transcription factor CREB, or the cyclic AMP response element binding protein, as an important activator gene transcription in response to cAMP signaling (Montminy et al., 1986). These discoveries provided investigators with a potential molecular handle to study the role of stimulus-responsive transcription in memory. The first evidence that CREB plays a role in learning and memory came from a study in which investigators showed that Aplysia neurons contained CREB-like proteins and that injection of oligonucleotides of the cyclic AMP response element to which these proteins bind into the nucleus of Aplysia sensory neurons blocked long-term heterosynaptic facilitation without affecting short-term heterosynaptic facilitation (Dash et al., 1990). Separate studies had shown that CREB was also as an important activator of c-Fos transcription in mammalian cells in response to extracellular stimuli (Sheng et al., 1988) (see section 1.3.1 Initial insights from the c-Fos promoter). Hence, the CREB protein appeared to be an important regulator of long-term adaptations in Aplysia as well activity-dependent transcription in the mammalian nervous system. Experiments done in genetically modified mice have supported a role for CREB in mammalian long-term plasticity and memory. Mutation of Creb in mice resulted in defective long-term memory and long lasting long-term potentiation (L-LTP), a cellular correlate of long-term memory, with no affect on short-term memory or short term synaptic plasticity (Bourtchuladze et al., 1994). Furthermore, transgenic mice overexpressing a constitutively active form of CREB exhibit L-LTP in response to weak stimuli that would not normally elicit L-LTP (Barco et al., 2002). These data suggest that CREB plays an important role in long-term
cellular adaptations to stimuli and long-term memory. Since these processes require new protein synthesis and since CREB is a transcription factor regulated by extracellular stimuli, these results also implicate stimulus-responsive transcription in long-term cellular adaptations to stimuli and long-term memory.

### 1.2.2 Role in dendritic and synaptic development

In addition to playing a role in long-term cellular adaptations to stimuli and long-term memory, stimulus-responsive transcription also has been shown to play important roles in development of dendrites and synapses that underlie these long-term phenomena. The most convincing evidence supporting a role of stimulus-responsive transcription in dendritic and synaptic development has come from detailed studies of these processes in the context of genetic alterations to stimulus-responsive genes. For example the activity-dependent transcription factors CREB, CREST, and NEUROD have all been shown to play important roles in dendritic development (Aizawa et al., 2004; Gaudilliere et al., 2004; Redmond et al., 2002; Wayman et al., 2006). Furthermore, genes whose transcription is regulated by neuronal activity, such as the microRNA miRNA132 and candidate plasticity gene 15 (Cpg15), have also been shown to regulate dendritic remodeling (Cantallops et al., 2000; Nedivi et al., 1998; Vo et al., 2005; Wayman et al., 2008). Similarly, neuronal activity-dependent genes have also been shown to play important roles in synapse development. The neuronal activity-dependent transcription factor MEF2 has been shown to negatively regulate excitatory synapse number (Barbosa et al., 2008; Flavell et al., 2006). Furthermore, the protein...
products of the activity-regulated genes *Arc*, *Homer1a*, *Npas4*, and *Plk2* have all been shown to regulate various aspects of synapse development (Chowdhury et al., 2006; Lin et al., 2008; Pak and Sheng, 2003; Rial Verde et al., 2006; Sala et al., 2003). These studies have demonstrated that neuronal stimulus-responsive transcription plays critical roles in the development of both dendrites and synapses in the nervous system.
1.3 Regulation of stimulus-responsive transcription in the nervous system

Because neuronal stimulus-responsive transcription plays important roles in the development and function of the nervous system, there has been great interest in understanding the molecular mechanisms underlying this program of gene expression. Early work focused on studying the promoters of stimulus-responsive genes. This yielded important insights into the proximal cis-acting elements and proximally bound trans-acting factors that regulate stimulus-responsive transcription. With the development of chromatin and transcription factor signatures that identify enhancers, and sequencing technologies that enable their unbiased genome-wide identification, recent studies have begun to identify distal enhancer elements that may also regulate stimulus-responsive transcription. Since enhancers are though to be the main drivers of specificity in gene expression, their identification and study is an important step in understanding the regulation of stimulus-responsive transcription in the nervous system.

1.3.1 Initial insights from the c-Fos promoter

Soon after the discovery of the stimulus-dependent transcription of the c-Fos gene, investigators sought to understand the mechanisms by which c-Fos was inducibly transcribed in response to stimuli. Initial studies demonstrated that plasmids containing several hundred base pairs of sequence upstream of the c-Fos transcriptional
start site were sufficient to generate stimulus-responsive transcription similar to that exhibited by \textit{c-Fos in vivo} (Deschamps et al., 1985; Treisman, 1985). Deletion analysis of the \textit{c-Fos} promoter revealed cis-acting regulatory elements that were critical for stimulus-dependent transcription of \textit{c-Fos}. Promoter deletion analyses demonstrated that \textit{c-Fos} induction from serum stimulation in fibroblasts required an element termed the serum response element (SRE) (Greenberg et al., 1987; Treisman, 1985). Subsequent experiments employing DNA affinity chromatography discovered the trans-acting transcription factor that bound to the SRE, the serum response factor (SRF) (Treisman, 1987). The SRE was subsequently shown to be required for calcium-dependent transcription of \textit{c-Fos} (Misra et al., 1994) and SRF was subsequently shown to be required for activity-dependent induction of \textit{c-Fos} in the nervous system (Ramanan et al., 2005). These experiments provided evidence that SRF plays an important role in regulating transcriptional responses to neuronal activity by binding to SRE sites near the promoters of target genes, such as \textit{c-Fos}.

Separate deletion analyses of the \textit{c-Fos} promoter revealed an additional cis-acting element required for calcium-dependent induction of the \textit{c-Fos} gene. Deletion experiments demonstrated that \textit{c-Fos} induction in response to depolarization in PC12 cells required an element termed the calcium response element (CaRE) (Sheng et al., 1988), which bound to the transcription factor CREB (Sheng et al., 1990). Previous experiments had established a role for cAMP responsive transcriptional activation by CREB at the cyclic AMP response element (CRE) (Montminy and Bilezikjian, 1987; Montminy et al., 1986). Together, these experiments demonstrated a role for CREB in transcriptional activation in response to elevations in both cAMP and calcium, and
provided evidence that CREB plays a role in regulating transcriptional responses to neuronal activity by binding to CaRE/CRE sites near the promoters of target genes, such as c-Fos.

Careful analyses of the sequences within the c-Fos promoter provided important insights into the cis-regulatory elements and trans-acting factors that drive c-Fos expression in response to extracellular stimuli. Importantly, the discovery of SRF and CREB proved to not only be important in understanding the stimulus-responsive transcription of c-Fos, but also of other stimulus-responsive genes. For example, the SRF knockout animal exhibited no induction of not only c-Fos but also the immediate early genes FosB, Egr1, Egr2, c-Jun, JunB, Arc, ActB, and Actg1 in response to electroconvulsive shock (Ramanan et al., 2005). This suggested that SRF is a broadly important regulator of activity-dependent transcription in the nervous system. Genome-wide analysis of CREB target genes has demonstrated hundreds of genes that require CREB for transcription in response to the cAMP agonist forskolin (Zhang et al., 2005). While these studies suggest that SRF and CREB are both broadly important in generating stimulus-responsive transcriptional programs, the genome-wide sets of transcriptional target genes for these factors in the nervous system have not been defined.

1.3.2 Discovery of enhancer elements

As initial understanding of the regulation of stimulus-responsive transcription from the promoter regions of c-fos and other genes was developing, the contribution of
Enhancers to transcriptional regulation was just beginning to be appreciated. Enhancers were first discovered through studies of β-globin gene regulation (Banerji et al., 1981). Investigators studying the expression of the β-globin gene from plasmids containing a 4.7 kb long segment of chromosomal DNA encompassing the β-globin gene found that expression of β-globin was increased a remarkable 200 fold when the plasmid also contained a 72 bp sequence of DNA derived from Simian virus 40 (SV40). Because of its remarkable ability to enhance β-globin gene transcription, this element was referred to as an enhancer. The investigators also showed that the SV40 enhancer functioned to increase β-globin gene transcription independent of its distance from the promoter and in either sequence orientation relative to the promoter (Banerji et al., 1981). These are now known to be important and defining properties of enhancers.

After the discovery of viral enhancers (Banerji et al., 1981; Moreau et al., 1981), metazoan enhancers were discovered (Banerji et al., 1983; Gillies et al., 1983) thus generalizing the relevance of enhancers to transcriptional regulation. Subsequently, several additional endogenous enhancers have been studied in detail, including the β-globin locus control region (Bender et al., 2000; Grosveld et al., 1987; Hardison et al., 1997; Moon and Ley, 1990), a limb bud enhancer for the Sonic hedgehog (Shh) gene (Lettice et al., 2003; Sagai et al., 2005), the interferon-β enhancer (Munshi et al., 1999; Panne, 2008), and the sparkling eye enhancer of the Drosophila dPax2 gene (Evans et al., 2012). These studies have established the importance of enhancers to gene regulation and have begun to elucidate the principles underlying enhancer function. However, a major hurdle in understanding the function of enhancers and the contribution of enhancers to specific programs of gene expression, such as neuronal
stimulus regulated transcription, has been the identification of enhancer elements within the genome. Enhancers are short DNA sequences, approximately several hundred base pairs in length, that are embedded within the genome. Because enhancers lack defining characteristics, they cannot be identified within the genome on the basis of their sequence alone. Recently, however, great advances have been made in the identification of enhancers within the genome (Buecker and Wysocka, 2012). These advances, described in the introduction to the next chapter of my thesis, have facilitated my work on the identification and characterization of functional stimulus-responsive enhancers in the nervous system.
2  GENOME-WIDE IDENTIFICATION OF STIMULUS-RESPONSIVE ENHANCERS IN THE NERVOUS SYSTEM
2.1 SUMMARY

Normal brain development and function require stimulus-responsive programs of gene expression, but the impact of neuronal stimuli on chromatin, and the specific enhancers that regulate stimulus-responsive programs of gene expression are poorly understood. Here, we discover that stimulation with neuronal activity and brain derived neurotrophic factor (BDNF) cause rapid, widespread, and distinct changes in the acetylation of histone H3 lysine 27 (H3K27Ac) at thousands of promoters and enhancers throughout the neuronal genome. We find that functional stimulus-responsive enhancers can be specifically identified by stimulus-inducible H3K27Ac, and we use this dynamic chromatin signature to discover enhancers that respond to activity, BDNF, or both. This discovery of these enhancers reveals the genome-wide basis for transcriptional specificity in response to distinct stimuli. Furthermore, this work provides a critical resource to begin understanding the cis-regulatory elements that transform sensory experience into specific transcriptional programs that facilitate long-lasting changes in neuronal function. Finally, stimulus-inducible H3K27Ac may aid in the identification and study of stimulus-regulated enhancers in other tissues.
2.2 INTRODUCTION

During development, transcriptional programs give rise to distinct cellular lineages through the establishment of cell type specific chromatin states (Davidson, 2010; Ho and Crabtree, 2010; Kouzarides, 2007; Lee and Young, 2013). These distinct chromatin signatures instruct gene expression primarily through the genome-wide demarcation of enhancers, cis regulatory-elements that act at a distance to regulate gene transcription (Buecker and Wysocka, 2012; Bulger and Groudine, 2011; Levine, 2010; Ong and Corces, 2011; Spitz and Furlong, 2012; Visel et al., 2009b). Recent efforts to identify enhancers using genome-wide sequencing techniques have led to the discovery of hundreds of thousands of cis-regulatory elements (Consortium et al., 2012; Heintzman et al., 2007; Shen et al., 2012). Despite this progress, relatively little is known about the influence of extracellular stimuli on chromatin state at enhancers, and it is not clear which enhancers among the hundreds of thousands that have been identified function to promote stimulus-dependent transcription.

In the nervous system, the chromatin state of terminally differentiated neurons must not only maintain neuronal identity but also allow for sensory-experience-dependent transcription (Borrelli et al., 2008; Day and Sweatt, 2011; Dulac, 2010; Graff and Tsai, 2013; Kandel, 2001). In response to sensory experience, strong bursts of synaptic activity induce a program of gene expression in excitatory neurons that is required for proper development and refinement of neural circuits and for long-lasting changes in neuronal function that underlie learning, memory, and behavior (Greer and Greenberg, 2008; Leslie and Nedivi, 2011). Studies of this neuronal activity-dependent
gene program have primarily focused on signaling to promoter bound transcription factors (Alberini, 2009; Lyons and West, 2011). The release of the neurotransmitter glutamate at synapses leads to membrane depolarization of the post-synaptic neuron, triggering calcium influx through L-type voltage gated calcium channels. The influx of calcium then leads to activation of a complex signaling network that induces the post-translational modification of promoter bound transcription factors that initiate multiple waves of gene expression.

In addition to these critical signaling events at the promoters of activity-regulated genes, signaling to enhancers contributes to activity-dependent gene expression, although there has been little progress towards characterizing the function of activity-regulated enhancers because it has been difficult to identify these elements within the genome. However, recent genome-wide studies in a variety of cell types have characterized chromatin and transcription factor signatures that now allow for the comprehensive identification of enhancers. For example, enhancers have been identified on the basis of their hypersensitivity to DNAseI digestion, enrichment of monomethylation of lysine 4 on histone H3 (H3K4Me1), enrichment of acetylation of lysine 27 on histone H3, and their ability to bind the transcriptional coactivators CBP/P300 (Consortium et al., 2012; Creyghton et al., 2010; Heintzman et al., 2009; Rada-Iglesias et al., 2011; Visel et al., 2009a). However, all enhancers are not functionally equivalent. The cellular repertoire of enhancers consists of both enhancers that are thought to be poised for future activation, and enhancers that are currently active (Rada-Iglesias et al., 2011). Poised enhancers are typically identified by their enrichment for H3K4me1, whereas active enhancers exhibit additional features (i.e.
CBP/P300 binding, eRNA transcription, H3K27Ac). H3K27Ac has been shown to be present specifically at active enhancers and not poised enhancers, and thus has been used in several studies of developmentally regulated enhancers to identify active enhancers genome-wide (Creyghton et al., 2010; Mikkelsen et al., 2010; Rada-Iglesias et al., 2012; Rada-Iglesias et al., 2011).

Several studies have recently identified enhancers within the nervous system (Kim et al., 2010; Shen et al., 2012; Visel et al., 2013). While most of these studies have not attempted to understand the enhancers within the genome that contribute to stimulus-dependent transcription in the nervous system, one recent study used H3K4Me1 enrichment and inducible CBP binding to identify nearly 12,000 putative neuronal activity-dependent enhancers (Kim et al., 2010). However, this number far exceeds the number of activity-regulated genes in excitatory neurons, suggesting that many of these enhancers may not directly contribute to activity-dependent transcription. Thus, it remains unclear whether all or a subset of these enhancers actually function to drive neuronal activity-dependent gene expression. The activity-regulated regulated enhancers identified in excitatory neurons thus far appear to be heterogeneous in terms of the transcription factors they bind and their ability to recruit RNA polymerase and drive transcription of enhancer associated RNAs (eRNAs). This suggests that these CBP-bound enhancers may not all function in the same manner. While some of these enhancers might mediate activity-dependent gene transcription, others could respond to distinct stimuli such as neurotrophic factors. It is also possible that some of the CBP-bound enhancers do not directly regulate stimulus-dependent gene transcription and instead might be constitutively active or inactive under the conditions studied.
Consistent with these possibilities, studies of developmentally regulated enhancers have shown that not all CBP/P300 bound enhancers are active (Rada-Iglesias et al., 2011; Visel et al., 2009a). It is not clear if these observations apply to stimulus-responsive enhancers in terminally differentiated cell types since to date no chromatin or transcription factor signatures have been defined that identify which of the tens of thousands of enhancers within a cell function to drive stimulus-responsive gene transcription.

In this study we determined the effects of neuronal activity on the active chromatin landscape of mouse cortical neurons. We find that neuronal activity can induce rapid changes in H3K27Ac at thousands of neuronal enhancers throughout the genome. Notably, increases in H3K27Ac occur specifically at enhancers that function to promote activity-dependent gene transcription. We observed overlapping but distinct changes in H3K27Ac at enhancers in response to stimulation with brain derived neurotrophic factor (BDNF), revealing the genome-wide basis for transcriptional specificity in response to distinct stimuli. Our studies reveal a previously underappreciated heterogeneity among active enhancers in terms of their responsiveness to stimuli. Furthermore, the comprehensive identification of neuronal activity and BDNF-dependent enhancers in cortical neurons provides a critical resource for elucidating the role of stimulus-responsive transcription in synaptic plasticity, behavior and disease. Finally, the epigenetic signature of acutely inducible H3K27Ac may aid in the identification and study of stimulus-regulated enhancers in other tissues.
2.3 RESULTS

2.3.1 H3K27Ac changes rapidly in response to neuronal activity

Despite progress in identifying enhancers in the nervous system, no study has systematically determined which of the tens of thousands of enhancers that have been identified function to drive stimulus-responsive transcription. We hypothesized that characterizing the active enhancer landscape before and after neuronal activity might enable us to identify the set of enhancers that respond to activity. To test this hypothesis, we used ChIP-Seq to measure the genomic distribution of the active chromatin associated chromatin mark H3K27Ac in mouse cortical neurons before and after two hours of membrane depolarization with elevated extracellular potassium chloride (KCl). Exposure of neuronal cultures to KCl is a well-established in vitro experimental paradigm that mimics neuronal activity in vivo by inducing membrane depolarization, calcium influx through L-type voltage-sensitive calcium channels, and calcium-dependent changes in gene transcription. We performed two independent bioreplicates of ChIP-Seq before and after membrane depolarization with KCl, and found that the signal for H3K27Ac was highly correlated in separate bioreplicates in each condition (Figure 2.1, Spearman’s rho $\rho=0.83$ between $-\text{KCl}$ experiments, $\rho=0.86$ between $+\text{KCl}$ experiments). This suggested that H3K27Ac signal in our experiments was replicable across experiments. As a result, we pooled both bioreplicates for subsequent analyses.
Figure 2.1: Comparison of H3K27Ac signal in two independent experiments before and after membrane depolarization with KCl

(a) H3K27Ac ChIP-Seq signal in mouse cortical neurons before membrane depolarization (-KCl) in two independent experiments (bioreplicate 1, B1; bioreplicate 2, B2), quantified at all H3K27Ac peaks in the genome. H3K27Ac signal was highly correlated between experiments, Spearman’s rho $\rho=0.83$. (b) H3K27Ac ChIP-Seq signal in mouse cortical neurons after 2 hours of membrane depolarization (+KCl) in two independent experiments (bioreplicate 1, B1; bioreplicate 2, B2), quantified at all H3K27Ac peaks in the genome. H3K27Ac signal was highly correlated between experiments, Spearman’s rho $\rho=0.86$. In both plots, each point represents the H3K27Ac signal quantified within a 2kb window surrounding an enhancer center.
After pooling both H3K27Ac bioreplicates (B1 and B2) in each condition (-KCl and +KCl), we identified regions of significant H3K27Ac enrichment in each condition (-KCl and +KCl) by calling H3K27Ac peaks using MACS with a significance threshold of \( p = 1 \times 10^{-5} \). We then asked how the genomic distribution of H3K27Ac peaks compared before and after membrane depolarization with KCl. To investigate this, we classified all H3K27Ac peaks in each condition based on their location relative to genes in the NCBI Reference Sequence Database (RefSeq). H3K27Ac peaks were classified as being proximal if they were within 1kb of an annotated transcriptional start site (TSS). H3K27Ac peaks were classified as being distal if they were greater than 1kb from an annotated transcriptional start site (TSS). Distal H3K27Ac peaks were further classified as intragenic if they occurred within a RefSeq gene, or as extragenic if they did not occurred within a RefSeq gene. We found that both before and after membrane depolarization, the majority of H3K27Ac peaks occurred distal to RefSeq TSSs (Figure 2.2, 65% distal peaks –KCl, 63% distal peaks +KCl). Furthermore, the genomic distribution of H3K27Ac peaks was similar before and after depolarization. Before depolarization (-KCl), 35% of H3K27Ac peaks were proximal, 32% of H3K27Ac peaks were distal intragenic, and 33% of peaks were distal extragenic. After depolarization (+KCl), 37% of H3K27Ac peaks were proximal, 33% of H3K27Ac peaks were distal intragenic, and 30% of peaks were distal extragenic. This suggested that the overall distribution of H3K27Ac peaks within the genome did not change with neuronal activity.
Figure 2.2: Genomic distribution of H3K27Ac peaks before and after membrane depolarization with KCl

(a) Genomic distribution of H3K27Ac peaks before membrane depolarization with KCl (-KCl). The majority of peaks occur distal to RefSeq TSSs, with 32% of distal peaks occurring within genes (intragenic) and 33% of distal peaks occurring outside of genes (extragenic). (b) Genomic distribution of H3K27Ac peaks after membrane depolarization with KCl (+KCl). The majority of peaks occur distal to RefSeq TSSs, with 33% of distal peaks occurring within genes (intragenic) and 30% of distal peaks occurring outside of genes (extragenic).
While the overall genomic distribution of H3K27Ac peaks did not change with neuronal activity, we asked whether the levels of H3K27Ac changed in response to neuronal activity at individual enhancer elements. This raises the question of how individual enhancer elements should be identified. H3K27Ac enrichment distal to promoter regions has been used to identify enhancers, but H3K27Ac enrichment often occurs over broad regions of the genome and can encompass multiple regulatory elements. Furthermore, the accurate localization of individual enhancers within regions of H3K27Ac enrichment can prove difficult. Distal regions of open chromatin identified by DNaseI hypersensitivity (DHS) have also been used to identify enhancers. Compared to H3K27Ac, DHS provides the benefit of narrow peaks that allow for accurate localization of regulatory elements. However, distal DNaseI hypersensitivity is not specific to enhancers and can indicate the presence of various distal cis-regulatory elements, such as silencers and insulators (Gross and Garrard, 1988). We found that 98.6% of all H3K27Ac peaks within our system overlapped with previously generated DHS data from brain tissues by the ENCODE consortium (Consortium, 2011), and reasoned that enhancers within our system could be comprehensively, specifically, and accurately identified by integrating our H3K27Ac data with this brain DHS data.

To identify individual enhancer elements within our system, we isolated the set of distal (>1kb from a RefSeq TSS) DHS sites that overlapped with our H3K27Ac peaks. To determine the effect of neuronal activity on H3K27Ac at individual enhancers, we quantified the levels of H3K27Ac at individual enhancer elements throughout the genome. While the enhancer-associated chromatin mark H3K4Me1 was largely similar at neuronal enhancers before and after neuronal activity (Figure 2.3a, Spearman’s rank
correlation coefficient $\rho = 0.96, 0.97$ in two independent experiments), we found that H3K27Ac levels at neuronal enhancers exhibited substantially more variability before and after neuronal activity (Figure 2.3b, $\rho=0.53, 0.58$ in two independent experiments). H3K27Ac levels at neuronal enhancers also exhibited substantial variability before and after neuronal activity in the merged dataset of both H3K27Ac bioreplicates (Figure 2.3c, $\rho=0.53$). H3K27Ac appeared to increase and decrease at thousands of neuronal enhancers and activity-dependent increases in H3K27Ac seemed to occur both at active enhancers that displayed H3K27Ac prior to stimulation and at poised enhancers that did not display H3K27Ac prior to stimulation (Figure 2.3c). Inspection of individual loci revealed strikingly different H3K27Ac behaviors at neuronal enhancers that appeared similar based on other chromatin modifications, transcription factor binding events, and DNaseI hypersensitivity (Figure 2.4). Neuronal enhancers that appeared otherwise similar exhibited increasing H3K27Ac, high unchanging H3K27Ac, decreasing H3K27Ac, or no H3K27Ac in response to neuronal activity (Figure 2.4). Hence, H3K27Ac levels changed rapidly and dramatically at thousands of neuronal enhancers in response to activity, while H3K4Me1 levels were largely constant. Furthermore, neuronal enhancers exhibit significant heterogeneity in both the levels of H3K27Ac and also the dynamics of H3K27Ac in response to neuronal activity. This suggested that neuronal enhancers might be heterogeneous in their function and in their responsiveness to neuronal activity.
Figure 2.3: Chromatin modifications at neuronal enhancers before and after membrane depolarization

Levels and correlation of histone modifications at neuronal enhancers (defined by H3K27Ac enrichment and DNaseI hypersensitivity) throughout the genome before membrane depolarization (-KCl) and after membrane depolarization (+KCl) in two independent experiments (bioreplicate 1, B1; bioreplicate 2, B2). (a) H3K24Me1 levels. (b) H3K27Ac levels. (c) Levels and correlation of H3K27Ac at neuronal enhancers (defined by H3K27Ac enrichment and DNaseI hypersensitivity) throughout the genome before membrane depolarization (-KCl) and after membrane depolarization (+KCl) in the pooled datasets (merged B1B2).
**Figure 2.4: Individual enhancers exhibit different H3K27Ac behaviors in response to membrane depolarization**

Genome browser views of ChIP-Seq and RNA-Seq tracks at examples of individual neuronal enhancers exhibiting increasing H3K27Ac, constant high H3K27Ac, decreasing H3K27Ac, or no H3K27Ac in response to membrane depolarization with KCl. Other chromatin marks displayed include the enhancer associated H3K4Me1, the promoter associated H3K4Me3, and the repressive chromatin associated H3K27Me3. Transcription factor binding events displayed include the transcriptional coactivator CREB-binding protein (CBP), and RNA polymerase II (RNAPII). For all chromatin marks and transcription factors, ChIP-Seq tracks display input-normalized reads from neurons stimulated with 0 hours (‘-’) or two hours (‘+’) of KCl. RNA sequencing tracks display sequencing reads aligning to forward (F) and reverse strands (R) of the genome from neurons stimulated with 0, 1, or 6 hours of KCl. Also displayed are DNaseI hypersensitivity signal from adult mouse cerebrum and vertebrate conservation by PhastCons.
To gain initial insight into how neuronal activity-dependent changes in H3K27Ac at enhancers might relate to activity-dependent programs of gene expression, we inspected the locus of the canonical activity-dependent gene c-Fos. Previous studies in neurons have observed increases in histone acetylation in response to physiological stimuli at the promoters of stimulus-responsive genes (Guan et al., 2009; Guan et al., 2002; Levenson et al., 2004). Consistent with previous studies, we found that levels of H3K27Ac increased at the c-Fos promoter in response to neuronal activity. We also found that levels of H3K27Ac increased at four nearby enhancers in response to neuronal activity (Figure 2.5). This suggested that neuronal activity-dependent gene expression might correlate with increasing H3K27Ac in response to neuronal activity and that neuronal enhancers that contribute to activity-dependent gene expression may exhibit increasing H3K27Ac in response to neuronal activity.
**Figure 2.5: Enhancers near the c-Fos gene exhibit increases in H3K27Ac in response to membrane depolarization**

Genome browser view of ChIP-Seq and RNA-Seq tracks at the c-Fos locus. Neuronal activity-dependent increases in H3K27Ac levels are seen at the c-Fos transcriptional start site (TSS) and at four nearby enhancers (e1,2,4,5). For all chromatin marks and transcription factors, ChIP-Seq tracks display input-normalized reads from neurons stimulated with 0 hours ('-') or two hours ('+') of KCl. RNA sequencing tracks display sequencing reads aligning to forward (F) and reverse strands (R) of the genome from neurons stimulated with 0, 1, or 6 hours of KCl.
2.3.2 Inducible H3K27Ac identifies functional neuronal activity-responsive enhancers

To determine if increases in H3K27Ac occurred at enhancers associated with activity-induced genes genome-wide, we first classified enhancers into different groups based on their differential H3K27Ac responses to neuronal activity. We selected three subsets of enhancers that exhibited the most distinct H3K27Ac dynamics in response to neuronal activity: enhancers with increasing H3K27Ac (n=2868), enhancers with decreasing H3K27Ac (n=3746), and enhancers with high constant levels of H3K27Ac before and after neuronal activity (n=1395) (Figure 2.6a; see methods). For comparison, we also included a fourth group of enhancers that had H3K4Me1, exhibited inducible binding of the transcriptional coactivator CBP in response to neuronal activity, but had no appreciable H3K27Ac before or after neuronal activity (n=3223) (Figure 2.6a). This fourth group was included because a previous report classified these elements as neuronal activity-dependent enhancers (Kim et al., 2010). Average profiles of H3K27Ac ChIP-Seq signal for each group of neuronal enhancers displayed clearly different H3K27Ac behaviors in response to neuronal activity (Figure 2.6b). This suggested that these enhancer groups could be meaningfully used to determine whether neuronal enhancers with different H3K27Ac behaviors are associated with different transcriptional functions within the nervous system.
Figure 2.6: Classification of neuronal enhancers with different H3K27Ac behaviors

(a) Classification of neuronal enhancers into subgroups with distinct H3K27Ac behaviors. Enhancers with increasing H3K27Ac, high constant H3K27Ac, decreasing H3K27Ac, or no H3K27Ac in response to neuronal activity were classified into separate groups. Each point represents the input normalized H3K27Ac signal quantified within a 2kb window surrounding an enhancer center, with the x-axis value representing the signal before membrane depolarization (-KCl) and the y-axis value representing the signal after two hours of membrane depolarization with KCl (+KCl). (b) Average profiles of H3K27Ac ChIP-Seq signal at each subgroup of enhancers before membrane depolarization (dashed lines) and after membrane depolarization (solid lines). The y-axis represents the level of H3K27Ac signal, displayed as the mean number of input-normalized H3K27Ac ChIP-Seq reads for each subgroup of enhancers. H3K27Ac signal at enhancers was aligned by the centers DNaseI hypersensitive sites.
We hypothesized that different H3K27Ac behaviors were likely to reflect different transcriptional functions for these enhancers, and that only neuronal enhancers with increasing H3K27Ac in response to neuronal activity would promote gene expression in response to neuronal activity. To determine the contribution of each subset of enhancers to neuronal gene expression, we performed transcriptome sequencing (RNA-Seq) in untreated or KCl-depolarized cortical neurons (0, 1, and 6 hours of KCl). Then, for each subset of enhancers, we assessed the expression of nearby genes and the expression of enhancer associated RNAs (eRNAs), which are thought to occur at enhancers actively engaged in transcriptional regulation (Kim et al., 2010; Wang et al., 2011). Strikingly, we found that both nearest gene expression and eRNA transcription closely paralleled the H3K27Ac behaviors observed at each subset of enhancers (Figure 2.7a,b). As expected enhancers with high unchanging H3K27Ac were near genes that exhibited significantly higher levels of expression and had higher levels of eRNA transcription than enhancers lacking H3K27Ac, consistent with their classification as active enhancers (Figure 2.7a,b). Notably, only enhancers with increasing H3K27ac in response to neuronal activity were associated with activity-dependent increases in the expression of nearby genes and eRNAs (Figure 2.7a,b), suggesting that this subset of enhancers may function specifically to promote neuronal activity dependent gene expression. Thus, while a large number of active enhancers can be identified by their enrichment for H3K27Ac, profiling H3K27Ac in response to neuronal activity revealed that these enhancers differ considerably from one another not only in their levels of H3K27Ac but also in the expression of nearby genes and in the transcription of eRNAs. By measuring H3K27Ac levels before and after a neuronal activity and classifying enhancers based on their H3K27Ac dynamics, we find that only enhancers with activity-
dependent increases in H3K27Ac appear to contribute to neuronal activity-dependent transcription. Our data further suggests that while inducible CBP binding in response to neuronal activity has previously been used to identify neuronal activity-dependent enhancers, this is clearly not a specific marker. While enhancers with inducible H3K27Ac exhibited greater CBP binding than other acetylated enhancer classes, enhancers from all four classes exhibited some level of inducible CBP binding (Figure 2.7c). Furthermore, enhancers that lacked H3K27Ac but exhibited inducible robustly CBP binding were not associated with genomic correlates of activity-dependent enhancer function (Figure 2.7c). This suggested that inducible H3K27Ac is a much more specific and predictive marker of functional neuronal activity-dependent enhancers than inducible coactivator binding.
Figure 2.7: Enhancers with different H3K27Ac behaviors correlate with different patterns of transcription in the genome

(a) Boxplot of expression of nearest genes for each H3K27Ac enhancer class. mRNA expression is displayed as reads per kilobase per million mapped reads (RPKM). Nearest genes with nonzero expression by RNA-SEQ were used for this analysis. Expression of the set of genes near each enhancer class was assessed at 0, 1, and 6 hours after membrane depolarization with KCl. (b) Boxplot of eRNA expression for each H3K27Ac enhancer class. eRNA expression is displayed as reads per kilobase per million mapped reads (RPKM). eRNA expression for each enhancer was calculated as the number of reads within a 4kb window surrounding the enhancer center. For this analysis, only extragenic enhancers were used. Expression of eRNAs for each enhancer class was assessed at 0, 1, and 6 hours after membrane depolarization with KCl. (c) Boxplot of CBP binding for each H3K27Ac enhancer class before membrane depolarization (0hr KCl) and after membrane depolarization (2hr KCl). CBP binding at each enhancer was calculated as the number of input normalized ChIP-Seq reads within an 800bp window surrounding the enhancer center. * indicates p < 2.2x10^{-16} in paired Wilcoxon test.
2.3.3 Functional testing of enhancers in response to neuronal activity

While genomic correlates of enhancer activity suggested that only enhancers with neuronal activity-dependent increases in H3K27Ac function to promote activity-dependent transcription, we sought to directly test the ability of each class of enhancers to drive transcription in an activity-dependent manner. To accurately measure activity-regulated transcriptional changes, we developed a neuronal activity-regulated luciferase reporter based on the upstream regulatory region of the neuronal activity-regulated gene neuronal pentraxin-2 (Nptx2, Figure 2.8a). Nptx2 encodes a secreted synaptic protein that can bind to and induce clustering of AMPA receptors to regulate homeostatic scaling of excitatory synapses within the nervous system (Chang et al., 2010). We discovered a putative enhancer ~3kb upstream of the Nptx2 transcriptional start site (TSS). The 4.4kb sequence upstream of the Nptx2 TSS was able to drive activity-dependent expression of a luciferase reporter gene in a manner that depended critically upon the presence of the upstream enhancer (Figure 2.8b). We used this reporter to test the ability of enhancers from each of the four H3K27Ac enhancer classes to promote neuronal activity-regulated transcription by replacing the Nptx2 enhancer with inducibly bound enhancers from each H3K27Ac enhancer class and measuring activity-dependent induction of the reporter. While our genomic analyses suggested that many enhancers inducibly bound by CBP may not contribute to neuronal activity-dependent gene expression, we sought to directly test this by selecting enhancers from each H3K27Ac enhancers class that were inducibly bound by CBP. As a result, we replaced the Nptx2 enhancer with inducibly CBP-bound enhancers from each H3K27Ac group. We then transfected the reporter constructs into mouse cortical neuron cultures, and
then measured reporter activity induced by 6 hours of depolarization with KCl. Strikingly, all enhancers that exhibited increasing H3K27Ac signal in response to neuronal activity drove robust activity-dependent transcription of the reporter (14/14; 100% with >2 fold induction with KCl; Figure 2.8b), whereas nearly all other enhancers failed to do so, despite their recruitment of CBP (1/28; 3.6% with >2 fold induction with KCl; Figure 2.8b).
Figure 2.8: Functional testing of neuronal enhancers with different H3K27Ac behaviors

(a) Schematic of Nptx2 reporter. The 4.4kb sequence upstream of the mouse Nptx2 gene was cloned into a luciferase reporter construct. Approximately 3kb upstream of the Nptx2 TSS is an enhancer element, identified by DNaseI hypersensitivity, CBP binding, and H3K27Ac. (b) Luciferase reporter data for the Nptx2 reporter, reported as the fold induction of reporter activity in mouse cortical neurons after 6 hours of depolarization with KCl relative to 0 hours. The wild-type Nptx2 reporter is able to induce activity-dependent reporter expression (Nptx2). However, if the Nptx2 enhancer is deleted, the reporter no longer induces activity-dependent reporter expression (Nptx2-enh). Inducibly CBP-bound enhancers from the four H3K27Ac enhancer classes were cloned into the Nptx2 reporter, replacing the Nptx2 enhancer, and tested for their ability to induce activity-dependent reporter expression. Only enhancers exhibiting increasing H3K27Ac with neuronal activity consistently drove reporter expression in response to neuronal activity. n ≥ 3 for all enhancers tested.
Across all enhancers tested, neuronal activity-dependent changes in H3K27Ac correlated much more strongly with reporter activity (Spearman’s rank correlation coefficient ρ=0.88; Figure 2.9a), than did changes in CBP (ρ=0.65; Figure 2.9b), or changes in RNAPII (ρ=0.34; Figure 2.9c), which has been previously shown to inducibly bind to enhancers (Kim et al., 2010). This suggested that only enhancers that exhibit activity-dependent increases in H3K27ac function to drive activity-regulated gene transcription and further supported inducible H3K27Ac as a much more specific and predictive marker of functional neuronal activity-dependent enhancers than inducible coactivator binding or RNAPII binding. Thus, we concluded that the dynamic chromatin signature of increasing H3K27Ac is a specific and predictive marker of functional neuronal activity-dependent enhancers.

**Figure 2.9: Correlation between reporter induction and genomic variables by ChIP-Seq**

The Nptx2 reporter fold induction of each enhancer displayed in Figure 2.8b was plotted against the change in H3K27Ac (a), change in CBP (b), or change in RNAPII (c) seen at that enhancer. Green, blue, magenta, and black points represent enhancers with increasing, constant, decreasing, or no H3K27Ac ChIP-Seq signal in response to neuronal activity. ρ indicates Spearman’s rank correlation coefficient for the two variables plotted in each panel.
During development, enhancer elements are thought to be the principal determinants of cell-type specific gene expression. However, numerous studies of neuronal activity-regulated transcription have demonstrated that the promoters of activity-regulated genes are sufficient to drive activity-dependent expression under some conditions (Alberini, 2009; Robertson et al., 1995). This brings into question whether activity-dependent enhancers serve as determinants of stimulus-responsiveness or if they simply amplify transcription from activity-regulated promoters. To gain some insight into this question, we asked whether the ability of activity-responsive enhancers to promote neuronal activity-regulated transcription depended upon their pairing with the activity-regulated Nptx2 promoter. We cloned four enhancers from each H3K27Ac class into two additional luciferase reporter vectors containing heterologous promoters not known to drive neuronal activity-dependent transcription: an SV40 promoter and a minimal TATA box containing promoter. We hypothesized that if activity-dependent enhancers instructed the transcriptional response to neuronal activity, they would be able to drive activity-dependent transcription from heterologous promoters. However, if activity-dependent enhancers simply amplified promoter driven transcriptional responses to neuronal activity, we hypothesized that they would not be able to drive activity-dependent transcription from heterologous promoters. We found that enhancers with increasing H3K27Ac in response to neuronal activity were able to drive robust activity-dependent reporter expression when paired with either the SV40 promoter (Figure 2.10c,d) or the minimal TATA box promoter (Figure 2.10e,f). While the minimal TATA box containing promoter was weaker than the other promoters (Figure 2.10e,f), enhancer-driven reporter expression for the Nptx2 reporter and the SV40 were strongly correlated with one
another ($\rho=0.95$; Figure 2.11), suggesting that the ability to activate transcription in response to neuronal activity was intrinsic to the enhancer element itself, with minimal contribution from the promoter. These results suggest that neuronal activity-dependent enhancers play a significant role in instructing activity-dependent gene expression, rather than functioning to merely amplify transcription from activity-regulated promoters. Furthermore, since enhancers not exhibiting increasing H3K27Ac levels in response to neuronal activity did not drive activity-dependent expression of the other reporter constructs tested (Figure 2.10c-f), these results provide additional evidence from independent reporter contexts that only enhancers with increasing H3K27Ac in response to neuronal activity are able to drive activity-dependent transcription.
Figure 2.10: Functional testing of neuronal enhancers in different reporter constructs

Luciferase data from neuronal enhancers from each of the four H3K27Ac enhancer classes cloned into either the Nptx2 reporter containing the Nptx2 enhancer (a, b), an SV40 promoter reporter (c, d), or a minimal TATA box containing promoter reporter (e, f). Reporter activity is reported as the fold induction of reporter activity in mouse cortical neurons after 6 hours of depolarization with KCl relative to 0 hours of depolarization with KCl. Panels a, c, and e show data for individual enhancers. Panels b, d, and f represent average reporter induction for all enhancers in each enhancer class. n ≥ 3 for all enhancers tested.
Figure 2.11: Enhancers drive very similar levels of activity-dependent reporter expression independent of the promoter

For each enhancer displayed in Figure 2.10, the fold induction of the SV40 reporter (displayed in Figure 2.10c) was plotted against the fold induction of the Nptx2 reporter (displayed in Figure 2.10a). Green, blue, magenta, and black points represent enhancers with increasing, constant, decreasing, or no H3K27Ac ChIP-Seq signal in response to neuronal activity. For greater clarity, the data is presented on a log-log scale. ρ indicates Spearman’s rank correlation coefficient between reporter inductions plotted.
2.3.4 Neuronal activity-dependent enhancers are increasingly used during brain development

While we were able to use H3K27Ac to identify neuronal activity-dependent enhancers in our in vitro culture system with KCl stimulation, we sought to evaluate whether these enhancers were also activity-dependent in vivo. To assess the in vivo utilization of our neuronal activity-dependent enhancers, we integrated our datasets with H3K27Ac and DNaseI hypersensitivity (DHS) datasets generated by other laboratories from mouse brain tissues at different stages in development (Consortium, 2011; Shen et al., 2012). We reasoned that since activity levels within the brain increase over development, evaluating the H3K27Ac and DNaseI hypersensitivity (DHS) signal at our enhancers at different stages in development could shed light on the utilization of these enhancers with increasing levels of activity exhibited over development in vivo. We found individual examples of enhancers that exhibited increasing H3K27Ac levels in response to KCl stimulation that also exhibited increasing H3K27Ac levels and DHS signal with brain development (Figure 2.12a). We quantified H3K27Ac levels and DHS signal at all activity-dependent enhancers we previously identified and found that these enhancers exhibited significantly more H3K27Ac (Figure 2.12b) and DHS (Figure 2.12c) in adult brain tissue compared to embryonic brain tissue. This suggested that, genomewide, neuronal activity-dependent enhancers identified in our system are not only utilized in the brain in vivo, but are increasingly utilized as the brain becomes more active during development.
Figure 2.12: Activity-dependent enhancers are increasing utilized during brain development

(a) Genome browser views of ChIP-Seq tracks at examples of activity-dependent enhancers that exhibit increasing H3K27Ac and DNaseI hypersensitivity (DHS) with brain development. (b) Quantification of H3K27Ac signal at all activity-dependent enhancers in the genome at two stages of brain development. (c) Quantification of DHS signal at all activity-dependent enhancers in the genome at two stages of brain development. In (b) and (c), * indicates P < 2.2x10^{-16} by paired Wilcoxon signed rank test.
2.3.5 Distinct yet overlapping enhancers drive activity-dependent and BDNF-dependent transcriptional programs

While the dynamic chromatin signature of stimulus-inducible H3K27Ac enabled us to identify the subset of neuronal enhancers that function to promote neuronal activity-dependent transcription, it is not clear whether this group of enhancers contains all stimulus-responsive enhancers in neurons or if different stimuli might induce distinct changes to the active enhancer landscape. Neurons must respond to a diverse array of stimuli with appropriate transcriptional changes to function properly in vivo. However, the mechanisms by which different extracellular stimuli instruct distinct programs of gene expression are poorly understood. Furthermore, how cis-regulatory elements at stimulus responsive genes activate transcription in response to diverse stimuli is not clear. For example, at genes that are upregulated in response to several distinct stimuli in neurons (such as c-Fos), it is not clear whether the response to all stimuli is driven by one multifunctional enhancer or if each stimulus activates a distinct enhancer element to drive transcription. At a mechanistic level, the cis-acting DNA sequences that determine why some enhancers respond to stimuli and others do not are poorly characterized (Evans et al., 2012). Attempts to understand these issues have been limited by challenges in the unbiased, genome-wide identification of the enhancers relevant for stimulus-responsive gene transcription. Our discovery of a dynamic chromatin signature that can accurately identify neuronal activity-regulated enhancers genome-wide provides a potential solution to this problem. Therefore, we sought to utilize this signature to identify neuronal enhancers that respond to an additional stimulus. This would enable a more detailed study of the general principles underlying
stimulus-responsive enhancer function and could help begin to decipher the specific mechanisms that drive transcriptional changes in response to neuronal activity.

Brain derived neurotrophic factor (BDNF) is a particularly important extracellular signal for nervous system development and functional plasticity (Hong et al., 2008; Park and Poo, 2013). Like neuronal activity, BDNF signaling can induce new gene transcription in neurons, although BDNF is thought to regulate a distinct program of gene expression (Gaiddon et al., 1996; Watson et al., 2001). We utilized the dynamic chromatin signature of stimulus-inducible H3K27Ac to identify the set of poised and active enhancers that respond to BDNF stimulation. We performed ChIP-Seq to measure genome-wide levels of H3K27Ac in mouse cortical neurons before and after stimulation with BDNF. In response to BDNF treatment we observed dynamic changes in H3K27Ac across the genome (Spearman rank correlation coefficient \( \rho = 0.39 \); Figure 2.13a). First, we selected a subset of enhancers that exhibited inducible H3K27Ac (n=2134) using the same criteria to what was used for the set identified for neuronal activity (Figure 2.13b; see methods). The 4.4kb sequence upstream of the Nptx2 TSS was able to drive BDNF-dependent expression of a luciferase reporter gene in a manner that depended critically upon the presence of the upstream enhancer (Figure 2.14). To test the ability of enhancers with increasing H3K27Ac with BDNF stimulation to promote neuronal BDNF-regulated transcription, we replaced the Nptx2 enhancer with enhancers with increasing H3K27Ac and measured BDNF-dependent induction of the reporter. Similar to what was observed for neuronal activity, we found that enhancers with increasing H3K27Ac levels in response to BDNF stimulation were able to drive BDNF-dependent expression of the Nptx2 reporter (Figure 2.14), providing direct
evidence that many of these enhancers function to promote BDNF-dependent transcription. Therefore, the dynamic chromatin signature of stimulus-inducible H3K27Ac was able to accurately identify BDNF responsive enhancers.

**Figure 2.13: H3K27Ac at neuronal enhancers before and after BDNF stimulation**

(a) Levels and correlation of H3K27Ac at neuronal enhancers (defined by H3K27Ac enrichment and DNaseI hypersensitivity) throughout the genome before BDNF stimulation (-BDNF) and after BDNF stimulation (+BDNF), Spearman’s rho $\rho=0.39$. (b) Classification of neuronal enhancers into subgroups with distinct H3K27Ac behaviors in response to BDNF stimulation. Enhancers with increasing H3K27Ac, high constant H3K27Ac, decreasing H3K27Ac, or no H3K27Ac in response to BDNF stimulation were classified into separate groups. Each point in both panels represents the input normalized H3K27Ac signal quantified within a 2kb window surrounding an enhancer center, with the x-axis value representing the signal before BDNF stimulation (-BDNF) and the y-axis value representing the signal after two hours of BDNF stimulation (+BDNF).
Figure 2.14: Functional testing of neuronal enhancers with increasing H3K27Ac with BDNF stimulation

Luciferase reporter data for the Nptx2 reporter, reported as the fold induction of reporter activity in mouse cortical neurons after 6 hours of BDNF stimulation relative to 0 hours. The wild-type Nptx2 reporter is able to induce BDNF-dependent reporter expression (Nptx2). However, if the Nptx2 enhancer is deleted, the reporter no longer induces BDNF-dependent reporter expression (Nptx2-enh). Enhancers exhibiting increasing H3K27Ac in response to BDNF stimulation were cloned into the Nptx2 reporter, replacing the Nptx2 enhancer, and tested for their ability to induce BDNF-dependent reporter expression. n ≥ 3 for all enhancers tested.
The ability to accurately identify stimulus-responsive enhancers also provides the novel opportunity to study the properties of BDNF enhancers and compare their properties with those of neuronal activity-dependent enhancers. Having identified two sets of stimulus-responsive enhancers in the nervous system, we next asked whether stimulus-responsive enhancers responded to a single stimulus or could respond to multiple stimuli. We reasoned that initial insight into the question of stimulus-responsiveness of enhancers might be obtained by examining the enhancers near genes that exhibit different stimulus-responsive patterns of expression. We investigated dynamics of H3K27Ac at enhancers near Npas4, a gene that is known to be preferentially induced by neuronal activity (Lin et al., 2008), Arc, which is preferentially induced in response to BDNF (Kawashima et al., 2009), and c-Fos, which is induced in response to both neuronal activity and BDNF (Bonni et al., 1995) (Figure 2.15). We discovered that the enhancers near these genes had H3K27Ac dynamics consistent with their stimulus-responsive patterns of expression. Several putative enhancers near the Npas4 gene exhibited inducible H3K27Ac only in response to neuronal activity (Figure 2.15, left). Similarly, a well-characterized enhancer (Kawashima et al., 2009) upstream of the Arc gene exhibited inducible H3K27Ac only in response to BDNF (Figure 2.15, center). Interestingly, enhancers near the c-Fos gene exhibited distinct patterns of H3K27Ac in response to different stimuli. Two enhancers upstream of the c-Fos TSS exhibited inducible H3K27Ac specifically in response to neuronal activity while one enhancer downstream of the TSS exhibited inducible H3K27Ac in response to both neuronal activity and BDNF (Figure 2.15, right). These loci demonstrate that while some neuronal stimulus-responsive enhancers respond to specific stimuli, others can respond to multiple stimuli.
Figure 2.15: Differential changes in H3K27Ac with KCl and BDNF stimulation

Genome browser views of H3K27Ac ChIP-Seq tracks at the Npas4 locus (left), the Arc locus (center), and the c-Fos locus (right). ChIP-Seq tracks display input-normalized H3K27Ac ChIP-Seq reads from neurons from the indicated stimulation conditions. Also displayed are DNaseI hypersensitivity signal from adult mouse cerebrum and vertebrate conservation by PhastCons. Dashed boxes identify regulatory elements with different stimulus-responsive changes in H3K27Ac.
From inspection of individual genetic loci, it was clear that while some neuronal stimulus-responsive enhancers respond to specific stimuli, others can respond to multiple stimuli. We sought to characterize neuronal enhancers throughout the genome that exhibited differential responses to neuronal activity and BDNF. At a genomic level, a distinct yet overlapping set of DNaseI hypersensitive sites exhibited increases in H3K27Ac in response to neuronal activity and BDNF. Thus, we hypothesized that by identifying all neuronal enhancers in the genome with different stimulus responsive properties, we could begin to understand the mechanisms by which enhancers respond to specific stimuli. In order to accomplish this, we first identified all neuronal activity-responsive (n=2261; Figure 2.16a), BDNF-responsive (n=1560; Figure 2.16b), or activity and BDNF responsive enhancers (n=404; Figure 2.16c) based on the inducibility of H3K27Ac at these enhancers in response to KCl, BDNF, or both. Average profiles of H3K27Ac ChIP-Seq signal for each group of neuronal enhancers displayed clearly different H3K27Ac behaviors in response to neuronal activity and BDNF (Figure 2.16).
**Figure 2.16: Identification of neuronal enhancers with differential responses to neuronal activity and BDNF stimulation**

Average profiles of H3K27Ac ChIP-Seq signal at KCl specific enhancers (a), BDNF specific enhancers (b), or KCl and BDNF responsive enhancers (c). The y-axis represents the level of H3K27Ac signal, displayed as the mean number of input-normalized H3K27Ac ChIP-Seq reads at each subgroup of enhancers. H3K27Ac signal at enhancers was aligned by the centers DNaseI hypersensitive sites. H3K27Ac signal at enhancers is displayed before KCl depolarization (dashed red line), after KCl depolarization (solid red line), before BDNF stimulation (dashed blue line), or after BDNF stimulation (solid blue line).
For each set of enhancers, the changes in H3K27Ac in response to each stimulus predicted the stimulus-specific changes in transcription, as assessed by functional testing of these enhancers in Nptx2 luciferase reporter assays (Figure 2.17). This suggested that dynamic stimulus-dependent changes in H3K27Ac can not only accurately identify stimulus-responsive enhancers but can also accurately identify enhancers throughout the genome that respond differentially to distinct stimuli.

Figure 2.17: Functional testing of enhancers with differential responses to neuronal activity and BDNF stimulation

Neuronal enhancers with differential H3K27Ac behaviors in response to neuronal activity and BDNF were cloned into the Nptx2 luciferase reporter and tested for their ability to induce reporter expression in response to KCl, BDNF, or both (a). (b) Average change in H3K27Ac for all enhancers in each stimulus-responsive enhancer class shown in (a). (c) Average reporter induction for all enhancers in each stimulus-responsive enhancer class shown in (a).
Having identified neuronal enhancers that respond to either neuronal activity or BDNF, we asked whether these different sets of enhancers contribute to stimulus-dependent transcriptional programs with different biological functions within the neuron. We did this by assessing whether activity-responsive and BDNF responsive enhancers were associated with genes of distinct functional annotations by performing ontology analysis with the Genomic Regions Enrichment of Annotations Tool (GREAT) (McLean et al., 2010). Each class of enhancers was associated with neuronal cellular components, biological processes, mouse phenotypes, and disease ontologies, consistent with specific functions of these enhancers in neurons (Table 1). However, there was little overlap in the specific categories enriched from each group (Table 1), consistent with the differences in the physiological functions of neuronal activity and BDNF. Closer analysis of the enriched terms for each class of enhancers revealed possible differences in the biological functions of stimulus-dependent transcriptional programs induced by neuronal activity and BDNF (Table 1). While activity-responsive enhancers were linked to genes that localize to dendritic compartments and play a role in behavior, BDNF-responsive enhancers were linked to genes that localize to vesicles and play a role in signaling. Enriched terms also revealed distinct mouse phenotypes and human diseases associated with each set of enhancers. While activity-responsive enhancers were linked to abnormal brain morphology and psychiatric disease, BDNF-responsive enhancers were linked to abnormal synapse function and cancer and neurodegenerative disease. These data suggest that dynamic stimulus-dependent changes in H3K27Ac can not only accurately identify enhancers throughout the genome that respond differentially to distinct stimuli, but that these different sets of enhancers may contribute to different biological functions within the neuron.
KCl and BDNF specific enhancers were analyzed using the Genomic Regions Enrichment of Annotations Tool (GREAT). Both KCl specific and BDNF specific enhancers were linked to genes with nervous system-related annotations but these classes of enhancers differed in the specific annotations that were most enriched within each ontology category. Only the top three most significant terms are displayed for each ontology category.

<table>
<thead>
<tr>
<th>Ontology Category</th>
<th>KCl-specific enhancers Term</th>
<th>P-value</th>
<th>BDNF-specific enhancers Term</th>
<th>P-value</th>
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</thead>
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<td>7.9E-18</td>
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<td></td>
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<td>cytoplasmic membrane-bounded vesicle</td>
<td>5.6E-15</td>
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<tr>
<td></td>
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<td>4.0E-10</td>
<td>membrane-bounded vesicle</td>
<td>5.9E-15</td>
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<td>5.4E-15</td>
<td>regulation of signaling</td>
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<td>9.8E-07</td>
<td>substance-related disease</td>
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</tbody>
</table>
2.4 **DISCUSSION**

In neurons, extracellular stimuli can induce specific and temporally regulated changes in gene expression that correlate with the formation and functional plasticity of synaptic connections in developing and adult brains (Loebrich and Nedivi, 2009). In order to identify and begin to understand the cis-regulatory elements that function to instruct the temporally and spatially circumscribed induction of activity-regulated transcription in neurons, we utilized genome-scale tools to map enhancer elements in cortical neurons. While previous studies have begun to identify neuronal enhancers, the enhancers that function to promote stimulus-responsive transcription within the nervous system have not been specifically identified. This is in part because no chromatin signature has been described for the specific identification of this subset of enhancers. We demonstrate that membrane depolarization with KCl and stimulation with BDNF can both induce rapid changes in H3K27Ac at thousands of enhancer elements within the genome. We further show that stimulus-responsive enhancers within the nervous system can be specifically identified on the basis of the dynamic chromatin signature of stimulus-inducible H3K27Ac. We utilized this dynamic chromatin signature to comprehensively identify and characterize functional KCl and BDNF responsive enhancer elements in mouse cortical neurons. These enhancers are sufficient to drive robust and stimulus-specific reporter expression in cortical neurons *in vitro*. Furthermore, these enhancers are associated with genes with distinct functional annotations, suggesting that KCl and BDNF responsive enhancers play distinct biological functions within the neuron. Finally, by integrating our data with epigenomic data from other studies, we have gained insight into the cis-regulatory logic.
of activity-regulated transcriptional control and have identified thousands of previously uncharacterized neuronal enhancer elements.

2.4.1 Rapid changes in H3K27Ac at neuronal enhancers

In the nervous system, it has been proposed that long-term changes in synaptic function associated with learning and memory might arise in part from stable changes in chromatin modifications that serve as a molecular “mnemonic” devices (Day and Sweatt, 2011; Graff and Tsai, 2013). Interestingly, global levels of histone acetylation occur in brain regions that are strongly activated during specific experimental behavioral paradigms (Day and Sweatt, 2011; Graff and Tsai, 2013; Peleg et al., 2010). Furthermore, reduction or removal of histone deacetylase activity using genetic methods or treatment with HDAC inhibitors has been shown to increase memory formation and long-term potentiation (Graff and Tsai, 2013; Guan et al., 2009). It will be interesting to explore the possibility that HDAC inhibitors might influence learning and memory by acting to increase the magnitude or half-life of inducible acetylation at stimulus-responsive enhancers, which could lead to changes in the kinetics of stimulus-regulated gene transcription, decreases in the levels of stimulation required to induce stimulus-regulated gene transcription, or ectopic activation of genes that are not activity-regulated under physiological conditions.
2.4.2 Heterogeneity among active neuronal enhancers

Previous studies that have identified active enhancers within the nervous system have using distal enrichment of H3K27Ac. However, these studies have treated neuronal enhancers as a homogeneous population. We find that neuronal enhancers are quite heterogeneous, both in terms of their response to extracellular stimuli as well as their stimulus specificity. Our data suggest a previously underappreciated diversity of cis-regulatory function among enhancers defined as “active” using H3K27Ac enrichment, and suggest that by profiling enhancer activity both during differentiation and in specific stimulus paradigms it will be possible to further classify active enhancers into discrete functional classes. A recent report suggests that in macrophages, prolonged stimulation with an inflammatory stimulus induces the activation of “latent” enhancer elements, which exhibit none of the chromatin marks associated with poised enhancer elements (e.g. H3K4me1, nucleosome depletion) prior to exposure to inflammatory stimuli (Ostuni et al., 2013). These latent enhancers were suggested to function to prime specific genes for increased expression following a transient stimulus, which the authors suggest might provide a chromatin-based mechanism for storing information about prior exposure to environmental stimuli. However, when tested in luciferase reporter assays, only ~50% of these regions can drive stimulus responsive transcription, suggesting that a substantial portion of these elements are not acutely involved in stimulus-regulated transcription and are potentially more important for stimulus-induced phenotypic plasticity. In the brain, sensory experience can induce long-lasting changes in neuronal morphology, synapse number and plasticity, and survival, but the potential for this limited plasticity is balanced by the need to tightly
and stably regulate neuronal fate for the entire lifespan of the organism (Dulac, 2010). Further studies will be required to unravel the connection between synaptic activity and the active enhancer repertoire during brain development and function in vivo to determine whether neurons also utilize stimulus-responsive “latent” enhancers. It will be similarly interesting to investigate whether any observed differences in stimulus-responsive enhancer “latency” represents a physiologically relevant difference between the function of stimulus responsive transcription in macrophages and neurons.

2.4.3 Novel tools to study the role of activity-regulated gene transcription in nervous system development and plasticity

The induction of stimulus-regulated transcription occurs specifically in neurons that are highly active during a defined behavior (Alberini, 2009; Lyons and West, 2011). The induction of immediate early genes such as c-Fos, FosB, JunB, Nur77, Zif268 and Npas4 in vivo has been used as a tool to specifically mark the neurons involved in the circuitry governing a defined behavior. The ability of enhancers to integrate various transcription factor inputs and signal responsive cues can also be repurposed to develop tools to drive reporter gene expression or Cre recombinase in specific cell populations in vivo, providing genetic access to specific neuronal circuits. Similarly, in order to rigorously study the function of stimulus regulated genes it will be necessary to remove the enhancer(s) that specifically control a gene’s induction in order to disentangle the stimulus-dependent and stimulus-independent effects on synaptic function of these often pleiotropic factors (e.g. BDNF, see Hong et al., 2008). Historically it has been
difficult to separate the activity-regulated function of many genes from their functions in neural development, so the ability to functionally separate these features will become critical for future studies to determine the contribution of specific activity-regulated genes to learning and memory in vivo. Given recent efforts to establish in vitro stem cell models of neurological disorders that affect synaptic plasticity and function in vivo, activity-responsive enhancers could provide useful tools for the characterization of the in vitro maturation of various types of neurons derived from embryonic stem cell differentiation protocols (Dolmetsch and Geschwind, 2011; Ming et al., 2011).
2.5 **MATERIALS AND METHODS**

2.5.1 **Mouse cortical cultures**

Embryonic day 16.5 (E16.5) C57BL/6 embryonic mouse cortices were dissected and then dissociated for 10 minutes in 1× Hank’s Balanced Salt Solution (HBSS) containing 20 mg/mL trypsin (Worthington Biochemicals) and 0.32 mg/mL L-cysteine (Sigma). Trypsin treatment was terminated by washing dissociated cells three times for two minutes each in dissociation medium consisting of 1× HBSS containing 10 mg/mL trypsin inhibitor (Sigma). Cells were then triturated using a flame-narrowed Pasteur pipette to fully dissociate cells. After dissociation, neurons in were kept on ice in dissociation medium until plating. Cell culture plates were pre-coated overnight with a solution containing 20 ug/mL poly-D-lysine (Sigma) and 4 ug/mL mouse laminin (Invitrogen) in water. Prior to plating neurons, cell culture plates were washed three times with sterile distilled water and washed once with Neurobasal Medium (Life Technologies). Neurons were grown in neuronal medium consisting of Neurobasal Medium containing B27 supplement (2%; Invitrogen), penicillin-streptomycin (50 g/ml penicillin, 50 U/mL streptomycin, Sigma) and glutamine (1 mM, Sigma). At the time of plating, cold neuronal medium was added to neurons in dissociation medium to dilute neurons to the desired concentration. Neurons were subsequently plated and placed in a cell culture incubator that maintained a temperature of 37 degrees C and a CO2 concentration of 5%. Two hours after plating neurons, medium was completely aspirated from cells and replaced with fresh warm neuronal medium. Neurons were grown *in vitro* until the seventh day *in vitro* (DIV7).
2.5.1.1 Mouse cortical cultures for ChIP-Seq experiments

For ChIP-Seq experiments, mouse cortical neurons were plated at an approximate density of $4 \times 10^7$ on 15-cm dishes. Neurons were plated in 30mL neuronal medium. Ten mL of the medium was replaced with 12ml fresh warm medium on DIV3 and DIV6.

2.5.1.2 Mouse cortical cultures for luciferase reporter assays

For luciferase reporter assays, mouse cortical neurons were plated at an approximate density of $3 \times 10^5$ per well on 24-well plates. Neurons were plated in 500 uL neuronal medium. On the DIV3, 100 uL fresh warm medium was added to neurons. On DIV5 neurons were transfected (see section on transfection). At the completion of transfection, conditioned medium containing 15% fresh medium was returned to neurons.

2.5.2 Stimulation with potassium chloride (KCl)

Prior to KCl depolarization, neurons were quieted with 1 µM tetrodotoxin (TTX, Fisher) and 100 µM DL-2-amino-5-phosphopentanoic acid (DL-AP5, Fisher). Neurons were subsequently stimulated by adding warmed KCl depolarization buffer (170 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES) directly to the neuronal culture, to final concentration of 31% in the neuronal culture medium within the culture plate or well.
2.5.2.1 KCl stimulation for ChIP-Seq experiments

For KCl depolarization of neurons for ChIP-Seq experiments, DIV 6 neurons were quieted overnight with 1 µM TTX and 100 µM DL-AP5. The next morning, neurons were left silenced (-KCl condition) or stimulated for 2 hours with KCl (+KCl condition).

2.5.2.2 KCl stimulation for luciferase reporter assays

For KCl depolarization of neurons for luciferase reporter assays, DIV 7 neurons were quieted for two hours with 1 µM TTX and 100 µM DL-AP5. Two hours later, neurons were left silenced (-KCl condition) or stimulated for 6 hours with KCl (+KCl condition).

2.5.3 Stimulation with brain derived neurotrophic factor (BDNF)

2.5.3.1 BDNF stimulation for ChIP-Seq experiments

For BDNF stimulation of neurons for ChIP-Seq experiments, the volume of conditioned medium on DIV6 neurons was reduced to 25 mL. On DIV7, neurons were left in a basal condition (-BDNF condition) or stimulated for 2 hours with 50ng/mL recombinant human BDNF (Fisher) (+BDNF condition).
2.5.3.2 BDNF stimulation for luciferase reporter assays

For BDNF stimulation of neurons for luciferase reporter assays, DIV7 neurons were left in a basal condition (-BDNF condition) or stimulated for 6 hours with 50ng/mL recombinant human BDNF (Fisher) (+BDNF condition).

2.5.4 H3K27Ac Chromatin immunoprecipitation sequencing (ChIP-Seq)

Forty million mouse cortical neurons cultured to in vitro day 7 were used for each ChIP-Seq library construction. Typically 20-40 million cortical neurons were used for a single ChIP experiment. To cross-link protein-DNA complexes, media was removed from neuronal cultures and crosslinking-buffer (0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 25 mM HEPES-KOH, pH 8.0) containing 1% formaldehyde was added for 10 minutes at room temperature. Cross-linking was quenched by adding 125 mM glycine for five minutes at RT. Cells were then rinsed three times in ice-cold PBS containing complete protease inhibitor cocktail tablets (Roche) and collected by scraping. Cells were pelleted and either stored at -80 degrees C until use or immediately processed. Cell pellets were lysed by 20 cell pellet volumes (CPVs) of buffer 1 (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, pH 8.0, 10 % Glycerol, 0.5 % NP-40, 0.25 % Triton X-100, complete protease inhibitor cocktail) for 10 min at 4 degrees C. Nuclei were then pelleted by centrifugation at 3000 rpm for 10 min at 4 degrees C. The isolated nuclei were rinsed with 20 CPVs of buffer 2 (200 mM NaCl, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0, 10 mM Tris-HCl, pH 8.0, complete protease inhibitor cocktail) for 10 min at RT and re-pelleted. Next, 4 CPVs of buffer 3 (1 mM EDTA, pH 8.0, 0.5 mM
EGTA, pH 8.0, 10 mM Tris-HCl, pH 8.0, complete protease inhibitor cocktail) were added to the nuclei and sonication was carried out using a Misonix 3000 Sonicator (Misonix) set at a power setting of 7.5 (equivalent to 24 watts). 20 pulses of 15 seconds each were delivered at this setting, which resulted in genomic DNA fragments with sizes ranging from 200 bp to 2 kb. Insoluble materials were removed by centrifugation at 20,000 rpm for 10 min at 4 degrees C. The supernatant was transferred to a new tube and the final volume of the resulting nuclear lysate was adjusted to 1 mL by adding buffer 3 supplemented with 0.3 M NaCl, 1 % Triton X-100, 0.1 % Deoxycholate. The lysate was pre-cleared by adding 100 uL of pre-rinsed Protein A/G Agarose (Sigma) per 1 ml of the lysate and incubating for 1 hour at 4 degrees C. After pre-clearing, ten percent of the ChIP sample (50 uL from 500 uL lysate) was saved as input material. The remaining lysate was incubated with 0.5 ug H3K27Ac antibody ab4729 (Abcam) for immunoprecipitation. The antibody incubation was carried out overnight at 4 degrees C. The next day, 30 uL of pre-rinsed Protein A/G PLUS Agarose beads (Santa Cruz Biotechnology) was added to each ChIP reaction and further incubated for 1 hour at 4 degrees C. The beads bound by immune-complexes were pelleted and washed twice with each of the following buffers: low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl) and LiCl buffer (0.25M LiCl, 1% IGEPAL CA630, 1% deoxycholic acid (sodium salt), 1mM EDTA, 10mM Tris, pH 8.1). In each wash, the beads were incubated with wash buffer for 10 min at 4 degrees C while nutating. The washed beads were then rinsed once with 1x TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The immunoprecipitated material was eluted from the beads twice by adding 100 uL of elution buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0,
1 % SDS) to each ChIP reaction and incubating the sample at 65 degree C for 30 min with brief vortexing every 2 min. 150 uL of elution buffer was also added to the saved input material (50 uL) and this sample was processed together with the ChIP samples. The eluates were combined and crosslinking was reversed by incubation at 65 degrees C overnight. The next day, 7 ug RNase A (affinity purified, 1mg/mL; Invitrogen) was added to each sample and samples were incubated for 37 degrees C for one hour. Then, 7 uL Proteinase K (RNA grade, 20mg/mL; Invitrogen) was added to each sample and samples were incubated at 55 degrees C for two hours. The immunoprecipitated genomic DNA fragments were then extracted once with Phenol:Chloroform:Isoamyl Alcohol (25:24:1, pH 7.9; Life Technologies) and then back extracted with water. The resulting genomic DNA fragments were then purified using the QIAquick PCR purification kit (Qiagen) and DNA fragments were eluted in 100 ul of Buffer EB (elution buffer consisting of 10 mM Tris-HCl, pH 8.5, Qiagen). Samples were assessed for enrichment by quantitative PCR using primers to different genomic regions. Samples with significant enrichment over negative regions were submitted to the Beijing Genomic Institute (BGI) for 50 base pair single end sequencing on the Illumina Hiseq 2000 platform. For each sample, over 20 million clean reads were obtained.

2.5.5 ChIP-Seq analysis

2.5.5.1 Initial processing

Sequencing data was obtained from BGI in gzipped fastq file format. Files were transferred and unzipped. Then, sequencing reads were aligned to the July 2007
assembly of the mouse genome (NCBI 37, mm9) using the Burrows-Wheeler Aligner (BWA) (Li and Durbin, 2009) with default settings. The resulting bwa files were then converted to sam files and uniquely mapped reads were extracted from the sam files. Sam files of the uniquely mapped reads were then converted to bam files. Bam files were then used for peak calling using Model-based Analysis of ChIP-Seq (MACS) (Zhang et al., 2008) with the following parameters: -f BAM -g mm --nomodel --shiftsize=150.

2.5.5.2 Visualizing ChIP-Seq data on the UCSC genome browser

ChIP-Seq bam files were converted to bigwig track format to display the number of input normalized ChIP-Seq reads, normalized to 20 million total reads.

2.5.5.3 Identifying enhancers by integrating DNaseI hypersensitivity data with H3K27Ac ChIP-Seq data

DNaseI hypersensitivity (DHS) identified by using ENCODE consortium DHS peaks data from embryonic day 14.5 whole brain, embryonic day 18.5 whole brain, and adult 8 week cerebrum was assessed for overlap with H3K27Ac peaks. First, the genomic ranges of DHS peaks from adult 8 week cerebrum in all three bioreplicates were concatenated and merged. Then, merged ranges that overlapped with peaks in three out of three DHS bioreplicates were computed (8wk_merged_overlappingwithB1B2B3). Similarly, merged ranges that overlapped with two out of two bioreplicates from embryonic day 18.5 (e18.5_merged_overlappingwithB1B2) and merged ranges that overlapped with two out of two bioreplicates from embryonic day 14.5 (e14.5_merged_overlappingwithB1B2) were obtained. Then DHS peaks overlapping
with H3K27Ac were obtained through iterations of assessing overlap between the datasets. First, the DHS peaks in 8wk_merged_overlappingwithB1B2B3 that overlapped with H3K27Ac peaks (in the –KCl, +KCl, -BDNF, or +BDNF conditions) were isolated. Then, the DHS peaks in e18.5_merged_overlappingwithB1B2 that overlapped with the remaining H3K27Ac peaks were isolated. Then, the DHS peaks in e14.5_merged_overlappingwithB1B2 that overlapped with the remaining H3K27Ac peaks were isolated. Then, the DHS peaks in the first bioreplicate from 8-week cerebrum that overlapped with the remaining H3K27Ac peaks were isolated. Then, the DHS peaks in the second bioreplicate from 8-week cerebrum that overlapped with the remaining H3K27Ac peaks were isolated. Then, the DHS peaks in the third bioreplicate from 8-week cerebrum that overlapped with the remaining H3K27Ac peaks were isolated. Then, the DHS peaks in the first bioreplicate from e18.5 whole brain that overlapped with the remaining H3K27Ac peaks were isolated. Then, the DHS peaks in the second bioreplicate from e18.5 whole brain that overlapped with the remaining H3K27Ac peaks were isolated. Then, the DHS peaks in the first bioreplicate from e14.5 whole brain that overlapped with the remaining H3K27Ac peaks were isolated. Then, the DHS peaks in the second bioreplicate from e14.5 whole brain that overlapped with the remaining H3K27Ac peaks were isolated. Then, CBP peaks previously identified in our system (Kim et al., 2010) that overlapped with the remaining H3K27Ac peaks were isolated. This iterative procedure was used rather than simply assessing overlap between H3K27Ac signal and either the union or intersection of all DHS data to increase accuracy of calling overlaps and to reduce the multiplicity of DHS peaks associated with H3K27Ac peaks. In the end, 98.6% of H3K27Ac peaks were found to overlap with DHS peaks, with an average of 1.39 DHS peaks per H3K27Ac peak. Each DHS peak that
overlapped with H3K27Ac and existed greater than 1kb from an annotated RefSeq TSS was taken to be an enhancer for subsequent analyses.

2.5.5.4 Characterization of genomic distribution of enhancers

H3K27Ac peaks were classified based on their location relative to genes in the NCBI Reference Sequence Database (RefSeq). H3K27Ac peaks were classified as being proximal if they were within 1kb of an annotated transcriptional start site (TSS). H3K27Ac peaks were classified as being distal if they were greater than 1kb from an annotated transcriptional start site (TSS). Distal H3K27Ac peaks were further classified as intragenic if they occurred within a RefSeq gene, or as extragenic if they did not occurred within a RefSeq gene.

2.5.5.5 Quantification of ChIP-Seq signal at enhancers

For chromatin modifications (H3K4Me1, H3K27Ac), the number of input-normalized ChIP-Seq reads within a two kb window centered on each enhancer was taken to be the ChIP-Seq signal at the enhancer. For transcription factors (CBP), the number of input-normalized ChIP-Seq reads within an 800 bp window centered on each enhancer was taken to be the ChIP-Seq signal at the enhancer.

2.5.5.6 Classification of enhancers with distinct H3K27Ac behaviors

Enhancers were classified into different categories based on the behavior of the quantified H3K27Ac signal at each enhancer. Enhancers were classified as having increasing H3K27Ac if they exhibited a two fold or greater increase in H3K27Ac signal
with stimulation and if the stimulated signal for H3K27Ac was not within the bottom quartile of H3K27Ac signal at all enhancers identified in the stimulated condition. Enhancers were classified as having decreasing H3K27Ac if they exhibited a two fold or greater decrease in H3K27Ac signal with stimulation and if the unstimulated signal for H3K27Ac was not within the bottom quartile of H3K27Ac signal at all enhancers identified in the unstimulated condition. Enhancers were classified as having constant H3K27Ac if they exhibited H3K27Ac signal in the top quartile of all enhancers identified in both the unstimulated and stimulated conditions and if the H3K27Ac signal changed by 10% or less with stimulation. Enhancers were classified as having no H3K27Ac if they were identified previously on the basis of inducible CBP binding and enrichment for H3K4Me1 (Kim et al., 2010) but did not overlap with H3K27Ac peaks within the H3K27Ac datasets generated in this study.

2.5.6 RNA-Seq analysis

RNA-Seq data from a previous study (Kim et al., 2010) was analyzed and integrated into this study. For nearest gene analyses, the nearest gene (with nonzero expression) to an enhancer was linked to that enhancer and the expression of the genes nearest to each class of enhancers was characterized. For eRNA analysis, the number of RNA-Seq reads within a four kb window centered on each extragenic enhancer was taken to be the eRNA signal at the enhancer.
2.5.7 Luciferase reporter assays

2.5.7.1 Nptx2 reporter plasmid design

All luciferase reporter plasmids used were newly developed for this study. Most luciferase reporter plasmids used were based on the Nptx2 gene, and hence this reporter was termed the Nptx2 reporter. To develop the Nptx2 reporter, we cloned the 4355 bp region upstream of the Nptx2 coding sequence from C57BL/6 purified mouse genomic DNA between the NheI and EcoRV restriction sites within the multiple cloning site of the promoterless pGL4.11 reporter plasmid (Promega) using the primers shown in Table 2, with 5' clamp shown in red, NheI and EcoRV sites shown in green.

Table 2: Primers used to clone the Nptx2 upstream regulatory region into pGL4.11

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>5’ GCCGCCTAGCTCTCCTGGCTGTAGTGACCT 3’</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5’ GCCCGATAATCCTCGTAGCTGACCTGTGCTCATTCA 3’</td>
</tr>
</tbody>
</table>

pGL4.11 was chosen as the host plasmid since it contained the luc2P reporter gene, which contains an hPEST protein destabilization sequence. We found that the luc2P reporter responded more quickly and with greater magnitude to stimuli than luc2 reporters. Using PCR driven overlap extension (Heckman and Pease, 2007), the Nptx2 reporter was then modified so that the 1216 bp Nptx2 upstream enhancer (located -3607 to -2391 relative to the start of the Nptx2 coding sequence) was replaced with a multiple cloning site containing SbfI, PacI, PmeI, and AscI restrictions sites. In order to do this, the primers shown in Table 3 were used (A, B, C, D nomenclature same as described in
(Heckman and Pease, 2007)), with 5' clamp shown in red; NheI and EcoRV sites shown in green; SbfI, PacI, PmeI, and AscI sites shown in blue; and spacers shown in orange.

Table 3: Primers used to replace the Nptx2 upstream enhancer with a multiple cloning site

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5' GCGCGCTAGCTTCCGGCTGGCTTAGGACCT 3'</td>
</tr>
<tr>
<td>B</td>
<td>5' GGCGCGCCACAGTTAAACGCCGCTGATTTCTCCAGGTGTGTGAGACACTGTITTCA 3'</td>
</tr>
<tr>
<td>C</td>
<td>5' CCTGCAAGTACATCTAAGGGCCGGCGCTGGTGTGCACTGGTGCATT 3'</td>
</tr>
<tr>
<td>D</td>
<td>5' GCGCGATATCCTCCTGACCTGTGCTGCACCTCA 3'</td>
</tr>
</tbody>
</table>

The multiple cloning site was inserted into the Nptx2 upstream regulatory region to create a modified Nptx2 reporter so that various enhancers could be easily cloned into this multiple cloning site. We verified that the modified Nptx2 reporter in which the Nptx2 enhancer had been cloned into the multiple cloning site had the same inducibility as the wild-type Nptx2 reporter (data not shown). This suggested that the multiple cloning site did not affect the function of the reporter and that other enhancers could be similarly cloned into this multiple cloning site without adverse affects on enhancer function.

2.5.7.2 Alternate reporter plasmid design

We generated 2 additional reporter constructs by modifying pGL4.24 (Promega), a luciferase reporter containing a minimal TATA box containing promoter but not containing any enhancer elements. To facilitate cloning of enhancers from the Nptx2 reporter plasmid into this plasmid, we first modified this plasmid by adding a multiple
cloning site containing SbfI, PacI, PmeI, and AscI sites between BamHI and SalI sites downstream of the firefly luciferase gene (pGL4.24_minP_MCS). This multiple cloning site was added by annealing together two oligonucleotides containing the multiple cloning site as well as overhanging BamHI and SalI restriction sites, and then ligating this annealed product into pGL4.24 backbone that had been cut with BamHI and SalI. The sequences of the oligonucleotides are shown in Table 4, with BamHI and SalI sites shown in green and SbfI, PacI, PmeI, and AscI sites shown in blue.

Table 4: Primers used to introduce a multiple cloning site into pGL4.24

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>5’ GATCCGACACCTGCAGGACACTTAATTAAAGCGCGTTTTAAACGTGTGGCGCGCCATCCG 3’</td>
</tr>
<tr>
<td>R</td>
<td>5’ TCGACGGAGCGCGGCCACAGTTAAAACGCGCTTAATTAAAGCGCTGTCCCTGAGG TGTCG 3’</td>
</tr>
</tbody>
</table>

We then further modified pGL4.24_minP_MCS by removing the minimal TATA box containing promoter from this plasmid and replacing this promoter with an SV40 promoter from another Promega luciferase reporter, the pGL3-Promoter Vector. This cloning was achieved by using BglII and NcoI restriction sites flanking both promoter regions. This resulted in the generation of a separate reporter construct, pGL4.24_SV40_MCS, with which enhancer activity could be assessed.

2.5.7.3 Enhancer sequences

Enhancer with different H3K27Ac behaviors were cloned between the SbfI and AscI sites within the multiple cloning site of the modified Nptx2 reporter. Cloned enhancer regions varied in size but were approximately 1kb in length. The following enhancers were cloned in this way using the primers listed in Table 5, with 5’ clamp shown in red and SbfI and AscI sites shown in blue.
Table 5: Primers to clone neuronal enhancers with different H3K27Ac behaviors

Note that enhancers e31, e32, e37, e39, all cloned initially because they displayed increasing H3K27Ac in response to KCl, also displayed increasing H3K27Ac in response to BDNF. As a result, these enhancers were subsequently used in BDNF experiments in Figure 2.14 and Figure 2.17.

<table>
<thead>
<tr>
<th>Enhancer category</th>
<th>Name</th>
<th>Forward primer (5' → 3')</th>
<th>Reverse primer (5' → 3')</th>
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<td>No H3K27Ac (with KCl stimulation)</td>
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<td>Increasing H3K27Ac (with BDNF stimulation)</td>
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</table>
2.5.7.4 Transfection

Mouse cortical neurons plated on 24 well plates at a density of approximately 3x10^5 neurons per well were transfected for luciferase reporter assays using Lipofectamine 2000 Reagent (Invitrogen), generally according to the manufacturer’s protocol. Briefly, DNA mixes were made immediately preceding the transfection consisting of 1ug total plasmid DNA/well diluted in Neurobasal medium (Life Technologies). DNA typically consisted of 450ng firefly luciferase reporter DNA, 50ng pGL4.74 renilla luciferase reporter DNA (Promega), and 500ng empty pCS2 plasmid (Rupp et al., 1994; Turner and Weintraub, 1994) as filler DNA. Lipofectamine was used at 2uL/well and was diluted in Neurobasal medium just before the transfection. Within each experiment, all conditions were transfected in two to three independent wells, for technical duplicates or triplicates. Thirty minutes prior to the addition of Lipofectamine to neurons, the culture medium was removed and replaced with warmed Neurobasal medium. At this time, neurons were returned to the incubator and DNA mixes were added to diluted Lipofectamine in a drop-wise manner. After thirty minutes of incubation, DNA-Lipofectamine mixes were added to neurons, again in a drop-wise manner. The cells were left to incubate with the DNA-Lipofectamine mix for two hours, after which the transfection medium was replaced with supplemented conditioned neuronal medium.

2.5.7.5 Sample collection and luciferase assay

After stimulation, neurons were lysed using Passive Lysis Buffer (Dual-Luciferase Reporter Assay System, Promega). Lysates were then collected in microcentrifuge tubes
and frozen at -20 degrees C. At the time of performing the luciferase assay, neuronal lysates were thawed, briefly vortexed, briefly spun down, and then 20uL of each sample was added to one well of Costar White Polystyrene 96-well Assay Plates (Corning). The reagents to run the luciferase assay, Luciferase Assay Reagent II (LARII) and Stop & Glo Reagent (Dual-Luciferase Reporter Assay System, Promega), were aliquoted and thawed according to the manufacturer's protocol. The luciferase assay was performed using the Synergy 4 Hybrid Microplate Reader (BioTek), with 100uL of LARII and Stop & Glo Reagent injected per well. Data was subsequently downloaded and analyzed using Microsoft Excel.

2.5.7.6 Statistical Analyses of Luciferase Assay Data

Using the Dual-Luciferase Reporter Assay System, we recorded Firefly (FF) and Renilla (Ren) luminescence from each well. To correct for variations in transfection efficiency and cell lysate generation, the Firefly values were normalized to Renilla luminescence within each well, generating a ratio of FF/Ren. The stimulus-dependent fold induction of each reporter plasmid was obtained by dividing the (+ stimulus) FF/Ren value by the (- stimulus) value. To isolate the induction due to the enhancer, the fold induction of an enhancer reporter was divided by the fold induction of the appropriate backbone into which the enhancer was cloned, giving fold induction relative to backbone. Fold induction relative to backbone is the value shown in all figures containing luciferase reporter data. All error bars shown are standard error of biological replicates.
2.5.8 GREAT analysis

Functional annotations of genes linked to stimulus-responsive enhancers was investigated with the Genomic Regions Enrichment of Annotations Tool (GREAT) (McLean et al., 2010). For the analysis, the following settings were used: species assembly: Mouse: NCBI build 37 (UCSC mm9, Jul/2007); test regions: BED file containing genomic locations of stimulus inducible enhancers; background regions: whole genome; association rule setting: basal plus extension including 20 kb upstream, 20 kb downstream, plus distal up to 250 kb, and including curated regulatory domains. Ontology categories investigated include cellular components, biological processes, mouse phenotypes, and disease ontologies.
3 TRANSCRIPTIONAL MECHANISMS UNDERLYING STIMULUS-RESPONSIVE ENHANCERS IN THE NERVOUS SYSTEM
3.1 SUMMARY

Normal brain development and function require stimulus-responsive programs of gene expression, but how neuronal stimuli interface with stimulus-responsive enhancers to regulate gene expression is poorly understood. We have recently shown that functional stimulus-responsive enhancers can be identified by stimulus-inducible H3K27Ac, and we used this dynamic chromatin signature to discover enhancers that respond to activity, BDNF, or both. Here, we investigate the transcriptional mechanisms that underlie stimulus-responsive enhancer function in the nervous system. We show that stimulus-responsive enhancers require the coordinated action of the stimulus-general transcription factor activator protein 1 (AP1) with additional stimulus-specific factors. This work provides important insights into the cis-acting elements at stimulus-responsive enhancers that together with the trans-acting factors that bind to these elements transform sensory experience into specific transcriptional programs that facilitate long-lasting changes in neuronal function.
3.2 INTRODUCTION

Enhancers consist of binding sites for multiple families of transcription factors, the binding of which function synergistically to promote transcription at promoters of target genes (Spitz and Furlong, 2012). The exact complement of transcription factors recruited to enhancers determines when and where an enhancer is active. Much of what we know about the transcriptional mechanisms comes from studies of a few enhancers in detail. Recently, genome-wide methods have been developed to comprehensively identify the enhancer elements within the genome. These methods have been used to catalog enhancer elements in various cells and tissues in different organisms. Furthermore, genomic characterization has led to new understanding of how enhancer elements function and contribute to the establishment and maintenance of cellular identity. However, enhancers also play an important role in transcriptional responses to extracellular stimuli. Only recently have studies attempted to identify and characterize enhancers that contribute to stimulus-responsive programs of gene expression.

Stimulus responsive enhancers play an important role in the nervous system. In response to sensory experience, strong bursts of synaptic activity induce a program of gene expression in excitatory neurons that is required for proper development and refinement of neural circuits and for long-lasting changes in neuronal function that underlie learning, memory, and behavior. Studies of this neuronal activity-dependent gene program have primarily focused on signaling to promoter bound transcription factors. The release of the neurotransmitter glutamate at synapses leads to membrane depolarization of the post-synaptic neuron, triggering calcium influx through L-type
voltage gated calcium channels. The influx of calcium then leads to activation of a complex signaling network that induces the post-translational modification of promoter bound transcription factors that initiate multiple waves of gene expression. In addition to these critical signaling events at the promoters of activity-regulated genes, signaling to enhancers contributes to activity-dependent gene expression, although there has been little progress towards characterizing the function of activity-regulated enhancers because it has been difficult to identify these elements within the genome.

Recently, we have discovered that the dynamic chromatin signature of stimulus-inducible H3K27Ac specifically identifies functional stimulus-responsive enhancers. We employed this dynamic chromatin signature to identify neuronal enhancers that respond to neuronal activity, BDNF stimulation, or both. Here, we characterize the transcriptional mechanism underlying these stimulus-responsive enhancers within the nervous system. We identify the transcription factor motifs enriched at neuronal stimulus-responsive enhancers. We further study the binding of activity-dependent transcription factors predicted to bind to stimulus-responsive enhancers. The motif for the stimulus-responsive transcription factor activator protein 1 (AP1) is enriched at neuronal enhancers that respond to activity and those that respond to BDNF stimulation. AP1 transcription factors within the Fos family of transcription factors bind to stimulus responsive enhancers in response to neuronal activity and BDNF stimulation. We show that AP1 is necessary but not sufficient at a subset of enhancers for their response to both neuronal activity and BDNF stimulation. These data demonstrate that AP1 functions as a stimulus-general transcription factor that coordinates stimulus-responsive transcription at enhancers with other stimulus-specific
factors. These findings begin to elucidate the transcriptional mechanisms underlying stimulus responsive enhancers within the nervous system. These mechanisms will be important to not only understand how these elements function, but also to begin to understand how sequence variation at stimulus-responsive enhancers affects their function and may contribute to human variation and disease.
3.3 RESULTS

3.3.1 Sequence determinants of stimulus-responsive enhancers

To begin to understand the sequence determinants and transcription factors that regulate the function of stimulus-responsive enhancers, we performed de novo motif analysis on enhancers with different stimulus-responsive properties. Given that we had previously shown that reporter activity driven by enhancer elements appeared to be intrinsic to the sequence of these elements rather than genomic context or chromatin structure, we reasoned that de novo motif discovery might enable us to find important sequence determinants that drive distinct patterns of enhancer activity. De-novo motif analysis was performed using Multiple Em for Motif Elicitation (MEME) (Bailey and Elkan, 1994), beginning with neuronal enhancers that respond to membrane depolarization with elevated extracellular potassium chloride (KCl). Enhancers with increasing H3K27Ac in response to neuronal depolarization showed enrichment for motifs for the neuronal-activity regulated transcription factors AP-1, MEF2, and CREB (Figure 3.1a), suggesting that these transcription factors may drive neuronal activity-regulated transcriptional programs via enhancer mediated mechanisms. The AP1 transcription factor complex generally consists of dimers of FOS and JUN family transcription factors, many of which are canonical immediate early genes that are rapidly expressed in a protein synthesis-independent manner in response to extracellular stimuli in diverse cell types (Eferl and Wagner, 2003; Greenberg and Ziff, 1984; Sheng and Greenberg, 1990). MEF2 family transcription factors (MEF2A-D) are known to be activated via dephosphorylation in response to neuronal activity and to
play an important role in activity-dependent transcription in the nervous system (Barbosa et al., 2008; Flavell et al., 2006; Potthoff and Olson, 2007). The transcription factor CREB is known to be activated by phosphorylation in response to neuronal activity and to play an important role in activity-dependent transcription in the nervous system (Gonzalez and Montminy, 1989; Sheng et al., 1991). Notably, none of these activity-dependent transcription factor motifs were significantly enriched among enhancers with constant or decreasing levels of H3K27Ac, suggesting that the transcription factors that bind these motifs may function specifically at activity-responsive enhancers within the genome.

In addition to these activity-dependent transcription factor motifs, we also noted enrichment at activity-responsive enhancers for additional motifs for developmental transcription factors (Figure 3.1b). For example, we found that the X-box motif and the E-box motif were both significantly enriched among stimulus-responsive enhancers. These motifs are known to be bound by RFX and proneural bHLH transcription factors, respectively, both of which have been implicated in neuronal differentiation and development. RFX1 binding (as well as the X-box motif) has been shown to be enriched at active enhancers in cultured neural progenitor cells (Creyghton et al., 2010). Numerous bHLH transcription factors have also been shown to be expressed in neural progenitor cells (e.g. ASCL1, NGN1, NGN2) and differentiated neurons (e.g. NGN2, NEUROD, NHLH) and in many cases have been shown to play critical roles during nervous system development (Bertrand et al., 2002). Thus, neuronal stimulus-responsive enhancers may achieve tissue specificity through the action of RFX1 and proneural bHLH transcription factors at these enhancers, although further studies will
be required to understand how these and other transcription factors establish the active enhancer landscape in neurons.

**Figure 3.1: Motifs enriched among neuronal activity-responsive enhancers**

To understand the sequence determinants that contribute to the function of neuronal activity-responsive enhancers, we performed de novo motif analysis of the DNA sequences from all neuronal enhancers exhibiting increasing H3K27Ac levels in response to neuronal activity. De-novo motif analysis was performed using Multiple Em for Motif Elicitation (MEME) (Bailey and Elkan, 1994). The position weight matrices (PWM) for top neuronal activity-dependent transcription factor motifs (a) and the top developmental transcription factor motifs (b) identified by MEME are displayed, along with the E value reported by MEME for each de novo PWM. In addition, for each de novo PWM, the most similar PWM in the JASPAR or UniPROBE transcription factor motif databases, identified using TOMTOM (Gupta et al., 2007), is displayed.
Having characterized the transcription factor motifs and binding events enriched at neuronal activity-responsive enhancers, we next investigated the transcription factor motifs present at neuronal BDNF-responsive enhancers by performing de novo motif analysis on this set of enhancers. Surprisingly, the most enriched motif at BDNF-responsive enhancers was the same motif that was enriched at neuronal activity-responsive enhancers, the AP-1 motif (Figure 3.2b, top). Furthermore, the AP1 motif was also the most significantly enriched motif at neuronal enhancers exhibiting increasing H3K27Ac in response to neuronal activity but not BDNF (activity-specific enhancers; Figure 3.2a, bottom), increasing H3K27Ac in response to BDNF but not neuronal activity (BDNF-specific enhancers; Figure 3.2b, bottom), and increasing H3K27Ac in response to neuronal activity and BDNF (KCl and BDNF inducible enhancers; Figure 3.2c). This suggested that AP1 transcription factors may play a critical role in mediating transcriptional responses at enhancers to multiple stimuli.

**Figure 3.2: AP1 is the most significantly enriched motif at all classes of neuronal stimulus-responsive enhancers**

De novo motif analysis of the DNA sequences from neuronal enhancers exhibiting increasing H3K27Ac levels in response to neuronal activity (a), BDNF (b), or both (c). De-novo motif analysis was performed using Multiple Em for Motif Elicitation (MEME) (Bailey and Elkan, 1994). The position weight matrices (PWM) for top neuronal transcription factor motifs are displayed, along with the E value reported by MEME for each de novo PWM. In addition, for each de novo PWM, the most similar PWM in the JASPAR or UniPROBE transcription factor motif databases, identified using TOMTOM (Gupta et al., 2007), is displayed.

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3.3.2 Transcription factor binding to activity-responsive enhancers

Given the enrichment of activity-regulated transcription factor motifs at enhancers with increasing H3K27Ac in response to activity, we asked whether the transcription factors corresponding to these motifs would show greater binding at these enhancers. First, we performed ChIP-Seq for MEF2A and MEF2D before and after neuronal depolarization and found that these activity-dependent transcription factors were enriched at enhancers with increasing H3K27Ac in response to neuronal depolarization (Figure 3.3). We further integrated our H3K27Ac data with previously generated ChIP-Seq data for CREB, NPAS4, CBP, and RNAPII (Kim, 2010) in order to more thoroughly investigate the contribution of activity-regulated transcription factor binding to inducible H3K27ac at enhancers. Interestingly, all of these transcription factors also showed significantly more binding to enhancers with increasing H3K27Ac compared to other enhancers (Figure 3.3). Binding of the stimulus regulated transcription factor SRF was not enriched at enhancers with increasing H3K27ac, in line with the SRF motif not being significantly enriched these enhancers. Hence, many but not all activity-dependent transcription factors in the nervous system show not only enrichment of their motifs but also increased binding to activity-dependent enhancers in the genome. This suggests that several activity-dependent transcription factors collaborate at enhancers to regulate neuronal activity-dependent transcription, and that many such transcription factors may drive activity-dependent transcription via enhancer-mediated mechanisms.
Figure 3.3: Neuronal activity-responsive enhancers are enriched for binding by neuronal activity-dependent transcription factors

ChIP-Seq was performed (MEF2A) or analyzed (CREB, NPAS4, CBP, RNAPII) for the displayed transcription factors. Input normalized ChIP-Seq reads within an 800bp window surrounding each enhancer center were quantified at enhancers with increasing H3K27Ac or other enhancers, before and after membrane depolarization with KCl. Data is displayed in boxplots and * indicates $p < 2.2 \times 10^{-16}$ in paired Wilcoxon test.
Given that our genomic analyses suggested that neuronal activity-responsive enhancers are enriched for binding by neuronal activity-dependent transcription factors, we asked whether this enrichment could be seen at any individual loci of neuronal activity regulated genes. Two intragenic enhancers within the genomic locus for the activity-regulated neuronal gene \textit{Prkg2} reflect the preferential and activity-dependent binding of several activity-dependent transcription factors at enhancers with increasing but not constant H3K27Ac in response to membrane depolarization (Figure 3.4).

**Figure 3.4: Activity-dependent transcription factor binding at the \textit{Prkg2} locus**

Genome browser view of ChIP-Seq tracks for the indicated factors at the activity-regulated \textit{Prkg2} locus. ChIP-Seq tracks display input-normalized H3K27Ac ChIP-Seq reads from neurons before membrane depolarization (-KCl) or after membrane depolarization (+KCl). Also displayed are DNaseI hypersensitivity signal from adult mouse cerebrum and vertebrate conservation by PhastCons. Dashed boxes identify regulatory elements with different stimulus-responsive changes in H3K27Ac. Activity-dependent transcription factors (MEF2A, CREB, Cs, NPAS4) and general transcription factors (CBP, RNAPII) exhibit activity-dependent binding at the enhancer with increasing H3K27Ac in response to neuronal activity (‘Inc.’) but not at the enhancer with constant H3K27Ac in response to neuronal activity (‘Con.’).
3.3.3 AP1 is required for both neuronal activity-dependent and BDNF-dependent enhancer function

Given the enrichment of the AP1 motif at enhancers that respond to neuronal activity or BDNF and the lack of enrichment for the AP1 motif in active enhancers at which H3K27Ac did not change in response to either stimuli, we reasoned that the AP1 transcription factor complex may be critical for stimulus-responsive enhancers to promote transcription in response to extracellular stimuli. This would represent a novel mechanism for AP1 transcription factors in the nervous system, as AP1 has long been assumed to function at the promoters of neuronal activity-regulated genes to regulate transcription. First, we sought to characterize AP1 transcription factor binding to stimulus-responsive enhancers. Since FOS transcription factors have been shown to be an important component of the AP1 complex with important roles in the nervous system (Brown et al., 1996; Fleischmann et al., 2003; Jin et al., 2002; Zhang et al., 2002), we focused on members of this family of transcription factors. We first asked which Fos genes exhibited significant inducibility in response to extracellular stimuli, reasoning that inducible FOS transcription factors are likely to contribute to stimulus-dependent transcriptional control mediated by the AP-1 complex. Using RNA-Seq data we generated from mouse cortical neurons after 0, 1, or 6 hours of stimulation with KCl or BDNF, we characterized the expression of the genes of the four FOS family transcription factors: c-Fos, FosB, Fosl1, and Fosl2. We found that c-Fos and FosB were the most robustly induced Fos family members in response to stimuli, being similarly induced by KCl and BDNF (Figure 3.5). As a result, we chose to focus on these transcription factors.
RNA-Seq data from mouse cortical neurons stimulated with 0, 1, 6 hours KCl or 0, 1, 6 hours BDNF was analyzed to assess the levels of expression of genes in the Fos family of transcription factors. c-Fos and FosB (displayed) were the most inducible transcription factors and were found to be similarly induced in response to both KCl and BDNF. mRNA is expressed as reads per kilobase per million mapped reads (RPKM).

Since c-Fos and FosB were the most inducible AP1 transcription factors within the Fos family of transcription factors (by both KCl and BDNF stimulation), we next sought to characterize the binding of these transcription factors by ChIP-Seq in response to both KCl and BDNF stimulation. We first characterized binding in response to KCl. Using two independent C-FOS antibodies, we identified only 654 C-FOS peaks prior to KCl stimulation but identified 16,131 C-FOS peaks after KCl stimulation, consistent with the low baseline levels of C-FOS expression and the strong inducibility of C-FOS in response to neuronal activity. Furthermore, levels of C-FOS binding at C-FOS peaks exhibited strong inducibility with KCl stimulation (Figure 3.6a). In order to further confirm the specificity of these peaks, we performed an additional ChIP-Seq experiment for C-FOS and found that the majority of these peaks replicated in an independent experiment (79%). We also performed ChIP-Seq for C-FOS from neuronal
cultures expressing a lentiviral shRNA knockdown construct targeting c-Fos. C-FOS ChIP-Seq signal was substantially reduced by c-Fos RNAi throughout the genome (Figure 3.6b) and the majority of C-FOS peaks identified exhibited significantly reduced C-FOS signal after c-Fos knockdown (71%). Finally, we performed de novo motif analysis on the set of 16,131 peaks and found that the AP1 motif was the most strongly enriched motif within this set of loci. These data suggest that we were able to identify a high confidence set of C-FOS binding sites within the genome that were reproducible and specific.

**Figure 3.6: ChIP-Seq signal for C-FOS is inducible and specific in response to KCl stimulation**

(a) C-FOS ChIP-Seq was performed in mouse cortical neurons before membrane depolarization with KCl (-KCl) and after membrane depolarization with KCl (+KCl). Average profiles of input-normalized C-FOS ChIP-Seq signal before membrane depolarization (dashed lines) and after membrane depolarization (solid lines) are shown. (b) C-FOS ChIP-Seq was also performed after membrane depolarization with KCl in mouse cortical neurons that had been infected with lentivirus expressing either a control shRNA or an shRNA targeting c-Fos, in two independent experiments. Average profiles of C-FOS ChIP-Seq signal from control RNAi lentivirus infected neurons (dashed lines) and C-FOS RNAi lentivirus infected neurons (solid lines) are shown.
Having identified a high confidence set of C-FOS binding sites, we asked where C-FOS binding occurred within the genome. While we found the AP1 motif to be enriched at enhancers, AP1 factors have been long thought to act at promoters of target genes. An analysis of the genomic location of our rigorously defined set of C-FOS binding sites indicated that C-FOS binding was observed predominantly at gene distal sites across the genome, with the vast majority of peaks occurring greater than 1kb from an annotated TSS (82% of all replicating peaks, 94% of replicated peaks reduced by RNAi; Figure 3.7a). Analysis of a similarly verified set of high confidence FOSB peaks (n=8687; see methods) indicated that FOSB binding also occurred almost exclusively at sites greater than 1kb from an annotated TSS (96% of replicating peaks; Figure 3.7b). This strongly suggested that in our system, the AP1 transcription factors C-FOS and FOSB generally do not bind to promoters but rather bind to regions of the genome distant to promoters.

**Figure 3.7: Genomic distribution of C-FOS and FOSB peaks after stimulation with KCl**

ChIP-Seq for C-FOS and FOSB were performed before and after stimulation with KCl and peaks were called with MACS (Zhang et al., 2008). Peaks called after stimulation with KCl were then classified as proximal if they occurred within 1kb of an annotated RefSeq TSS, and as distal if they occurred outside of this window. The majority of C-FOS (a) and FOSB (b) peaks occur distal to TSSs.
Since the AP1 motif was enriched at stimulus-responsive enhancers, we hypothesized that C-FOS and FOSB binding to gene distal regions may be occurring at enhancers. Characterization of the chromatin modifications and transcription factor binding at distal C-FOS peaks demonstrated enrichment for enhancer associated H3K4Me1 (Figure 3.8a), H3K27Ac (Figure 3.8b), and CBP (Figure 3.8c), suggesting that C-FOS and binds to enhancers. Thus, in our system, FOS transcription factors appear to bind to enhancers rather than promoters in response to neuronal activity.

**Figure 3.8: Distal C-FOS peaks induced by KCl exhibit features of enhancers**

Chromatin modifications and transcription factor binding was assessed at distal C-FOS peaks within the genome before membrane depolarization (-KCl, dashed lines) and after membrane depolarization (+KCl, solid lines). (a) C-FOS peaks exhibit enrichment for the enhancer associated chromatin mark H3K4Me1 but not the promoter associated chromatin mark H3K4Me3. (b) C-FOS peaks exhibit enrichment for the active enhancer associated chromatin mark H3K27Ac, and H3K27Ac increases at C-FOS peaks with KCl stimulation. (c) C-FOS peaks exhibit enrichment for the transcriptional coactivator CBP with KCl stimulation. The y-axis for all plots represents the level of ChIP-Seq signal for the indicated factor, displayed as the mean number of input-normalized H3K27Ac ChIP-Seq reads at C-FOS peaks within the genome.
Given the enrichment of the AP1 motif among not only activity-responsive enhancers but also BDNF-responsive enhancers, and given our ability to detect bona fide C-FOS and FOSB binding by ChIP-Seq, we sought to characterize the binding of each of these factors in response to BDNF. We hypothesized that C-FOS and FOSB binding in response to BDNF would also occur at enhancers within the neuronal genome. We performed ChIP-Seq for each factor in response to BDNF. In BDNF stimulated neurons, we identified 15057 C-FOS peaks (93% gene distal; Figure 3.9a) and 13362 FOSB peaks (96% gene distal; Figure 3.9b), indicating that FOS transcription factors function primarily at gene distal regulatory elements in response to both neuronal activity and BDNF. These data strongly suggest that the current model of FOS function through promoters needs to be revisited.

Figure 3.9: Genomic distribution of C-FOS and FOSB peaks after stimulation with BDNF

ChIP-Seq for C-FOS and FOSB were performed before and after stimulation with BDNF and peaks were called with MACS (Zhang et al., 2008). Peaks called after stimulation with BDNF were then classified as proximal if they occurred within 1kb of an annotated RefSeq TSS, and as distal if they occurred outside of this window. The majority of C-FOS (a) and FOSB (b) peaks occur distal to TSSs.
Having shown that FOS transcription factors bind predominantly to gene distal regions within the genome likely to be enhancers, we next determined the extent to which FOS transcription factors bound to neuronal stimulus-responsive enhancers. We assessed C-FOS binding at neuronal stimulus-responsive enhancers. We found significant enrichment for C-FOS binding at KCl and BDNF responsive enhancers (Figure 3.10). Furthermore, we found that a significant percentage of stimulus-responsive enhancers overlapped with C-FOS or FOSB peaks (39% of activity-responsive enhancers, 61% of BDNF-responsive enhancers). These large percentages further suggest that FOS transcription factors likely contribute significantly to the function of both activity-responsive and BDNF-responsive enhancers.

**Figure 3.10: C-FOS binding at stimulus-responsive enhancers**

(a) Heatmap of H3K27Ac and C-FOS binding before and after KCl stimulation at KCl responsive enhancers, sorted by magnitude of H3K27Ac after KCl stimulation. (b) Average profile of input-normalized C-FOS ChIP-Seq signal before membrane depolarization (dashed lines) and after membrane depolarization (solid lines). (c) Heatmap of H3K27Ac and C-FOS binding before and after BDNF stimulation at BDNF responsive enhancers, sorted by magnitude of H3K27Ac after BDNF stimulation. (d) Average profile of input-normalized C-FOS ChIP-Seq signal before BDNF stimulation (dashed lines) and after BDNF stimulation (solid lines).
Importantly, a large percentage of stimulus-responsive enhancers were not bound by FOS transcription actors, suggesting that FOS-independent mechanisms may exist to activate enhancers in response to stimuli. This is to be expected since enhancers that are needed to express Fos transcription factors in response to stimuli are unlikely to require FOS for this response. There are likely distinct enhancers that function to activate the distinct waves of transcription that have been shown to occur in response to extracellular stimulation.

Interestingly, we found that a significant percentage of C-FOS and FOSB peaks in the genome did not occur at stimulus-responsive enhancers or at regions with any appreciable H3K27Ac. We asked whether these FOS binding sites functioned to regulate stimulus responsive transcription. We cloned several such loci into reporter constructs and found that none of these loci were able to drive transcription in an activity-dependent manner. This suggested that only a subset of all C-FOS and FOSB binding sites within the genome might contribute to stimulus-responsive transcription.

Since the AP1 transcription factors C-FOS and FOSB bind to stimulus-responsive enhancers within the genome, we hypothesized that AP1 may play a critical role in the function of these enhancers. To directly assess whether AP1 contributes to neuronal stimulus-inducible enhancer activity, we asked whether binding of the AP1 complex is necessary for the function of these enhancers. To accomplish this, we tested the function of Nptx2 reporter plasmids containing eight separate stimulus-inducible enhancers with canonical AP1 motifs that also exhibited high levels of binding by AP1 components (measured by ChIP-Seq). We disrupted the function of AP1 at these functional enhancers by either subtly mutating the AP1 motifs to block AP-1 binding or
by co-transfecting the dominant negative AP1 protein A-FOS (Ahn et al., 1998; Olive et al., 1997) with the Nptx2 reporter. We found that both manipulations significantly reduced activity-dependent and BDNF-dependent expression of each reporter tested, often down to levels exhibited by the reporter in the absence of any enhancer, suggesting that in some cases the AP1 motif is strictly required to achieve any enhancer activity.

**Figure 3.11: AP1 is critical for the function of KCl and BDNF responsive enhancers**

The impact of AP1 motif mutations (a, c) and expression of the AP1 dominant negative A-Fos (b, d) on the function of eight enhancers containing AP1 sites and exhibiting binding by FOS transcription factors were assessed. Luciferase reporter data of each enhancer within the Nptx2 reporter is shown, reported as the fold induction of reporter activity in mouse cortical neurons after 6 hours of stimulation with KCl (a, b) or BDNF (c, d) relative to 0 hours. n=3 in panels a and c. n=1 in panels b and d.

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Importantly, not all enhancers exhibiting inducible H3K27Ac in response to extracellular stimuli were sensitive to AP1 manipulations. Several enhancers with increasing H3K27Ac and robust reporter expression in response to neuronal activity were not affected by the AP1 dominant negative A-Fos in reporter assays (Figure 3.12). This not only provided evidence of the specificity of the function of the A-FOS dominant negative protein, but also suggested that AP1 independent mechanisms likely exist to enable enhancers to respond to neuronal activity. This is consistent with our earlier observations that not all enhancers with increasing H3K27Ac were bound by FOS transcription factors.

**Figure 3.12: AP1 is not required at all KCl responsive enhancers**

The expression of the AP1 dominant negative A-Fos has no impact on the function of seven enhancers that exhibit increasing H3K27Ac in response to neuronal activity and display responsiveness to KCl when cloned into the Nptx2 luciferase reporter. Luciferase reporter data of each enhancer within the Nptx2 reporter is shown, reported as the fold induction of reporter activity in mouse cortical neurons after 6 hours of stimulation with KCl relative to 0 hours. n=3 for all plasmids tested.
These data reveal a critical requirement for AP1 transcription factors at a subset of stimulus-responsive enhancers. Furthermore, these data suggest that AP1 plays a critical role in the function of enhancers with diverse stimulus-responsive properties, such as responsiveness to activity or BDNF. Given that AP1 transcription factors are expressed in a wide variety of cells in response to numerous extracellular stimuli, AP1 may play a critical role in the acquisition of stimulus-responsiveness of enhancers in multiple cellular contexts.

3.3.4 C-FOS regulates stimulus-responsive gene expression in the nervous system

C-FOS and other activity-regulated components of the AP1 complex bind to neuronal activity-dependent enhancers and abrogation of the binding of these components to enhancers through AP1 motif mutations leads to significant reductions in activity-dependent reporter induction. These data suggest that C-FOS may be important for neuronal activity-dependent enhancers. Since neuronal activity-dependent enhancers are associated with neuronal activity-dependent gene expression (Figure 2.7), we hypothesized that C-FOS may play a critical role in driving neuronal activity-dependent gene expression. To test this hypothesis directly, we performed genome-wide microarray analysis on neuronal RNA obtained from mouse cortical neurons that had been stimulated with 0, 1, 3, or 6 hours of KCl and infected with lentivirus expressing either a control RNAi or c-Fos RNAi. Western blot analysis of protein lysates obtained from simultaneously infected and stimulated cortical neurons
demonstrated that c-Fos RNAi was effective and specific. At each stimulus timepoint tested, c-Fos RNAi significantly reduced levels of C-FOS protein without affecting levels of the related FOSB protein, levels of activity-dependent posttranslational modifications, or cell viability as assessed by levels of β-actin (Figure 3.13).

Figure 3.13: c-Fos RNAi effectively and specifically reduces levels of C-FOS protein

Western blot analysis of protein lysates obtained from mouse cortical neurons that had been stimulated with 0, 1, 3, or 6 hours of KCl and infected with lentivirus expressing either a control RNAi or c-Fos RNAi. At each stimulus timepoint tested, c-Fos RNAi significantly reduced levels of C-FOS protein without affecting levels of the related FOSB protein, levels of activity-dependent posttranslational modifications, or cell viability as assessed by levels of β-actin.
Since C-FOS protein levels appear greatest after 3 hours of stimulation among the
timepoints tested in this experiment (Figure 3.13), we hypothesized that the greatest
effects on activity-dependent gene expression would be seen after 3 or 6 hours of KCl
stimulation. Analysis of the 1, 3, and 6 hour fold inductions of all expressed genes
revealed, with the greatest misregulation seen after 6 hours of KCl stimulation (Figure
6B, an increasing number of genes that were less inducible with c-Fos RNAi compared
to control RNAi red).

![Graphs showing fold induction of gene expression](image)

**Figure 3.14: Genome-wide microarray analysis with c-Fos RNAi**

Genome-wide microarray analysis was performed on neuronal RNA obtained from
mouse cortical neurons that had been stimulated with 0, 1, 3, or 6 hours of KCl and
infected with lentivirus expressing either a control RNAi or c-Fos RNAi. Scatterplots
show fold induction of gene expression at 1, 3, or 6 hrs after KCl stimulation relative to 0
hrs (unstimulated neurons) for all expressed genes (n=17767). In order to be considered
expressed, a gene must have been detected by a probe in both microarray bioreplicates
at at least one of the timepoints assessed and must have achieved a minimal expression
value of 250. The x-axis displays the fold induction exhibited in neurons infected with
control RNAi. The y-axis displays the fold induction exhibited in neurons infected with
c-Fos RNAi. The diagonal black line represents unity (y=x). The vertical red line
represents a three-fold cutoff for fold induction with control RNAi. Red points
represent genes induced at least three fold with control RNAi and exhibited a reduced
fold induction by at least 1.75 fold with c-Fos RNAi. An increasing number of genes
were less inducible with c-Fos RNAi compared to control RNAi with increasing time
after KCl stimulation (red numbers).
Investigation of genes misregulated with c-Fos RNAi revealed several genes with known functions in the nervous system including the postsynaptic scaffolding protein *Grasp* that has been shown to regulate metabotropic glutamate receptors (Kitano et al., 2002), the secreted protein *Nptx2* that has been shown to regulate AMPA receptor clustering (O’Brien et al., 2002; O’Brien et al., 1999) and homeostatic scaling (Chang et al., 2010), the hormone *Igf1* that has been shown to be important for brain development (Beck et al., 1995; Fernandez and Torres-Aleman, 2012), and the chromatin regulator *Hdac9* that has been shown to be highly expressed within the nervous system (Zhou et al., 2001) and regulate dendrite development (Sugo et al., 2010) (Figure 3.15a). While these and other important neuronal genes were misregulated with c-Fos RNAi, we sought to determine which genes may be direct targets of C-FOS within neurons. We hypothesized that direct targets may be identifiable not only on the basis of misexpression with c-Fos RNAi but also on the basis of a nearby activity-dependent enhancer bound by C-FOS. To investigate this possibility, we integrated our microarray data with our ChIP-Seq data and assessed the genomic regions surrounding these putative C-FOS target genes. We found that each of these genes was near an activity-regulated enhancer bound by C-FOS and other activity-dependent AP1 transcription factors (Figure 3.15b). This suggested that these genes may indeed be direct transcriptional targets of C-FOS. The identification of high confidence C-FOS target genes using integrated analysis of gene expression and ChIP-Seq data enables us to begin to understand the effector genes regulated by immediate early genes in the nervous system and how immediate early genes may mediate adaptive changes in the nervous system in response to neuronal activity.
Figure 3.15: High confidence C-FOS target genes with important functions in the nervous system

(a) Lineplots of fold induction of *Grasp*, *Nptx2*, *Igf1*, and *Hdac9* after 1, 3, or 6 hours of KCl stimulation (relative to 0 hrs) in neurons that had been infected with lentivirus expressing control RNAi (black line) or c-Fos RNAi (red line). Data is plotted as the average of two microarray bioreplicates +/- SEM. (b) Genome browser views of ChIP-Seq data at *Grasp*, *Nptx2*, *Igf1*, and *Hdac9* loci. All four of these genes are near enhancers that display increasing H3K27Ac in response to KCl stimulation and are bound by C-FOS and other inducible AP1 transcription factors. For all chromatin marks and transcription factors, ChIP-Seq tracks display input-normalized reads from neurons stimulated with 0 hours (‘-’) or two hours (‘+’) of KCl.
3.3.5 Functional dissection of stimulus responses of the Nptx2 enhancer

While our data reveal that AP1 is required for enhancer responses to neuronal activity and BDNF, it is not clear if AP1 is sufficient to generate these responses or if other transcription factors are also required. We sought to understand this issue by dissecting the function of a neuronal stimulus-responsive enhancer. We hypothesized that an enhancer that responded to both neuronal activity and BDNF might require AP1 to respond to each stimulus and might require additional stimulus-specific transcription factors to induce H3K27Ac at enhancers that respond to only one stimulus. We further hypothesized that mechanisms that enable an enhancer to respond to neuronal activity might be distinct from the mechanisms that enable an enhancer to respond to BDNF. To test these hypotheses, we chose to study the Nptx2 enhancer, which served as the basis for our luciferase reporter construct. The Nptx2 gene is robustly expressed in cortical neurons in response to both neuronal activity and BDNF, with highest expression at 6hrs, and greater expression in response to BDNF stimulation than KCl stimulation (Figure 3.16a). The enhancer ~3kb upstream of the Nptx2 gene exhibits inducible H3K27Ac in response to neuronal activity and BDNF (Figure 3.16b) and is required for the response of the Nptx2 reporter to both stimuli in a reporter assay (Figure 3.11a,c). The Nptx2 enhancer also exhibits robust binding of the AP1 transcription factors C-FOS and FOSB in response to each stimulus (Figure 3.16b and data not shown). All of these data suggest that the Nptx2 enhancer may be useful for determining how neuronal stimulus-responsive enhancers function to contribute to stimulus-responsive transcription in the nervous system.
Figure 3.16: Characterization of Nptx2 expression and Nptx2 locus

(a) RNA-Seq data from mouse cortical neurons stimulated with 0, 1, 6 hours KCl or 0, 1, 6 hours BDNF was analyzed to assess the levels of expression of Nptx2. Nptx2 is induced in response to both KCl and BDNF stimulation but is more induced in response to BDNF. mRNA is expressed as reads per kilobase per million mapped reads (RPKM).

(b) Genome browser view of ChIP-Seq tracks for the indicated factors at the stimulus-regulated Nptx2 locus. ChIP-Seq tracks display input-normalized ChIP-Seq reads from neurons before membrane depolarization (-KCl), after two hours of membrane depolarization (+KCl), before BDNF stimulation (-BDNF), or after two hours of BDNF stimulation (+BDNF). Also displayed are DNaseI hypersensitivity signal from adult mouse cerebrum and vertebrate conservation by PhastCons. Dashed boxes identify nearby enhancer elements.
We utilized the *Nptx2* reporter to dissect the function of the *Nptx2* enhancer. We first sought to identify the core element within the enhancer that was sufficient to generate transcriptional responses to neuronal activity and BDNF. To do this, we first generated truncation mutants of the *Nptx2* enhancer and measured the ability of each mutant to respond to neuronal activity and BDNF in reporter assays. We found that a core 180bp region retained the full activity of the full-length enhancer, suggesting that the sequence determinants that give rise to neuronal activity-dependent and BDNF-dependent responses from this enhancer reside within this region of the enhancer (Figure 3.17).

**Figure 3.17: Identification of the minimal sequence within the *Nptx2* upstream enhancer that drives responses to both KCl and BDNF**

Luciferase reporter data for truncation mutants of the *Nptx2* upstream enhancer (NUe) cloned into the *Nptx2* reporter, reported as the fold induction of reporter activity in mouse cortical neurons after 6 hours of KCl or BDNF stimulation relative to 0 hours. Error bars represent standard error of the mean (S.E.M.). \( n \geq 3 \) for all enhancers tested.
To discover motifs in an unbiased fashion that contribute to the function of the Nptx2 enhancer, we comprehensively scrambled the sequence of a minimized version of the enhancer and measured the ability of mutant enhancer sequences to respond to neuronal activity and BDNF in reporter assays. We scrambled approximately 7-10 base pairs at a time by converting all adenine nucleotides to cytosine nucleotides (A→C), all cytosine nucleotides to adenine nucleotides (C→A), all thymine nucleotides to guanine nucleotides (T→G), and all guanine nucleotides to thymine nucleotides (G→T). We found several regions within the enhancer whose mutation reduced reporter expression by more than 50% compared to the wild type sequence (Figure 3.18). Some stretches of sequence appeared to be important for the response of the Nptx2 enhancer to both KCl and BDNF, while other sequences appeared to be important for the response to either KCl or BDNF. Closer inspection of the critical sequences revealed three AP1 motifs within the enhancer that appeared to be critical for the responses to both KCl and BDNF (Figure 3.18). This suggested that AP1 might be important for generating responses to both KCl and BDNF at the Nptx2 enhancer, consistent with our earlier findings about AP1.
Figure 3.18: Comprehensive scrambling of the *Nptx2* enhancer reveals sequences important for responsiveness to KCl and BDNF

A minimized version of the *Nptx2* enhancer (schematic, top) was scrambled 7-10 base pairs at a time and the ability of each mutant to drive luciferase reporter expression in response to KCl (top bar graph) and BDNF (bottom bar graph) was assessed. Sequences whose mutation reduced reporter expression by more than 50% are highlighted in red for KCl and blue for BDNF. Luciferase activity is reported as the fold induction of reporter activity in mouse cortical neurons after 6 hours of KCl or BDNF stimulation relative to 0 hours. Error bars represent standard error of the mean (S.E.M.). n ≥ 3 for all enhancers tested with KCl, n ≥ 3 for all enhancers tested with BDNF.
To determine the functional contribution of each AP1 site while minimally impacting the sequence of the Nptx2 enhancer, we subtly mutated each AP1 motif within the full length Nptx2 enhancer, which included four AP1 motifs in total. A single base pair mutation of the second nucleotide of the AP1 motif from G to A has previously been shown to abrogate AP1 activity, and we independently confirmed that this subtle manipulation of AP1 sites phenocopied the effect of deletion of the entire AP1 site. Using AP1 single base pair point mutations, we found that both AP1 motifs that appeared to be critical for generating responses of the Nptx2 enhancer to KCl and BDNF in our scrambling experiments were critical to generate responses to neuronal activity and BDNF (AP1 #2,3; Figure 3.19a, b). The AP1 site not contained within the minimal enhancer region (AP1 #1) and the AP1 site that appeared to impact BDNF but not KCl responsiveness of the Nptx2 enhancer in our scrambling experiments (AP1 #4) did not appear to contribute significantly to the responsiveness of the Nptx2 enhancer to either KCl or BDNF. Mutation of individual AP1 motifs reduced reporter expression to different extents, and mutation of all four AP1 motifs within the Nptx2 enhancer eliminated nearly all reporter expression induced by both activity and BDNF. These data indicate that AP1 is required for responses of the Nptx2 enhancer to both neuronal activity and BDNF. Interestingly, while the AP1 motifs within the Nptx2 enhancer appear to contribute variably to the magnitude of the stimulus response, they do not appear to be used differentially in the context of different stimuli. This suggests that AP1 may function similarly in the context of different stimuli, and that other factors may collaborate with AP1 to generate responses to specific stimuli.
Figure 3.19: Functional contribution of individual AP1 sites to the responsiveness of the Nptx2 enhancer to KCl and BDNF

AP1 sites within the Nptx2 enhancer were subtly mutated (TGAGTCA→TAAGTCA) individually (AP#1, AP1#2, AP1#3, AP1#4) or all together (AP1#1-4) and the ability of each mutant to drive luciferase reporter expression in response to KCl (a) and BDNF (b) was assessed. Luciferase activity is reported as the fold induction of reporter activity in mouse cortical neurons after 6 hours of KCl or BDNF stimulation relative to 0 hours. Error bars represent standard error of the mean (S.E.M.). n ≥ 3 for all enhancers tested.
Having demonstrated that AP1 plays a critical role in the responsiveness of the Nptx2 enhancer to both activity and BDNF, we asked whether AP1 was sufficient to drive responses to these stimuli. To test this possibility, we mutated the entire sequence of a minimized version of the Nptx2 enhancer outside of the critical AP1 sites and tested whether the AP1 sites (retained with their native spacing and GC content) were sufficient to retain responsiveness to neuronal activity or BDNF. We found that scrambling the non-AP1 sequence of the enhancer eliminated responses to both activity and BDNF, suggesting that AP1 motifs are not sufficient to drive stimulus-responses of this enhancer and that additional sequences within the enhancer are required to generate stimulus responses.

To discover additional motifs that may contribute to the stimulus-responsive function of this enhancer, we searched the sequences identified in our scrambling experiment (Figure 3.18) for known transcription factor motifs other than AP1. Interestingly, we found that a sequence within the Nptx2 enhancer that appeared to be important for responses of this enhancer to KCl but not BDNF contained a “TCGTG” motif that has been shown to be the preferred motif for NPAS4 (Ooe et al, 2004 PMID: 14701734). ChIP-Seq data also revealed that the Nptx2 enhancer is bound by NPAS4 in response to neuronal activity (Figure 3.16b). NPAS4 is a basic helix loop helix PAS domain containing (bHLH-PAS) transcription factor that is expressed specifically in the nervous system in response to neuronal activity (Lin et al., 2008). Thus, we hypothesized that NPAS4 might also contribute to the ability of stimulus-responsive enhancers to respond to neuronal activity. To test this hypothesis, we abrogated the
function of NPAS4 at the Nptx2 enhancer by mutating the NPAS4 motif from the enhancer and by depleting NPAS4 levels by RNAi (Figure 3.20).

Figure 3.20: NPAS4 contributes to the KCl but not BDNF responsiveness of the Nptx2 enhancer

(a) The putative NPAS4 motif within the Nptx2 enhancer was deleted (NPAS4 del) and the ability of this mutant to drive luciferase reporter expression in response to KCl and BDNF was assessed. (b) The activity of the Nptx2 reporter in response to KCl and BDNF was assessed in neurons that had been transfected with scrambled shRNA or Npas4 shRNA. Luciferase activity is reported as the fold induction of reporter activity in mouse cortical neurons after 6 hours of KCl or BDNF stimulation relative to 0 hours. Error bars represent standard error of the mean (S.E.M.). n ≥ 3 for all enhancers tested.
We found that both manipulations significantly reduced the activity-dependent expression of the reporter, but neither manipulation significantly reduced the BDNF-dependent expression of the reporter. This suggested that the Nptx2 enhancer requires NPAS4 to respond to neuronal activity but not to respond to BDNF. Consistent with this possibility, data from a previous study shows that Nptx2 is less induced by neuronal activity after Npas4 RNAi (Lin et al., 2008). On the genome scale, analysis of the NPAS4 ChIP-Seq data indicated that Npas4 was bound preferentially to neuronal activity-dependent enhancers compared to other neuronal enhancers, providing further evidence that NPAS4 may be important for neuronal activity-dependent enhancer function.

These data together reveal that AP1 family transcription factors and NPAS4 collaborate to promote stimulus responsive transcription through enhancers. AP1 is required for both activity-dependent and BDNF dependent responses while NPAS4 is required for only the activity-dependent response. These experiments begin to elucidate how stimulus-dependent and stimulus specific transcriptional responses are generated within the nervous system. Further experiments will be required to reveal the additional determinants of stimulus-responsiveness of neuronal enhancers.

### 3.3.6 NPAS4 and CBP collaborate with C-FOS to drive activity-dependent enhancers

Our data from the functional dissection of the Nptx2 enhancer revealed a critical role for NPAS4 is driving enhancer responses to neuronal activity. To evaluate whether
NPAS4 and other factors collaborated with C-FOS to drive neuronal activity-dependent enhancers throughout the genome, we assessed the binding of NPAS4 and other transcription factors to C-FOS bound loci throughout the genome, reasoning that significant binding of transcription factors with C-FOS may indicate a function with C-FOS. Using previously generated data from our system (Kim et al., 2010), we found significant binding for both NPAS4 and CBP at C-FOS peaks within the genome. This suggested that NPAS4 and CBP may collaborate with C-FOS to drive neuronal activity-dependent enhancers.

![Figure 3.21: ChIP-Seq signal for NPAS4 and CBP and C-FOS peaks](image)

**Figure 3.21: ChIP-Seq signal for NPAS4 and CBP and C-FOS peaks**

Previously generated ChIP-Seq data for CBP and NPAS4 (Kim et al., 2010) was analyzed to evaluate the binding of these factors at sites of C-FOS binding throughout the genome. Average profiles of input-normalized CBP (black) and NPAS4 (red) ChIP-Seq signal before KCl stimulation (dashed lines) and after KCl stimulation (solid lines) are shown. Here, sites of C-FOS binding are defined as regions of the genome that exhibited a called C-FOS peak in three or more out of four separate C-FOS ChIP-Seq bioreplicates that were reduced by 50% or more in two out of two c-Fos RNAi ChIP-Seq bioreplicates.
We hypothesized that if CBP and NPAS4 collaborated with C-FOS to drive neuronal activity-dependent enhancers, we may expect a higher percentage of loci bound by C-FOS/NPAS4/CBP to exhibit increasing H3K27Ac in response to neuronal activity than loci bound by C-FOS but not NPAS4 or CBP. To investigate this possibility, we evaluated the H3K27Ac behaviors of C-FOS peaks that were bound without NPAS4 and CBP or were bound with NPAS4 and CBP. We found that while only 5% of C-FOS peaks that were not bound by NPAS4 and CBP exhibited increasing H3K27Ac in response to neuronal activity, a striking 47% of C-FOS peaks that were bound by NPAS4 and CBP exhibited increasing H3K27Ac in response to neuronal activity. This suggested that sites within the genome that are bound by C-FOS/NPAS4/CBP are nearly 10 times more likely to function as neuronal activity-dependent enhancers than sites within the genome bound by C-FOS but not NPAS4 or CBP. This suggested that NPAS4 and CBP may collaborate with C-FOS to drive neuronal activity-dependent enhancers throughout the genome. Additional experiments will have to be done to understand the specific function of each of these factors in neuronal activity-dependent enhancer function.
The H3K27Ac behaviors of C-FOS peaks that were bound without NPAS4 and CBP (left panel) or were bound with NPAS4 and CBP (right panel) were investigated. While only 5% of C-FOS peaks that were not bound by NPAS4 and CBP exhibited increasing H3K27Ac in response to neuronal activity, a striking 47% of C-FOS peaks that were bound by NPAS4 and CBP exhibited increasing H3K27Ac in response to neuronal activity.

Figure 3.22: H3K27Ac behaviors at loci bound by different complements of transcription factors
3.4 DISCUSSION

We have recently discovered that stimulus-responsive enhancers can be identified on the basis of rapidly increasing H3K27Ac levels in response to stimulation. We employed this dynamic chromatin signature to identify stimulus-responsive enhancers within the nervous system that respond to neuronal activity and BDNF stimulation. Here, we characterized the transcriptional mechanisms underlying activity-responsive and BDNF-responsive neuronal enhancers. We find that the AP1 transcription factor complex plays an important role at neuronal enhancers that respond to neuronal activity, BDNF, or both. We show that the AP1 motif is the most significantly enriched motif at all subsets of neuronal stimulus-responsive enhancers, that the AP1 transcription factors C-FOS and FOSB bind to stimulus-responsive enhancers, and that AP1 is required for responses to both neuronal activity and BDNF stimulation at a subset of neuronal enhancers. By dissecting the function of the activity and BDNF responsive Nptx2 enhancer, we confirm the necessity of the AP1 transcription factor in coordinating responses to activity and BDNF and also show that AP1 is not sufficient to generate responses to these stimuli. AP1 must coordinate with other stimulus-specific transcription factors to generate stimulus-responses at enhancers. In the case of the Nptx2 enhancer, AP1 coordinates with the activity-specific transcription factor NPAS4 to generate responses to neuronal activity. These results begin to elucidate the transcriptional mechanisms underlying stimulus-responsive enhancers within the nervous system.
3.4.1 Multiple transcriptional mechanisms underlying stimulus responsive enhancers

While AP1 transcription factors play a critical role at a subset of neuronal enhancers, transcriptional mechanisms independent of AP1 also exist to drive stimulus responses at enhancers. We show that not all stimulus responsive enhancers are bound by FOS transcription factors and not all stimulus responsive enhancers are affected by the expression of a potent AP1 dominant negative transcription factor. Hence, other mechanism likely exists at these enhancers. This is consistent with the observation that multiple waves of transcription occur in response to extracellular stimuli. Extracellular stimuli rapidly activate pre-existing transcription factors (such as CREB, MEF2, and SRF) within the cell that drive a first wave of transcription. Many genes expressed in this first wave of transcription encode transcription factors (such as FOS, JUN, and NPAS4) that, once expressed, activate a second wave of transcription in response to extracellular stimuli. It is possible that the assessment of H3K27Ac 2 hours after stimulation with KCl or BDNF best reveals active enhancers mediating the second wave of transcription. This may be why AP1 appears to play a dominant role within the enhancers characterized in this study. Assessment of inducible H3K27Ac at earlier time points after stimulation may better enable the understanding of AP1-independent mechanisms at stimulus-responsive enhancers.
3.4.2 Role of AP1 at neuronal stimulus-responsive enhancers

While we find AP1 to play a critical role at neuronal stimulus-responsive enhancers, this is in stark contrast to historical studies of AP1. Since enhancers have been difficult to identify until recently, historically much of the work on AP1 has focused on promoter elements that contain AP1 motifs. The presence of AP1 motifs within promoter regions, the importance of these motifs in reporter assays, and the ability of AP1 transcription factors to bind these motifs in gel-shift assays was all taken as evidence that AP1 functioned predominantly at promoters of target genes to regulate transcription. However, the studies that proposed this mechanism of function, being done long ago, were not able to demonstrate binding of AP1 transcription factors to promoters in neurons in vivo. Here, we present for the first time ChIP-Seq data from mouse cortical neurons that clearly demonstrates that the vast majority of C-FOS and FOSB binding does not occur at promoter regions. Furthermore, we find no evidence of C-FOS or FOSB binding at the promoters of reported FOS target genes in the nervous system. For example, the first reported target gene for FOS in the nervous system was proenkephalin (Sonnenberg et al., 1989c). The study that reported this target gene suggested that FOS bound to AP1 motifs near the promoter to regulate expression of this gene. However, we find no evidence of C-FOS or FOSB binding at the promoter of this gene in any of our ChIP-Seq bioreplicates in response to neuronal activity or BDNF. While proenkephalin or other proposed target genes may be regulated by FOS transcription factors, our data suggests that the mechanisms of such regulation do not occur at promoters but likely occur at enhancers.
3.4.3 Genetic control of activity-regulated enhancers and human disease

We have found that the human versions of many of our identified mouse activity-regulated enhancers can drive reporter expression in response to depolarization when introduced into cultured mouse neurons (data not shown), which suggests that many of these regions might be functionally conserved between mouse and human. Thus, the ability to identify a refined set of functional stimulus responsive enhancers should permit an accurate identification of activity-regulated enhancers in human neurons. Instances in which function is not conserved could provide insights into how the transcriptional responses of human neurons to synaptic activity have evolved. Changes in the activity-regulated gene program during human evolution might allow for adaptations in synaptic plasticity and function that are thought to be critical for human specific cognitive abilities (Dolmetsch and Geschwind, 2011; Konopka and Geschwind, 2010). We have begun to elucidate how these elements are regulated by activity-dependent transcription factors in mouse and how various transcription factor motifs correlate with inducible acetylation. These data could be potentially utilized to better interpret the potential functional consequences of human single nucleotide polymorphisms (SNPs) in these regulatory regions (Maurano et al., 2012). Indeed, the majority of variants within the genome appear to be in noncoding regions. As a result, human variation at enhancer elements may contribute to different traits, common diseases, and rare diseases. Exome sequencing studies of autistic individuals and their unaffected relatives have largely failed to identify recurrent de novo coding mutations that can explain the disease burden, indicating that non-coding variation could potentially play an important role in the genetics of autism (Buxbaum et al., 2012).
Given the proposed importance of synaptic signaling in autism spectrum disorders (Ebert and Greenberg, 2013), the identification of the cis-regulatory control of human activity-regulated transcription could provide a critical resource for the further elucidation of the genetics of these disorders.
3.5 MATERIALS AND METHODS

3.5.1 Mouse cortical cultures

Embryonic day 16.5 (E16.5) C57BL/6 embryonic mouse cortices were dissected and then dissociated for 10 minutes in 1× Hank’s Balanced Salt Solution (HBSS) containing 20 mg/mL trypsin (Worthington Biochemicals) and 0.32 mg/mL L-cysteine (Sigma). Trypsin treatment was terminated by washing dissociated cells three times for two minutes each in dissociation medium consisting of 1× HBSS containing 10 mg/mL trypsin inhibitor (Sigma). Cells were then triturated using a flame-narrowed Pasteur pipette to fully dissociate cells. After dissociation, neurons in were kept on ice in dissociation medium until plating. Cell culture plates were pre-coated overnight with a solution containing 20 ug/mL poly-D-lysine (Sigma) and 4 ug/mL mouse laminin (Invitrogen) in water. Prior to plating neurons, cell culture plates were washed three times with sterile distilled water and washed once with Neurobasal Medium (Life Technologies). Neurons were grown in neuronal medium consisting of Neurobasal Medium containing B27 supplement (2%; Invitrogen), penicillin-streptomycin (50 g/ml penicillin, 50 U/mL streptomycin, Sigma) and glutamine (1 mM, Sigma). At the time of plating, cold neuronal medium was added to neurons in dissociation medium to dilute neurons to the desired concentration. Neurons were subsequently plated and placed in a cell culture incubator that maintained a temperature of 37 degrees C and a CO2 concentration of 5%. Two hours after plating neurons, medium was completely aspirated from cells and replaced with fresh warm neuronal medium. Neurons were grown in vitro until the seventh day in vitro (DIV7).
3.5.1.1 Mouse cortical cultures for ChIP-Seq experiments

For ChIP-Seq experiments, mouse cortical neurons were plated at an approximate density of $4 \times 10^7$ on 15-cm dishes. Neurons were plated in 30mL neuronal medium. Ten mL of the medium was replaced with 12ml fresh warm neuronal medium on DIV3 and DIV6.

3.5.1.2 Mouse cortical cultures for luciferase reporter assays

For luciferase reporter assays, mouse cortical neurons were plated at an approximate density of $3 \times 10^5$ per well on 24-well plates. Neurons were plated in 500 ul neuronal medium. On the DIV3, 100 uL fresh warm medium was added to neurons. On DIV5 neurons were transfected (see section on transfection). At the completion of transfection, conditioned medium containing 15% fresh neuronal medium was returned to neurons.

3.5.1.3 Mouse cortical cultures for microarray experiments

For microarray experiments, mouse cortical neurons were plated at an approximate density of $3 \times 10^5$ per well on 24-well plates. Neurons were plated in 500 ul neuronal medium. On the DIV5, neurons were infected for 6 hours with viral supernatant containing lentivirus expressing either a control RNAi or c-Fos RNAi. At the completion of infection, wells were washed three times with warmed neuronal medium, and then conditioned medium containing 15% fresh medium was returned to neurons. Four wells were used for each condition: one well was used to obtain protein lysates for western blot, three wells were used to obtain RNA for microarrays.
3.5.2 Stimulation with potassium chloride (KCl)

Prior to KCl depolarization, neurons were quieted with 1 µM tetrodotoxin (TTX, Fisher) and 100 µM DL-2-amino-5-phosphopentanoic acid (DL-AP5, Fisher). Neurons were subsequently stimulated by adding warmed KCl depolarization buffer (170 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES) directly to the neuronal culture, to final concentration of 31% in the neuronal culture medium within the culture plate or well.

3.5.2.1 KCl stimulation for ChIP-Seq experiments

For KCl depolarization of neurons for ChIP-Seq experiments, DIV 6 neurons were quieted overnight with 1 µM TTX and 100 µM DL-AP5. The next morning, neurons were left silenced (-KCl condition) or stimulated for 2 hours with KCl (+KCl condition).

3.5.2.2 KCl stimulation for luciferase reporter assays

For KCl depolarization of neurons for luciferase reporter assays, DIV 7 neurons were quieted for two hours with 1 µM TTX and 100 µM DL-AP5. Two hours later, neurons were left silenced (-KCl condition) or stimulated for 6 hours with KCl (+KCl condition).

3.5.2.3 KCl stimulation for microarray experiments

For KCl depolarization of neurons for microarray experiments, DIV 7 neurons were quieted for two hours with 1 µM TTX and 100 µM DL-AP5. Two hours later, neurons were left silenced (0 hour condition) or stimulated for 1, 3, or 6 hours with KCl.
3.5.3 Stimulation with brain derived neurotrophic factor (BDNF)

3.5.3.1 BDNF stimulation for ChIP-Seq experiments

For BDNF stimulation of neurons for ChIP-Seq experiments, the volume of conditioned medium on DIV6 neurons was reduced to 25 mL. On DIV7, neurons were left in a basal condition (-BDNF condition) or stimulated for 2 hours with 50ng/mL recombinant human BDNF (Fisher) (+BDNF condition).

3.5.3.2 BDNF stimulation for luciferase reporter assays

For BDNF stimulation of neurons for luciferase reporter assays, DIV7 neurons were left in a basal condition (-BDNF condition) or stimulated for 6 hours with 50ng/mL recombinant human BDNF (Fisher) (+BDNF condition).

3.5.4 Lentivirus infection

The pLLx3.7 backbone was used for generation of lentivirus as previously described (Lois et al., 2002; Rubinson et al., 2003).

3.5.5 H3K27Ac Chromatin immunoprecipitation sequencing (ChIP-Seq)

Forty million mouse cortical neurons cultured to in vitro day 7 were used for each ChIP-Seq library construction. Typically 20-40 million cortical neurons were used for a single ChIP experiment. To cross-link protein-DNA complexes, media was removed...
from neuronal cultures and crosslinking-buffer (0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 25 mM HEPES-KOH, pH 8.0) containing 1% formaldehyde was added for 10 minutes at room temperature. Cross-linking was quenched by adding 125 mM glycine for five minutes at RT. Cells were then rinsed three times in ice-cold PBS containing complete protease inhibitor cocktail tablets (Roche) and collected by scraping. Cells were pelleted and either stored at -80 degrees C until use or immediately processed. Cell pellets were lysed by 20 cell pellet volumes (CPVs) of buffer 1 (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, pH 8.0, 10 % Glycerol, 0.5 % NP-40, 0.25 % Triton X-100, complete protease inhibitor cocktail) for 10 min at 4 degrees C. Nuclei were then pelleted by centrifugation at 3000 rpm for 10 min at 4 degrees C. The isolated nuclei were rinsed with 20 CPVs of buffer 2 (200 mM NaCl, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0, 10 mM Tris-HCl, pH 8.0, complete protease inhibitor cocktail) for 10 min at RT and re-pelleted. Next, 4 CPVs of buffer 3 (1 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0, 10 mM Tris-HCl, pH 8.0, complete protease inhibitor cocktail) were added to the nuclei and sonication was carried out using a Misonix 3000 Sonicator (Misonix) set at a power setting of 7.5 (equivalent to 24 watts). 20 pulses of 15 seconds each were delivered at this setting, which resulted in genomic DNA fragments with sizes ranging from 200 bp to 2 kb. Insoluble materials were removed by centrifugation at 20,000 rpm for 10 min at 4 degrees C. The supernatant was transferred to a new tube and the final volume of the resulting nuclear lysate was adjusted to 1 mL by adding buffer 3 supplemented with 0.3 M NaCl, 1 % Triton X-100, 0.1 % Deoxycholate. The lysate was pre-cleared by adding 100 ul of pre-rinsed Protein A/G Agarose (Sigma) per 1 ml of the lysate and incubating for 1 hour at 4 degrees C. After pre-clearing, ten percent of the ChIP sample (50 ul from 500 ul lysate) was saved as input material. The
remaining lysate was incubated with 4 ug antibodies for immunoprecipitation. The antibodies used in this study are shown in Table 6:

**Table 6: Antibodies used for ChIP-Seq experiments**

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Antibody or antibodies used</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEF2A</td>
<td>Custom</td>
</tr>
<tr>
<td>MEF2D</td>
<td>Custom</td>
</tr>
<tr>
<td>C-FOS</td>
<td>sc52, sc7202 (Santa Cruz)</td>
</tr>
<tr>
<td>FOSB</td>
<td>sc7203 (Santa Cruz)</td>
</tr>
</tbody>
</table>

The antibody incubation was carried out overnight at 4 degrees C. The next day, 30 ul of pre-rinsed Protein A/G PLUS Agarose beads (Santa Cruz Biotechnology) was added to each ChIP reaction and further incubated for 1 hour at 4 degrees C. The beads bound by immune-complexes were pelleted and washed twice with each of the following buffers: low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl) and LiCl buffer (0.25M LiCl, 1% IGEPAL CA630, 1% deoxycholic acid (sodium salt), 1mM EDTA, 10mM Tris, pH 8.1). In each wash, the beads were incubated with wash buffer for 10 min at 4 degrees C while nutating. The washed beads were then rinsed once with 1x TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The immunoprecipitated material was eluted from the beads twice by adding 100 ul of elution buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 1 % SDS) to each ChIP reaction and incubating the sample at 65 degree C for 30 min with brief vortexing every 2 min. 150 ul of elution buffer was also added to the saved input material (50 ul) and this sample was processed together with the ChIP samples. The eluates were combined and crosslinking was reversed by incubation at 65 degrees C overnight. The next day, 7 ug RNase A (affinity purified, 1mg/mL; Invitrogen) was added to each
sample and samples were incubated for 37 degrees C for one hour. Then, 7 uL Proteinase K (RNA grade, 20mg/mL; Invitrogen) was added to each sample and samples were incubated at 55 degrees C for two hours. The immunoprecipitated genomic DNA fragments were then extracted once with Phenol:Chloroform:Isoamyl Alcohol (25:24:1, pH 7.9; Life Technologies) and then back extracted with water. The resulting genomic DNA fragments were then purified using the QIAquick PCR purification kit (Qiagen) and DNA fragments were eluted in 100 ul of Buffer EB (elution buffer consisting of 10 mM Tris-HCl, pH 8.5, Qiagen). Samples were assessed for enrichment by quantitative PCR using primers to different genomic regions. Samples with significant enrichment over negative regions were submitted to the Beijing Genomic Institute (BGI) for 50 base pair single end sequencing on the Illumina Hiseq 2000 platform. For each sample, over 20 million clean reads were obtained.

3.5.6 ChIP-Seq analysis

3.5.6.1 Initial processing

Sequencing data was obtained from BGI in gzipped fastq file format. Files were transferred and unzipped. Then, sequencing reads were aligned to the July 2007 assembly of the mouse genome (NCBI 37, mm9) using the Burrows-Wheeler Aligner (BWA) (Li and Durbin, 2009) with default settings. The resulting bwa files were then converted to sam files and uniquely mapped reads were extracted from the sam files. Sam files of the uniquely mapped reads were then converted to bam files. Bam files
were then used for peak calling using Model-based Analysis of ChIP-Seq (MACS) (Zhang et al., 2008) with the following parameters: -f BAM -g mm.

3.5.6.2 Visualizing ChIP-Seq data on the UCSC genome browser

ChIP-Seq bam files were converted to bigwig track format to display the number of input normalized ChIP-Seq reads, normalized to 20 million total reads.

3.5.6.3 Characterization of genomic distribution of FOS binding sites

C-FOS and FOSB peaks were classified based on their location relative to genes in the NCBI Reference Sequence Database (RefSeq). Peaks were classified as being proximal if they were within 1kb of an annotated transcriptional start site (TSS). H3K27Ac peaks were classified as being distal if they were greater than 1kb from an annotated transcriptional start site (TSS).

3.5.6.4 Quantification of ChIP-Seq signal at enhancers

For transcription factors (MEF2A, MEF2D, C-FOS, FOSB), the number of input-normalized ChIP-Seq reads within an 800 bp window centered on each enhancer was taken to be the ChIP-Seq signal at the enhancer.
3.5.7 Luciferase reporter assays

3.5.7.1 Nptx2 reporter plasmid design

All luciferase reporter plasmids used were newly developed for this study. Most luciferase reporter plasmids used were based on the Nptx2 gene, and hence this reporter was termed the Nptx2 reporter. To develop the Nptx2 reporter, we cloned the 4355 bp region upstream of the Nptx2 coding sequence from C57BL/6 purified mouse genomic DNA between the NheI and EcoRV restriction sites within the multiple cloning site of the promoterless pGL4.11 reporter plasmid (Promega) using the primers shown in Table 7, with 5' clamp shown in red, NheI and EcoRV sites shown in green:

Table 7: Primers used to clone the Nptx2 upstream regulatory region into pGL4.11

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>5' GCGCGCTAGCTTCCCTGGCITGATAGTGACCT 3'</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5' GCGCGATATCCCTGCTGACCTGTCGTCTCACTCA 3'</td>
</tr>
</tbody>
</table>

pGL4.11 was chosen as the host plasmid since it contained the luc2P reporter gene, which contains an hPEST protein destabilization sequence. We found that the luc2P reporter responded more quickly and with greater magnitude to stimuli than luc2 reporters. Using PCR driven overlap extension (Heckman and Pease, 2007), the Nptx2 reporter was then modified so that the 1216 bp Nptx2 upstream enhancer (located -3607 to -2391 relative to the start of the Nptx2 coding sequence) was replaced with a multiple cloning site containing SbfI, PacI, PmeI, and AscI restrictions sites. In order to do this, the primers shown in Table 8 were used (A, B, C, D nomenclature same as described in (Heckman and Pease, 2007)), with 5' clamp shown in red; NheI and EcoRV sites shown in green; SbfI, PacI, PmeI, and AscI sites shown in blue; and spacers shown in orange.
Table 8: Primers used to replace the Nptx2 upstream enhancer with a multiple cloning site

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5’ GCGCGCATAGCTGCCTCGCTGGCTGTAGTGACCT 3’</td>
</tr>
<tr>
<td>B</td>
<td>5’ GGCGCGCCAGTTAAGACGCGCTTAATTAGTGGCTGCAGCTGGTGTAGAAGCTACCTTGTTCCA 3’</td>
</tr>
<tr>
<td>C</td>
<td>5’ CCTCGAGGAAGCTTTAGGCTCAAGCTGTTCGGTGGCCAGCTAGTAACAGTTGGCATT 3’</td>
</tr>
<tr>
<td>D</td>
<td>5’ GCGCCATATGCTCGACCTGTGTGCTACCTTA 3’</td>
</tr>
</tbody>
</table>

The multiple cloning site was inserted into the Nptx2 upstream regulatory region to create a modified Nptx2 reporter so that various enhancers could be easily cloned into this multiple cloning site. We verified that the modified Nptx2 reporter in which the Nptx2 enhancer had been cloned into the multiple cloning site had the same inducibility as the wild-type Nptx2 reporter (data not shown). This suggested that the multiple cloning site did not affect the function of the reporter and that other enhancers could be similarly cloned into this multiple cloning site without adverse effects on enhancer function.

3.5.7.2 Enhancer sequences for wild-type of mutated C-FOS bound enhancers

Activity-dependent enhancers containing AP1 sites, exhibiting reproducible and specific binding by the AP1 transcription factor C-FOS, were cloned between the SbfI and AscI sites within the multiple cloning site of the modified Nptx2 reporter. For most enhancers, we observed that the trough between H3K27Ac peak shoulders as well as DNaseI hypersensitivity typically could be localized to a region less than 500bp. As a result, we synthesized approximately 500 bp genomic blocks for each enhancer of interest using gBlocks Gene Fragments (IDT). For each enhancer, we designed a wild-type version and a version of the enhancer in which the AP1 sites were mutated by one
base pair (TGAGTCA\textrightarrow TAAGTCA), which has been shown previously to abrogate AP1 binding (Risse et al., 1989). These gBlocks are shown in Table 9, with the clamp shown in bold, the SbfI site in green, and the AscI site in red. In the case where no gBlock is shown, the sequence was obtained via PCR.
<table>
<thead>
<tr>
<th>Table 9: gBlocks designed to create wild-type and AP1 mutant versions of 8 neuronal enhancers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enb</strong></td>
</tr>
<tr>
<td>e4</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>e61</td>
</tr>
<tr>
<td>e7</td>
</tr>
<tr>
<td>e8</td>
</tr>
<tr>
<td>e9</td>
</tr>
<tr>
<td>e10</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
Due to a low quantity of gBlock DNA provided, we also designed primers to PCR amplify the desired sequence from gBlock fragments. These primers are shown in Table 10, with the clamp shown in red, and the SbfI and AscI sites shown in blue.

**Table 10: PCR primers to amplify gBlock fragments**

<table>
<thead>
<tr>
<th>Enh</th>
<th>Forward primer (5’→3’)</th>
<th>Reverse primer (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>e4</td>
<td>GCGTTCCTGAGCGAGGAGAGGTGTC</td>
<td>GTAGGCGCCCTGAAGTAAGTGTTTT</td>
</tr>
<tr>
<td>e16</td>
<td>GCGTTCCTGAGGTTAGTGCTCCAGAGAAAG</td>
<td>GTAGGCGCCCTCTCCTAAAGGACCCAGCTCA</td>
</tr>
<tr>
<td>e17</td>
<td>GCGTTCCTGAGGGATACATCTTCTGGAATT</td>
<td>GTAGGCGCGCCGGAATTTCACAGTTTAAAC</td>
</tr>
<tr>
<td>e18</td>
<td>GCGTTCCTGAGGCATTATTTTTGGAAAATT</td>
<td>GTAGGCGCGCCCTCTCAGACTTGAG</td>
</tr>
<tr>
<td>e28</td>
<td>GCGTTCCTGAGGACCTTCTTCTCTCCCA</td>
<td>GTAGGCGCGGCCGACCTCTCCTCCTCCTC</td>
</tr>
<tr>
<td>e29</td>
<td>GCGTTCCTGAGGAGATGCTAAAAGCGTACAG</td>
<td>GTAGGCGCGCCATTAGTTTAAAGCCCATATT</td>
</tr>
<tr>
<td>e30</td>
<td>GCGTTCCTGAGGGCATTGCTACTAGGTGATAT</td>
<td>GTAGGCGCGCCCTGTAAGCAAAATTTCGGTTT</td>
</tr>
<tr>
<td>e31</td>
<td>GCGTTCCTGAGGAAACCCGTAGACAGCAGGAA</td>
<td>GTAGGCGCGCCCTGCTACTACTAAGCAAGAA</td>
</tr>
</tbody>
</table>

**3.5.7.3 Generation and use of the AP1 dominant negative A-Fos**

A plasmid containing N-terminal Flag-tagged A-Fos (Ahn et al., 1998; Olive et al., 1997) was obtained from Addgene (Plasmid #33353, Addgene). The A-Fos gene was PCR amplified and cloned into a custom designed pLenti-GB-hUb-IRES-EGFP lentiviral vector between the human Ubiquitin C promoter and the IRES sequence using AscI and EcoR1 sites. For use in a luciferase assay, the 500ng filler pCS2 DNA was replaced with 250ng pCS2, 125 ng empty pLenti-GB-hUb-IRES-EGFP vector, and 125ng A-Fos. This was compared to a condition transfected with 250ng pCS2, 250 ng empty pLenti-GB-hUb-IRES-EGFP vector.

**3.5.7.4 Nptx2 upstream enhancer truncations**

To identify the minimal enhancer element within the Nptx2 upstream enhancer capable of driving stimulus-responsive transcription, we first minimized the Nptx2 reporter to 560bp, removing the regions further outside the H3K27Ac trough and DNaseI hypersensitive site. In an attempt to truncate the Nptx2 enhancer further, we...
sequentially removed about 50bp at a time. We designed eight constructs to clone from the Nptx2 reporter, the primers for which are listed in Table 11.

Table 11: Primers to truncate the Nptx2 enhancer to identify the minimal sequence capable of driving stimulus-responsive transcription

<table>
<thead>
<tr>
<th>Forward primer (5’ -&gt; 3’)</th>
<th>Reverse primer (5’ -&gt; 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NUe560bp</td>
<td>GCGTCTTCGTGCAGGAAATTCAGCACCGAGGAT</td>
</tr>
<tr>
<td>mNUe1del1</td>
<td>geatgtCTGCGACGGCTCTTGAAATTCAGCACGAGGAT</td>
</tr>
<tr>
<td>mNUe1del2</td>
<td>geatgtCTGCGACGGCTCTTGAAATTCAGCACGAGGAT</td>
</tr>
<tr>
<td>mNUe1del3</td>
<td>geatgtCTGCGACGGCTCTTGAAATTCAGCACGAGGAT</td>
</tr>
<tr>
<td>mNUe1del4</td>
<td>geatgtCTGCGACGGCTCTTGAAATTCAGCACGAGGAT</td>
</tr>
<tr>
<td>mNUe1del5</td>
<td>geatgtCTGCGACGGCTCTTGAAATTCAGCACGAGGAT</td>
</tr>
<tr>
<td>mNUe1del6</td>
<td>geatgtCTGCGACGGCTCTTGAAATTCAGCACGAGGAT</td>
</tr>
<tr>
<td>mNUe1del7</td>
<td>geatgtCTGCGACGGCTCTTGAAATTCAGCACGAGGAT</td>
</tr>
<tr>
<td>mNUe1del8</td>
<td>geatgtCTGCGACGGCTCTTGAAATTCAGCACGAGGAT</td>
</tr>
<tr>
<td>NUe180bp</td>
<td>geatgtCTGCGACGGCTCTTGAAATTCAGCACGAGGAT</td>
</tr>
</tbody>
</table>

3.5.7.5 Nptx2 reporter scrambling

To identify the crucial sequences within the minimized Nptx2 enhancer that contributed to the responsiveness of this enhancer to KCl and BDNF stimulation, we sequentially scrambled ~7-10 bases at a time from an already minimized reporter. As before, we designed genomic blocks (gBlocks Gene Fragments, IDT) containing the scrambled mutations that we used as PCR templates. gBlocks were PCR amplified using a common forward primer (5’ ttctggtgtgacgagct attacg 3’) and a common reverse primer (5’ agcaacgttcggcttttcg 3’). The gBlocks are shown in Table 12.
## Table 12: gBlock sequences synthesized to comprehensively scramble the minimal Nptx2 enhancer

<table>
<thead>
<tr>
<th>Name</th>
<th>primer/gblock</th>
</tr>
</thead>
<tbody>
<tr>
<td>5_mNUeSC RAtoCG9-10</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
</tr>
<tr>
<td>6_mNUeSC RAtoCG9</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
</tr>
<tr>
<td>7_mNUeSC RAtoCG10-10</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
</tr>
<tr>
<td>8_mNUeSC RAtoCG20-20</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
</tr>
<tr>
<td>9_mNUeSC RAtoCG30-30</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
</tr>
<tr>
<td>10_mNUeSC RAtoCG40-40</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
</tr>
<tr>
<td>11_mNUeSC RAtoCG50-50</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
</tr>
<tr>
<td>12_mNUeSC RAtoCG60-60</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
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<tr>
<td>13_mNUeSC RAtoCG69-80</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
</tr>
<tr>
<td>14_mNUeSC RAtoCG81-82</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
</tr>
<tr>
<td>15_mNUeSC RAtoCG90-97</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
</tr>
<tr>
<td>17_mNUeSC RAtoCG10-10</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
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<tr>
<td>18_mNUeSC RAtoCG18-18</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
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<tr>
<td>19_mNUeSC RAtoCG28-28</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
</tr>
<tr>
<td>20_mNUeSC RAtoCG38-38</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
</tr>
<tr>
<td>21_mNUeSC RAtoCG48-48</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
</tr>
<tr>
<td>22_mNUeSC RAtoCG58-58</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
</tr>
<tr>
<td>23_mNUeSC RAtoCG68-68</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
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<tr>
<td>24_mNUeSC RAtoCG78-78</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
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<tr>
<td>25_mNUeSC RAtoCG88-88</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
</tr>
<tr>
<td>26_mNUeSC RAtoCG98-98</td>
<td>tctggtgtagactgGCTTGCAGGTTAATCACGGGATGATGATGGTATATATACTATGCTGTTGCAATTTCTCAAGTAGCAGAAAAATCTGTGGGCCCTGGGCTCTGTCACTCGCACAGCCCTTCTCATGGGTAGGGTGAGTCATGCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA</td>
</tr>
</tbody>
</table>

GCGTGGCAGTGCGTTTGCTTGATACCGAGTCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA

GCGTGGCAGTGCGTTTGCTTGATACCGAGTCACGCAGGAGTCATGCACGCAGGATCTTTCAGGTGGACGGTTTCTGTGCTGTGAGGAAGAA
3.5.7.6 Mutating AP1 motifs within the Nptx2 enhancer

We observed four AP1 motifs within the full-length 1216bp Nptx2 enhancer. Using PCR driven overlap extension (Heckman and Pease, 2007), we sequentially mutated the second base pair of each AP1 motif with a mutation (TGAGTCA→TAAGTCA) that has been previously shown to abrogate the binding of AP1 (Risse et al., 1989). In order to do this, the primers shown in Table 13 were used (A, B, C, D nomenclature same as described in (Heckman and Pease, 2007)), with 5’ clamp shown in red.

Table 13: Primers to subtly mutate each AP1 motif present within the sequence of the Nptx2 enhancer

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5’GGTCCTGCAGCAGAAAACAAACAGCTGCT 3’</td>
</tr>
<tr>
<td>D</td>
<td>5’GTATGGCCCGCCCTACTAGCTAGTGGTGGG 3’</td>
</tr>
<tr>
<td>AP1 #1 B</td>
<td>CTTCCCTGCAAAACATGACTTTAGGAACATCCTCCGGTGGCTGA</td>
</tr>
<tr>
<td>AP1 #1 C</td>
<td>CGAGGATAGTCTCATTGAGGCTATGCTGCTGAGCTGCTCT</td>
</tr>
<tr>
<td>AP1 #2 B</td>
<td>GATCTGGTTATGACTTAACCTCCACAGAGGGCT</td>
</tr>
<tr>
<td>AP1 #2 C</td>
<td>CTCATGGGTAAGGTTAAGTCAAGCAGCAGGCAGCTTCAGG</td>
</tr>
<tr>
<td>AP1 #3 B</td>
<td>AGATCTGACAGGATGAACTACTGCACATTTCTTATCT</td>
</tr>
<tr>
<td>AP1 #3 C</td>
<td>GATAGGGGCTATGACTGACTCTGCTGAGATCTGACAGC</td>
</tr>
<tr>
<td>AP1 #4 B</td>
<td>CCAGCCCGACATAGTAAATGAGTGGGTTTACTATGCTGT</td>
</tr>
<tr>
<td>AP1 #4 C</td>
<td>AGTACCACCCACTATTTACTATGGCGCTGGTGCCCAGG</td>
</tr>
</tbody>
</table>

3.5.7.7 Mutating the NPAS4 motif within the Nptx2 reporter

Using PCR driven overlap extension (Heckman and Pease, 2007), we deleted a putative NPAS4 binding site located directly under the ChIP-Seq peak. In order to do this, the primers shown in Table 14 were used (A, B, C, D nomenclature same as described in (Heckman and Pease, 2007)), with 5’ clamp shown in red.
Table 14: Primers used to delete NPAS4 motif from Nptx2 enhancer

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5' GCGTCTGCAGGCAGAAAAACAAAGGCTTGCT 3'</td>
</tr>
<tr>
<td>B</td>
<td>5' TTTTCGTACCTTGAGAAAAGCAGATCTGAGCAGATGGAATC 3'</td>
</tr>
<tr>
<td>C</td>
<td>5' GATTCGCTTCAGATCTGCTTCTCAAGTACGACAGAAAA 3'</td>
</tr>
<tr>
<td>D</td>
<td>5' GTATGGCCGGCCCTACTAGCTAAGTGGTTGGG 3'</td>
</tr>
</tbody>
</table>

3.5.7.8 Transfection

Mouse cortical neurons plated on 24 well plates at a density of approximately 3x10^5 neurons per well were transfected for luciferase reporter assays using Lipofectamine 2000 Reagent (Invitrogen), generally according to the manufacturer’s protocol. Briefly, DNA mixes were made immediately preceding the transfection consisting of 1ug total plasmid DNA/well diluted in Neurobasal medium (Life Technologies). DNA typically consisted of 450ng firefly luciferase reporter DNA, 50ng pGL4.74 renilla luciferase reporter DNA (Promega), and 500ng empty pCS2 plasmid (Rupp et al., 1994; Turner and Weintraub, 1994) as filler DNA. Lipofectamine was used at 2uL/well and was diluted in Neurobasal medium just before the transfection. Within each experiment, all conditions were transfected in two to three independent wells, for technical duplicates or triplicates. Thirty minutes prior to the addition of Lipofectamine to neurons, the culture medium was removed and replaced with warmed Neurobasal medium. At this time, neurons were returned to the incubator and DNA mixes were added to diluted Lipofectamine in a drop-wise manner. After thirty minutes of incubation, DNA-Lipofectamine mixes were added to neurons, again in a drop-wise manner. The cells were left to incubate with the DNA-Lipofectamine mix for two hours, after which the transfection medium was replaced with supplemented conditioned neuronal medium.
3.5.7.9 Sample collection and luciferase assay

After stimulation, neurons were lysed using Passive Lysis Buffer (Dual-Luciferase Reporter Assay System, Promega). Lysates were then collected in microcentrifuge tubes and frozen at -20 degrees C. At the time of performing the luciferase assay, neuronal lysates were thawed, briefly vortexed, briefly spun down, and then 20µL of each sample was added to one well of Costar White Polystyrene 96-well Assay Plates (Corning). The reagents to run the luciferase assay, Luciferase Assay Reagent II (LARII) and Stop & Glo Reagent (Dual-Luciferase Reporter Assay System, Promega), were aliquoted and thawed according to the manufacturer's protocol. The luciferase assay was performed using the Synergy 4 Hybrid Microplate Reader (BioTek), with 100µL of LARII and Stop & Glo Reagent injected per well. Data was subsequently downloaded and analyzed using Microsoft Excel.

3.5.7.10 Statistical Analyses of Luciferase Assay Data

Using the Dual-Luciferase Reporter Assay System, we recorded Firefly (FF) and Renilla (Ren) luminescence from each well. To correct for variations in transfection efficiency and cell lysate generation, the Firefly values were normalized to Renilla luminescence within each well, generating a ratio of FF/Ren. The stimulus-dependent fold induction of each reporter plasmid was obtained by dividing the (+ stimulus) FF/Ren value by the (- stimulus) value. To isolate the induction due to the enhancer, the fold induction of an enhancer reporter was divided by the fold induction of the appropriate backbone into which the enhancer was cloned, giving fold induction relative to backbone. Fold induction relative to backbone is the value shown in all figures.
containing luciferase reporter data. All error bars shown are standard error of biological replicates.

3.5.8 DNA Microarrays and data analysis

For all microarray experiments, total RNA was purified using RNeasy mini kits (Qiagen). Ten micrograms of RNA was hybridized to Affymetrix Mouse Genome 430 2.0 Array with Affymetrix processing. To identify activity-regulated genes regulated by C-FOS, mouse cortical neurons were cultured for 5 days, infected with lentivirus expressing control or c-Fos RNAi, and then depolarized with mM KCl for 0, 1, 3, or 6 hours. Two microarray bioreplicates were performed for all conditions. The DNA-Chip (dChip) software package (Li and Wong, 2001) was used to analyze the microarray data. Genes were considered C-FOS targets if they met the following criteria: (1) the gene was present and expressed at a level of at least 250 at at least one of the timepoints in both bioreplicates of the experiment, (2) there was at least a 3-fold induction of the gene relative to the 0 hour timepoint, (3) there was at least a 1.75 fold reduction in the induction of the gene with c-Fos RNAi compared to control RNAi.

3.5.9 de novo motif analysis

For de novo motif analysis, MEME (Bailey and Elkan, 1994) was used to analyze the 200 bps surrounding enhancer centers. The following parameters were used: -dna -mod anr -nmotifs 15 -minw 5 -maxw 10 -revcomp.
4 GENERAL DISCUSSION

The work presented in this thesis advances our understanding of stimulus responsive enhancers in the nervous system. By discovering that H3K27Ac changes rapidly at neuronal enhancers, and that stimulus-dependent increases in H3K27Ac at neuronal enhancers can be used to identify enhancers that function to promoter stimulus-responsive transcription, we now have a much more accurate picture of the enhancers that respond to stimuli. We used the dynamic chromatin signature of stimulus-inducible H3K27Ac to identify and characterize neuronal enhancers that respond to neuronal activity (modeled in vitro by the application of KCl) as well as neuronal enhancers that respond to BDNF. We now know that neuronal enhancers can respond to specific stimuli or to multiple stimuli and that activity and BDNF responsive enhancers are linked to genes with distinct functions. We also know that the function of neuronal stimulus responsive enhancers appears to be intrinsic to the DNA sequence of these enhancers. Because of this, understanding the sequences that give rise to stimulus responsiveness of enhancers should be computationally and experimentally tractable. We have begun to understand the sequence determinants of stimulus responsive enhancers by performing de novo motif finding and studying the function of neuronal stimulus responsive enhancers in luciferase reporter assays in vitro, a system in which stimulus-responsive enhancer function can be effectively recapitulated. These approaches have shown that AP1 transcription factors play a critical role in the function of a subset of stimulus-responsive enhancers. One enhancer context in which AP1 is required is the enhancer upstream of the neuronal stimulus-regulated gene Nptx2. We
have begun to understand the sequence determinants underlying the *Nptx2* enhancer. This work has revealed the critical importance of AP1 for the KCl and BDNF-dependent responses of this enhancer, but has also revealed that AP1 is insufficient to drive the responses of this enhancer to either KCl or BDNF. At the *Nptx2* enhancer, both AP1 and NPAS4 are required for responses to neuronal activity, but these two factors are still insufficient to drive activity-dependent responses from the *Nptx2* enhancer. Further work will be required to understand the additional factors that collaborate with AP1 and NPAS4 to drive responses to activity, and the factors that collaborate with AP1 to drive responses to BDNF. These advances in our understanding of stimulus-responsive enhancers in the nervous system were possible because of the approaches that we employed to study this biology. However, there are limitations to the experiments we have performed and conclusions we are able to draw from these experiments. We would like to discuss some of the advantages and limitations of the methods we have used to study stimulus-responsive transcription in the nervous system.
4.1 ADVANTAGES AND LIMITATIONS OF THE EXPERIMENTS PERFORMED IN THIS THESIS

4.1.1 In vitro studies

4.1.1.1 Advantages of in vitro studies

While all of the work described in this thesis has been performed in primary mouse cortical neurons, all experiments have been done using these neurons in vitro. Studying neuronal stimulus-responsive transcriptional regulation in vitro has several advantages. First, neurons from a single source can be plated onto multiple cell culture dishes that can be treated differently and then compared with one another. In studying stimulus-responsive transcription, this is of critical importance. All stimulus-responsive phenomena require the comparison of a stimulated state with an unstimulated state. To isolate the effects of the stimulus, stimulated and unstimulated cells need to be as similar to one another as possible. In practice, the only way for this to be achieved is through in vitro experiments. In experiments that I did not describe in this thesis, I have characterized stimulus-responsive transcription in vivo using kainic acid-induced seizures in mice. All experiments were performed on mice from a pure C57BL/6 genetic background that were litter-mates born and raised to the time of the experiment in the same cage. Despite their identical genetic background and identical environment from birth, these mice exhibited significant variability in the levels of immediate-early gene protein products (such as CFOS, FOSB, JUNB, and NPAS4) induced in response to seizure, assessed by western blot. This could be due to differences between the mice,
such as differences is nourishment and development. It is often observed that some mice within a litter are larger and better nourished than others. Alternatively, differences in the inducibility of immediate early genes could be due to differences in the stimulation of different mice. This brings me to the second major advantage of studying neuronal stimulus-responsive transcription in vitro: the ability to uniformly and reproducibly stimulate cells.

Stimulating neurons in vitro by adding the stimulus directly to the neuronal cell culture medium allows for uniform and reproducible stimulation. In contrast, stimulating mice to study neuronal stimulus-responsive transcription in vivo is extremely challenging. To study robust transcriptional responses, we and others have employed kainic acid induced seizures in juvenile or adult mice. However, the delivery of kainate by subcutaneous injections, is inherently variable. The site of injection, the depth of injection, the volume of injected liquid that remains within the animal, can all vary considerably and lead to significant differences in the level of seizure induced in the animal. After subcutaneous kainate injections, some mice begin to visibly seize after 10 minutes, other mice begin to seize after 30 minutes, and some mice never display visible seizure-related behaviors. This variability contributes to variability in seizure induced transcriptional programs activated in the nervous system, thus making it extremely difficult to achieve reproducible results. This challenge in achieving reproducible stimulation brings me to the third major advantage of studying neuronal stimulus-responsive transcription in vitro: the ability to perform and compare experimental manipulations with one another.
In vitro neuronal cultures can be easily experimentally manipulated to study mechanisms underlying stimulus-responsive transcription. By employing lentivirus containing shRNA to knockdown particular protein products, or cDNAs to overexpress particular protein products, the role of candidate proteins in stimulus-responsive transcription can be studied. In this thesis, we have begun using these tools to understand the role of AP1 transcription factors in neuronal stimulus-responsive transcription. While methods exist to study gene function in vivo (such as the generation of transgenic mouse lines, in utero electroporation of knockdown or overexpressing plasmids, or stereotactic viral injections), these methods are more challenging and time consuming than in vitro approaches. Furthermore, the inability to consistently and reproducibly stimulate animals in vivo becomes a major issue. For example, if a gene is knocked down via virally delivered shRNA in one animal in vivo, the relevant control is another animal that has been infected with a control shRNA. However, if these two animals cannot be similarly stimulated in vivo, the control animal differs from the experimental animal, and the effects of the knockdown cannot be rigorously assessed.

4.1.1.2 Limitations of in vitro studies

While there are many advantages of employing an in vitro approach to study stimulus responsive transcription within the nervous system, as detailed above, there are also important limitations to this approach. While in vitro neuronal cultures recapitulate many aspects of neuronal biology, including stimulus-responsive transcription, this is clearly an artificial system. Normally neurons form and maintain precise synaptic contacts with other neurons, and precise cellular contacts with glial
cells, through a carefully orchestrated process of development within the three dimensional environment of a brain that is nourished by blood and cerebrospinal fluid. In contrast, when neurons are cultured in vitro, they are stripped of their synaptic and cellular contacts through harsh trituration, plated on a two dimensional plastic surface, and grown in culture medium in an incubator. This artificiality in vitro will cause differences in neuronal function compared to in vivo. As a result, any phenomenon observed in vitro should be demonstrated in vivo in to ensure that it is not a artifact of the in vitro system.

Another disadvantage of in vitro studies of stimulus responsive transcription is that there is no way to physiologically stimulate cells in vitro. Normally, the brain is activated by sensory experience which in turn activates specific neural circuits within the brain. When studying cortical neurons in vitro, not only are there no sensory inputs to speak of, neural circuits and synaptic connectivity are completely disorganized. As a result, any stimulation of neurons in vitro is artificial. We have utilized the extracellular potassium chloride (KCl) to depolarize neurons and mimic neuronal activity. While KCl does depolarize neurons, it does so by depolarizing the neuronal membrane as a whole rather than by activating neurotransmitter gated ion channels at synapses as occurs in vivo. Hence, KCl is an artificial stimulus of neurons grown in an artificial system. Nonetheless, KCl has proved to be a useful discovery tool for many activity-dependent phenomena that have been recapitulated in vivo with physiological stimuli.
4.1.2 Chromatin immunoprecipitation (ChIP)

4.1.2.1 Advantages of ChIP

ChIP has emerged as one of the most powerful tools available to survey transcription factor binding and chromatin modifications in the genome. ChIP enables transcription factor binding and chromatin modifications to be assessed in vivo. When combined with high throughput sequencing, ChIP enables genome-wide assessments of transcription factor binding events and chromatin modifications. Furthermore, ChIP is a relatively simple assay that can be easily scaled to investigate multiple proteins of histone modifications simultaneously.

4.1.2.2 Limitations of ChIP

While ChIP has significant advantages, it also has limitations that need to be kept in mind. First of all, ChIP is normally performed under crosslinking conditions to maintain protein-DNA interactions through the experiment. Crosslinking can introduce artifacts causing a locus within the genome to appear artificially enriched for certain transcription factor binding events or chromatin modifications, when it is only in proximity to a locus truly exhibiting these features. ChIP also relies on antibodies that recognize the desired epitope within the cell. All antibodies bind nonspecifically to proteins within the cell, as any silver stain of an immunoprecipitation will demonstrate. Hence, it is not always clear if a signal in a ChIP experiment is due to the immunoprecipitation of the desired epitope or of another protein. For these reasons, it is preferable to utilize multiple pieces of evidence to confirm a result by ChIP. In the
case of ChIP for transcription factors, it can be useful to assess whether known motifs for the transcription factor are enriched underneath ChIP signal and if the signal is specific to the protein of interest by performing ChIP using the same antibody in cells in which that protein has been knocked down or knocked out. We have utilized both motif enrichment and ChIP from shRNA infected cells to increase our confidence in our ChIP results for C-FOS and FOSB in this work.

Another limitation of ChIP is the fact that it is a biochemical assay that provides the average signal over millions of cells (ChIP experiments done for the work described in this thesis were done with 20 million neurons). As a result ChIP does not provide information about the variability that may exist between cells processed together or at the two alleles of each locus within a cell. If the two alleles of a locus vary in sequence, allele specificity can be studied using high throughput sequencing.

Finally, ChIP, in particular ChIP-SEQ, is a variable assay. It is essential that bioreplicates be performed to ensure that a given peak or region of enrichment is reproducible. We have performed multiple bioreplicates of our H3K27Ac and transcription factor ChIP experiments in this work.

4.1.3 Luciferase reporter assays

4.1.3.1 Advantages of luciferase reporter assays

Luciferase reporter assays provide several advantages for studying gene expression and the function of DNA sequences. Luciferase reporter assays provide high sensitivity, a wide dynamic range, and easy methodology. Furthermore, luciferase
reporter assays enable direct investigations into the function of sequences of regulatory DNA, unlike studies of endogenous gene expression. Finally, since luciferase reporter plasmids can be delivered to cells via transfection or infection, luciferase reporter assays enable signaling to DNA sequences to be studied in the context of the cell and its environment. In comparison, studies of transcription using reconstituted systems in vitro do not enable one to study transcriptional regulation in the context of normal cellular signaling.

4.1.3.2 Limitations of luciferase reporter assays

While luciferase reporter assays offer several advantages, these assays are artificial. DNA cloned into a plasmid is very different than DNA that exists within the cell as part of chromatin. In the cell, modifications of chromatin during development instruct programs of gene expression. Luciferase plasmids lack the normal chromatin structure of cellular DNA and as a result lack this important regulatory component. As a result, DNA sequences that are normally not available for transcription factor binding can be exposed in luciferase reporters causing ectopic activity to be observed from a DNA sequence. As a result, luciferase data alone should not be taken to indicate activity in the context of the cell. In the case of our work, we couple luciferase reporter assays with ChIP-Seq data obtained in vivo to increase our confidence in our findings. It is only because the stimulus-responsive enhancers we have identified exhibit both increasing H3K27Ac within the neuron as well as the ability to drive stimulus responsive reporter expression within a plasmid that we take these enhancers to promote stimulus-responsive gene expression with the neuron. Still, luciferase reporter assays do not inform us of endogenous gene expression directly. For example, while many of the
enhancers cloned in this work are active in luciferase reporter assays, we still do not know which genes within the cell these enhancers may regulate. Further studies will be required to understand the specific contributions of the stimulus responsive enhancers we have identified to stimulus-responsive transcription within the neuron.
4.2 FUTURE DIRECTIONS

The work I have performed for my thesis has advanced our understanding of stimulus-responsive enhancers in the nervous system. It is bittersweet that my work has opened more questions than it has answered. It is sweet because I take this as an indication that these findings will have importance for developing further understanding of this biology. However, it is also bitter because I will not be able to myself continue performing these experiments to find out the answers. Hopefully others will be motivated to do so! Here, I outline the most interesting future directions that I see from this point that I would pursue if I could have more time in the laboratory.

4.2.1 Demonstrating stimulus-inducible H3K27Ac in the nervous system in vivo

As mentioned above, there are limitations to the findings we have obtained from our experiments in vitro. It will be important to determine whether we detect changes in H3K27Ac in the nervous system in vivo. This can be done using strong, artificial stimuli such as kainic acid induced seizures, or using more physiological stimuli, such as enriched environment or light exposure after dark rearing. Investigating stimulus-inducible H3K27Ac in vivo also opens the door to the exciting possibility of identifying and studying enhancers that are activated in vivo in response to specific stimuli or within particular brain regions.
4.2.2 Performing H3K27Ac at different time points after stimulation

As mentioned earlier in the thesis, multiple waves of transcription occur in response to extracellular stimulation. A first wave of transcription mediated by pre-existing transcription factors within the cell (such as CREB, MEF2, and SRF) occurs rapidly. Among the genes expressed in this first wave of transcription are transcription factors (such as C-FOS, FOSB, JUNB, and NPAS4) that activate a subsequent wave of transcription. In this work, we have only investigated H3K27Ac at 0 hours or 2 hours after sustained stimulation. It could be interesting to perform H3K27Ac ChIP-Seq at several additional time points after sustained stimulation or a brief pulse of stimulation. Such experiments may demonstrate that some enhancers exhibit inducible H3K27Ac early after a stimulus, while other enhancers may exhibit inducible H3K27Ac later after a stimulus. Such a finding may enable us to distinguish the enhancers that function to promote the first wave of transcription from those that promote the second wave of transcription, and study these two classes of enhancers separately. We have observed that only a subset of neuronal stimulus-responsive enhancers are bound by AP1 transcription factors and appear sensitive to the AP1 dominant negative A-Fos. It is possible that enhancers mediating the first wave of transcription do not require AP1 while enhancers mediating the second wave of transcription do require AP1. These interesting possibilities can only be explored if better temporal resolution of changes in H3K27Ac after stimulation is achieved. It may be the case that stimulus-responsive enhancers exhibit even greater diversity of responses than just early and late inducibility. Some enhancers may be activated transiently while others may exhibit longer lasting activity after a stimulus. Performing H3K27Ac ChIP-Seq at additional time points will
not only reveal if this is the case but will also enable us to begin understanding the sequences and transcription factors that give rise to diverse temporal responses from enhancers.

### 4.2.3 Better understanding the mechanisms underlying stimulus-responsive enhancers

While we have shown that AP1 plays critical roles at a subset of activity and BDNF responsive neuronal enhancers, the exact contribution of AP1 to enhancer function is not clear. Whether AP1 binding is necessary for recruitment of other transcription factors, recruitment of CBP/P300, activation of CBP/P300, deposition of H3K27Ac, or looping to target promoters is not clear. Insight into these questions can be achieved by delivering the AP1 dominant negative A-Fos to neurons via lentiviral infection, thereby disrupting the activity of AP1, and assessing any number of variables from transcription factor binding, to looping interactions, to gene expression. Furthermore, more work will be needed to understand the additional transcription factors that drive stimulus-responsive enhancers, and the contribution of each of these factors to enhancer function. Additional factors could be identified computationally based on presence of motifs, biochemically based on mass spectrometry of factors bound to AP1 factors or to stimulus responsive enhancers, or based on a candidate approach. Hopefully, detailed investigations at individual enhancers, such as the Nptx2 enhancer, will shed light onto not only the additional transcription factors that drive stimulus-responsive enhancers, but also the cis regulatory logic of enhancer function and stimulus-specific transcriptional responses.
4.2.4 Performing H3K27Ac ChIP-Seq in response to stimuli in human neurons

Recently, it has become feasible to obtain and culture human neurons in vitro. This provides the exciting possibility of performing H3K27Ac ChIP-Seq in human neurons in response to stimuli. This would not only enable the identification of stimulus-responsive enhancers in human neurons, it would also enable comparisons to be made between mouse and human. Such comparisons would not only be interesting from an evolutionary standpoint, they could also shed light onto the cis-regulatory logic of stimulus-responsive enhancers. Any variations in the H3K27Ac behaviors at orthologous enhancers in human and mouse could then be attempted to be understood on the basis of the variation in the underlying orthologous sequences. Similarly, if H3K27Ac ChIP-Seq is done from human neurons obtained from different individuals for whom whole genome sequencing is also obtained, the contribution of human variation to enhancer function can be studied using H3K27Ac as a readout. This need not be limited to stimulus responsive enhancers, and could be done for constitutively active enhancers as well. Studies performed in human neurons may enable us to begin to understand the functional contribution of sequence variation to gene regulation and human cognition.
5 REFERENCES


APPENDIX A: STIMULUS-RESPONSIVE GENES IN THE NERVOUS SYSTEM
6.1 Top KCl Responsive Genes

Table 15: Top 25 KCl-induced early response genes

RNA-Seq was performed from mouse cortical neurons that were stimulated with 0, 1, or 6 hours (h) of KCl or BDNF. Displayed are the top 25 KCl-induced early response genes, defined as genes with greater induction at 1 h than at 6 h after KCl stimulation. Only genes expressed in the top 50th percentile of all expressed genes within at least one of the time points (0 h, 1 h, 6 h KCl) were considered for this analysis. Genes are displayed in decreasing order of fold induction at the 1 h time point after KCl stimulation relative to the 0 h time point after KCl stimulation. mRNA expression values are reported as reads per kilobase per million mapped reads (RPKM). For each gene, gene name (Gene), NCBI GeneID, chromosome number (Ch), DNA strand (St), start and stop position are also displayed.

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Table 16: Top 25 KCl-induced late response genes

RNA-Seq was performed from mouse cortical neurons that were stimulated with 0, 1, or 6 hours (h) of KCl or BDNF. Displayed are the top 25 KCl-induced late response genes, defined as genes with greater induction at 6 h than at 1 h after KCl stimulation. Only genes expressed in the top 50th percentile of all expressed genes within at least one of the time points (0 h, 1 h, 6 h KCl) were considered for this analysis. Genes are displayed in decreasing order of fold induction at the 6 h time point after KCl stimulation relative to the 0 h time point after KCl stimulation. mRNA expression values are reported as reads per kilobase per million mapped reads (RPKM). For each gene, gene name (Gene), NCBI GeneID, chromosome number (Ch), DNA strand (St), start and stop position are also displayed.

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<td>Fox2</td>
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<td>45009111</td>
<td>45038256</td>
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<td>Sgc2</td>
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<td>79436665</td>
<td>24.41 44.14 157.97 1.81 6.47 46.02 78.42 218.83 1.70 4.76</td>
<td></td>
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</tbody>
</table>
6.2 Top BDNF Responsive Genes

Table 17: Top 25 BDNF-induced early response genes

RNA-Seq was performed from mouse cortical neurons that were stimulated with 0, 1, or 6 hours (h) of KCl or BDNF. Displayed are the top 25 BDNF-induced early response genes, defined as genes with greater induction at 1 h than at 6 h after BDNF stimulation. Only genes expressed in the top 50th percentile of all expressed genes within at least one of the time points (0 h, 1 h, 6 h BDNF) were considered for this analysis. Genes are displayed in decreasing order of fold induction at the 1 h time point after BDNF stimulation relative to the 0 h time point after BDNF stimulation. mRNA expression values are reported as reads per kilobase per million mapped reads (RPKM). For each gene, gene name (Gene), NCBI GeneID, chromosome number (Ch), DNA strand (St), start and stop position are also displayed.

<table>
<thead>
<tr>
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<th>Stop</th>
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<th>BDNF</th>
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<td>19883394</td>
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<td>+</td>
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<td>135975735</td>
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<td>145312455</td>
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</table>

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Table 18: Top 25 BDNF-induced late response genes

RNA-Seq was performed from mouse cortical neurons that were stimulated with 0, 1, or 6 hours (h) of KCl or BDNF. Displayed are the top 25 BDNF-induced late response genes, defined as genes with greater induction at 6 h than at 1 h after BDNF stimulation. Only genes expressed in the top 50th percentile of all expressed genes within at least one of the time points (0 h, 1 h, 6 h BDNF) were considered for this analysis. Genes are displayed in decreasing order of fold induction at the 6 h time point after BDNF stimulation relative to the 0 h time point after BDNF stimulation. mRNA expression values are reported as reads per kilobase per million mapped reads (RPKM). For each gene, gene name (Gene), NCBI GeneID, chromosome number (Ch), DNA strand (St), start and stop position are also displayed.
6.3 UCSC Genome Browser views of ChIP-seq and RNA-seq data

Near Stimulus Responsive Genes in the Nervous System

All genes listed in Appendix A are displayed on the subsequent pages, in alphabetical order. The window displayed ranges from 50kb upstream of the gene to 50kb downstream of the gene.

For each gene, the following tracks are displayed:

1. RNA-Seq after 0 hr of KCI stimulation, silenced conditions
2. RNA-Seq after 1 hr of KCI stimulation
3. RNA-Seq after 6 hr of KCI stimulation
4. RefSeq gene
5. PhastCons vertebrate conservation
6. DNaseI hypersensitivity from embryonic 14.5 whole brain*
7. DNaseI hypersensitivity from embryonic 18.5 whole brain*
8. DNaseI hypersensitivity from 8 week adult cerebrum*
9. H3K27Ac ChIP-seq from embryonic 14.5 whole brain*
10. H3K27Ac ChIP-seq from 8 week adult cortex*
11. H3K27Ac ChIP-seq in silenced conditions
12. H3K27Ac ChIP-seq after 2hrs of KCI stimulation
13. H3K4Me1 ChIP-seq in silenced conditions**
14. H3K4Me1 ChIP-seq after 2hrs of KCI stimulation**
15. H3K4Me3 ChIP-seq in silenced conditions**
16. H3K4Me3 ChIP-seq after 2hrs of KCI stimulation**
17. H3K27Me3 ChIP-seq in silenced conditions**
18. H3K27Me3 ChIP-seq after 2hrs of KCI stimulation**
19. CBP ChIP-seq in silenced conditions**
20. CBP ChIP-seq after 2hrs of KCI stimulation**
21. CBP ChIP-seq in silenced conditions
22. CBP ChIP-seq after 2hrs of KCI stimulation
23. P300 ChIP-seq in silenced conditions
24. P300 ChIP-seq after 2hrs of KCI stimulation
25. RNAPII ChIP-seq in silenced conditions**
26. RNAPII ChIP-seq after 2hrs of KCI stimulation**
27. C-FOS ChIP-seq in silenced conditions
28. C-FOS ChIP-seq after 2hrs of KCI stimulation
29. C-FOS ChIP-seq after 2hrs of KCI stimulation from control shRNA lentivirus infected neurons
30. C-FOS ChIP-seq after 2hrs of KCI stimulation from c-Fos shRNA lentivirus infected neurons
31. FOSB ChIP-seq in silenced conditions
32. FOSB ChIP-seq after 2hrs of KCI stimulation
33. FOSB ChIP-seq after 2hrs of KCI stimulation from control shRNA lentivirus infected neurons
34. FOSB ChIP-seq after 2hrs of KCI stimulation from FosB shRNA lentivirus infected neurons
35. NPAS4 ChIP-seq in silenced conditions**
36. NPAS4 ChIP-seq after 2hrs of KCI stimulation**
37. CREB ChIP-seq in silenced conditions**
38. CREB ChIP-seq after 2hrs of KCI stimulation**
39. SRF ChIP-seq in silenced conditions**
40. SRF ChIP-seq after 2hrs of KCI stimulation**
41. MEF2A ChIP-seq in silenced conditions
42. MEF2A ChIP-seq after 2hrs of KCI stimulation
43. SNPs
44. RepeatMasker
45. RNA-Seq after 0 hr of BDNF stimulation, basal conditions
46. RNA-Seq after 1 hr of BDNF stimulation
47. RNA-Seq after 6 hr of BDNF stimulation
48. UCSC gene
49. PhastCons mammalian conservation
50. H3K27Ac ChIP-seq in basal conditions
51. H3K27Ac ChIP-seq after 2hrs of BDNF stimulation
52. C-FOS ChIP-seq in basal conditions
53. C-FOS ChIP-seq after 2hrs of BDNF stimulation
54. FOSB ChIP-seq in basal conditions
55. FOSB ChIP-seq after 2hrs of BDNF stimulation

* denotes data from ENCODE, ** denotes data from Kim et al., 2010
Figure 6.1: 4930431Jo8Rik locus
Figure 6.2: 4931440P22Rik locus
Figure 6.3: 5430433G21Rik locus
Figure 6.4: Acan locus
Figure 6.5: Arc locus
Figure 6.6: Areg locus
Figure 6.7: Arl4d locus
Figure 6.8: *Atf3* locus
Figure 6.9: Axudt locus
Figure 6.10: Bdnf locus
Figure 6.11: Btg2 locus
Figure 6.12: Co30019I05Rik locus
Figure 6.13: C1ql2 locus
Figure 6.14: Cartpt locus
Figure 6.15: Cckbr locus
Figure 6.16: Ccl2 locus
Figure 6.17: Cenu locus
Figure 6.18: Citedt locus
Figure 6.19: *Col10a1* locus
Figure 6.20: Cort locus
Figure 6.21: Crem locus
Figure 6.22: Cyr61 locus
Figure 6.23: *Dusp1* locus

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Figure 6.24: Dusp4 locus
Figure 6.25: Dusp5 locus
Figure 6.26: *Dusp6* locus
Figure 6.27: *E530001K10Rik* locus
Figure 6.28: Egr1 locus
Figure 6.29: Egr2 locus
Figure 6.30: *Egr3* locus
Figure 6.31: Egr4 locus
Figure 6.32: Emp1 locus
Figure 6.33: Fbxo33 locus
Figure 6.34: Fos locus
Figure 6.35: FosB locus
Figure 6.36: Fosl2 locus
Figure 6.37: Gadd45b locus
Figure 6.38: Galr1 locus
Figure 6.39: Gpr3 locus
Figure 6.40: Hsd11b1 locus
Figure 6.41: *Hspb3* locus
Figure 6.42: Ier2 locus
Figure 6.43: Il33 locus
Figure 6.44: JunB locus
Figure 6.45: Klf10 locus
Figure 6.46: *Klf4* locus
Figure 6.47: LOC100038842 locus
Figure 6.48: LOC100043802 locus
Figure 6.49: LOC668604 locus
Figure 6.50: Maff locus
Figure 6.51: Map3k5 locus
Figure 6.52: Ngfb locus
Figure 6.53: Npas4 locus
Figure 6.54: Nptx1 locus
Figure 6.55: Nptx2 locus
Figure 6.56: Nr4a1 locus
Figure 6.57: Nr4a2 locus
Figure 6.58: Nr4a3 locus
Figure 6.59: Nrm1 locus
Figure 6.60: Pcsk1 locus
Figure 6.61: Popdc3 locus
Figure 6.62: Rasl11a locus
Figure 6.63: Rgs2 locus
Figure 6.64: *Rrad* locus
Figure 6.65: S100a3 locus
Figure 6.66: Scg2 locus
Figure 6.67: Sema3e locus
Figure 6.68: Serpinb2 locus
Figure 6.69: Sertad1 locus
Figure 6.70: Spty2d1 locus
Figure 6.71: Tac1 locus
Figure 6.72: Tiparp locus
Figure 6.73: Tnfrsf12a locus
Figure 6.74: *Trib1* locus
Figure 6.75: Vgf locus
Figure 6.76: Wnt2 locus
APPENDIX B: NEURONAL ENHANCERS WITH DIFFERENT H3K27AC BEHAVIORS
7.1 UCSC Genome Browser views of ChIP-Seq and RNA-Seq data

AT NEURONAL ENHANCERS WITH DIFFERENT H3K27Ac Behaviors

All enhancers tested in luciferase assays within this thesis are displayed on the subsequent pages, in order of enhancer number. A 20kb window surrounding each enhancer center is displayed.

For each enhancer, the following tracks are displayed:
1) RNA-Seq after 0 hr of KCl stimulation, silenced conditions
2) RNA-Seq after 1 hr of KCl stimulation
3) RNA-Seq after 6 hr of KCl stimulation
4) RefSeq gene
5) PhastCons vertebrate conservation
6) DNaseI hypersensitivity from embryonic 14.5 whole brain*
7) DNaseI hypersensitivity from embryonic 18.5 whole brain*
8) DNaseI hypersensitivity from 8 week adult cerebrum*
9) H3K27Ac ChIP-seq from embryonic 14.5 whole brain*
10) H3K27Ac ChIP-seq from 8 week adult cortex*
11) H3K27Ac ChIP-seq in silenced conditions
12) H3K27Ac ChIP-seq after 2hrs of KCl stimulation
13) H3K4Me1 ChIP-seq in silenced conditions**
14) H3K4Me1 ChIP-seq after 2hrs of KCl stimulation**
15) H3K4Me3 ChIP-seq in silenced conditions**
16) H3K4Me3 ChIP-seq after 2hrs of KCl stimulation**
17) H3K27Me3 ChIP-seq in silenced conditions**
18) H3K27Me3 ChIP-seq after 2hrs of KCl stimulation**
19) CBP ChIP-seq in silenced conditions**
20) CBP ChIP-seq after 2hrs of KCl stimulation**
21) CBP ChIP-seq in silenced conditions
22) CBP ChIP-seq after 2hrs of KCl stimulation
23) P300 ChIP-seq in silenced conditions
24) P300 ChIP-seq after 2hrs of KCl stimulation
25) RNAPII ChIP-seq in silenced conditions**
26) RNAPII ChIP-seq after 2hrs of KCl stimulation**
27) C-FOS ChIP-seq in silenced conditions
28) C-FOS ChIP-seq after 2hrs of KCl stimulation
29) C-FOS ChIP-seq after 2hrs of KCl stimulation from control shRNA lentivirus infected neurons
30) C-FOS ChIP-seq after 2hrs of KCl stimulation from c-Fos shRNA lentivirus infected neurons
31) FOSB ChIP-seq in silenced conditions
32) FOSB ChIP-seq after 2hrs of KCl stimulation
33) FOSB ChIP-seq after 2hrs of KCl stimulation from control shRNA lentivirus infected neurons
34) FOSB ChIP-seq after 2hrs of KCl stimulation from FosB shRNA lentivirus infected neurons
35) NPAS4 ChIP-seq in silenced conditions**
36) NPAS4 ChIP-seq after 2hrs of KCl stimulation**
37) CREB ChIP-seq in silenced conditions**
38) CREB ChIP-seq after 2hrs of KCl stimulation**
39) SRF ChIP-seq in silenced conditions**
40) SRF ChIP-seq after 2hrs of KCl stimulation**
41) MEF2A ChIP-seq in silenced conditions
42) MEF2A ChIP-seq after 2hrs of KCl stimulation
43) SNPs
44) RepeatMasker
45) RNA-Seq after 0 hr of BDNF stimulation, basal conditions
46) RNA-Seq after 1 hr of BDNF stimulation
47) RNA-Seq after 6 hr of BDNF stimulation
48) UCSC gene
49) PhastCons mammalian conservation
50) H3K27Ac ChIP-seq in basal conditions
51) H3K27Ac ChIP-seq after 2hrs of BDNF stimulation
52) C-FOS ChIP-seq in basal conditions
53) C-FOS ChIP-seq after 2hrs of BDNF stimulation
54) FOSB ChIP-seq in basal conditions
55) FOSB ChIP-seq after 2hrs of BDNF stimulation

* denotes data from ENCODE, ** denotes data from Kim et al., 2010
Figure 7.1: Enhancer 1 locus
Figure 7.2: Enhancer 2 locus
Figure 7.3: Enhancer 3 locus
Figure 7.4: Enhancer 4 locus
Figure 7.5: Enhancer 5 locus
Figure 7.6: Enhancer 6 locus
Figure 7.7: Enhancer 7 locus
Figure 7.8: Enhancer 8 locus
Figure 7.9: Enhancer 9 locus
Figure 7.10: Enhancer 10 locus
Figure 7.11: Enhancer 11 locus
Figure 7.12: Enhancer 12 locus
Figure 7.13: Enhancer 13 locus
Figure 7.14: Enhancer 14 locus
Figure 7.15: Enhancer 15 locus
Figure 7.16: Enhancer 16 locus
Figure 7.17: Enhancer 17 locus
Figure 7.18: Enhancer 18 locus
Figure 7.19: Enhancer 19 locus
Figure 7.20: Enhancer 20 locus
Figure 7.21: Enhancer 21 locus
Figure 7.22: Enhancer 22 locus
Figure 7.23: Enhancer 23 locus
Figure 7.24: Enhancer 24 locus
Figure 7.25: Enhancer 25 locus
Figure 7.26: Enhancer 26 locus
Figure 7.27: Enhancer 27 locus
Figure 7.28: Enhancer 28 locus
Figure 7.29: Enhancer 29 locus
Figure 7.30: Enhancer 30 locus
Figure 7.31: Enhancer 31 locus
Figure 7.32: Enhancer 32 locus
Figure 7.33: Enhancer 33 locus
Figure 7.34: Enhancer 34 locus
Figure 7.35: Enhancer 35 locus
Figure 7.36: Enhancer 36 locus
Figure 7.37: Enhancer 37 locus
Figure 7.38: Enhancer 38 locus
Figure 7.39: Enhancer 39 locus
Figure 7.40: Enhancer 40 locus
Figure 7.41: Enhancer 41 locus
Figure 7.42: Enhancer 42 locus
Figure 7.43: Enhancer 43 locus
Figure 7.44: Enhancer 44 locus
Figure 7.45: Enhancer 45 locus
Figure 7.46: Enhancer 46 locus
Figure 7.47: Enhancer 47 locus
Figure 7.48: Enhancer 48 locus
Figure 7.49: Enhancer 49 locus
Figure 7.50: Enhancer 50 locus
Figure 7.51: Enhancer 51 locus
Figure 7.52: Enhancer 52 locus
Figure 7.53: Enhancer 53 locus
Figure 7.54: Enhancer 54 locus
Figure 7.55: Nptx2 upstream enhancer locus
8 APPENDIX C: ACTIVITY-DEPENDENT LATE RESPONSE GENES
LESS INDUCED WITH C-FOS RNAI

On the subsequent pages are displayed all microarray probesets (n=128) that detected activity-dependent genes that were three fold or more induced (6hr/0hr expression>=3) in neurons infected with control RNAi but whose fold induction was reduced by at least 1.75 fold with c-Fos RNAi (fold induction c-Fos RNAi/fold induction control RNAi <= 1/1.75). Probesets are sorted alphabetically by the gene to which they correspond. Fold inductions from control RNAi and c-Fos RNAi infected cells are reported as the average fold induction of two microarray bioreplicates.
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<td>3.5</td>
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</tr>
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<td>12227</td>
<td>5.1</td>
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</tr>
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APPENDIX D: WORK RESULTING FROM COLLABORATIONS
Activity-dependent regulation of inhibitory synapse development by Npas4

Yingxi Lin1, Brenda L. Bloodgood1, Jessica L. Hauser1, Ariya D. Lapan2, Alex C. Koon1, Tae-Kyung Kim1, Linda S. Hu1, Athar N. Malik1,2 & Michael E. Greenberg3

Neuronal activity regulates the development and maturation of excitatory and inhibitory synapses in the mammalian brain. Several recent studies have identified signalling networks within neurons that control excitatory synapse development. However, less is known about the molecular mechanisms that regulate the activity-dependent development of GABAergic synapses. Here we report the identification of a transcription factor, Npas4, that plays a role in the development of inhibitory synapses by regulating the expression of activity-dependent genes, which in turn control the number of GABA-releasing synapses that form on excitatory neurons. These findings demonstrate that the activity-dependent gene program regulates inhibitory synapse development, and suggest a new role for this program in controlling the homeostatic balance between synaptic excitation and inhibition.

The density of inhibitory synapses in brain regions such as primary sensory cortex, hippocampus and cerebellum is regulated by the level of excitatory synaptic activity and sensory input1-3. In addition, initiation of the critical period for synaptic plasticity in the visual cortex is dependent on visual activity and strongly influenced by the maturation of inhibitory synapses4, suggesting that the activity-dependent regulation of GABAergic synapses is important for the plasticity of the nervous system. Finally, recent studies indicate that regulation of GABAergic synapses in response to neuronal activity may be a critical component of the homeostatic mechanism that maintains a balance between excitation and inhibition in the face of fluctuations in the level of sensory input into neural circuits5,6. Despite the accumulating evidence that neuronal activity regulating the development and maintenance of inhibitory synapses, the molecular mechanisms that control these processes remain to be characterized.

Here we identify a transcription factor, Npas4, that is critical for activity-dependent regulation of GABAergic synapse development. Npas4 expression is rapidly activated by excitatory synaptic activity and turns on a program of gene expression that triggers the formation and/or maintenance of inhibitory synapses on excitatory neurons. These findings provide a molecular link between neuronal excitation and GABAergic synapse development, and suggest a new role for the activity-dependent gene program in controlling inhibitory synapse formation/maintenance on excitatory neurons.

Npas4 is regulated by neuronal activity

The formation of inhibitory synapses onto excitatory neurons is regulated by neuronal activity, takes place over several days, and is a cell-wide process that results in the formation of synapses onto both the cell body and dendrites7-10. These features led us to hypothesize that activity-dependent development of inhibitory synapses might be controlled posttranslationally by one or more activity-regulated genes. To test this hypothesis, we used DNA microarrays to identify genes that are induced by membrane depolarization in mouse cortical neurons at the time when inhibitory synapses are developing.

We identified more than 300 genes whose expression levels were altered upon membrane depolarization (Gene Expression Omnibus accession number GSE11256), a third of which were novel activity-regulated genes not seen in previous screens11-13. We looked for genes predicted to encode transcription factors, reasoning that, through genome-wide characterization of the targets of an activity-regulated transcription factor that controls inhibitory synapse number, we could gain insight into the biological program that is important for inhibitory synapse development. Among the approximately 20 known or putative transcription factors identified, we focused on genes that are selectively induced by Ca2+ influx in neurons but not other cell types, that are transcribed in response to excitatory

1-4. Many of the effects of neuronal activity are mediated by the release of glutamate at excitatory synapses and the subsequent influx of calcium (Ca2+) into the postsynaptic neuron. This results in changes in the number and strength of synapses, a process that underlies learning and memory as well as animal behaviour.

Neurons in the central nervous system receive excitatory synaptic input from glutamatergic neurons and inhibitory input from GABA-releasing (GABAergic) interneurons, except during early development when the first GABAergic synapses are depolarizing and provide the excitatory drive critical for the subsequent development of glutamatergic synapses7. The proper balance between excitatory and inhibitory synapses is crucial for representation of sensory information7, execution of motor commands8,9 and higher-order cognitive functions10. Neurodevelopmental disorders such as autism, schizophrenia and epilepsy are associated with an imbalance between excitatory and inhibitory synapses7,10-12. The number or strength of excitatory synapses can be modified in response to changes in activity, and the molecular mechanisms of these processes have been extensively investigated11-14. Less is known about the activity-dependent regulation of inhibitory synapses.

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Npas4 is induced in cultured neurons by the GABAA receptor antagonist bicuculline, which increases action-potential firing and excitatory synaptic transmission (Fig. 1e). This induction requires an influx of extracellular Ca2+ through L-type voltage-sensitive calcium channels (L-VSCCs) and is partly dependent on the activation of N-methyl-D-aspartate (NMDA) and 3-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (Fig. 1e). Npas4 expression is also induced in pertinent brain regions in vivo in response to specific stimuli: visual stimulation of mice after a period of dark-rearing results in an increase in Npas4 messenger RNA (mRNA) and protein levels specifically in the visual cortex (Fig. 1f, g).

**Npas4 regulates the development of inhibitory synapses**

The effect of Npas4 on inhibitory synapse development was investigated by RNA interference (RNAi)-mediated knockdown in excitatory neurons of rat dissociated hippocampal cultures, before synapse formation was underway. A small hairpin RNA targeting Npas4 (Npas4-RNAi) effectively reduced Npas4 expression (Fig. 2a) without affecting the overall health of the neurons (Supplementary Fig. 1). To measure inhibitory synapse number, neurons were immunostained for presynaptic GABA-producing enzyme GAD65 and postsynaptic GABARα2β2 (CNQX, Fig. 2g). Co-localization of GAD65 and GABARα2β2 puncta on a green fluorescent protein (GFP)-transfected glutamatergic neuron was considered indicative of a synapse (Fig. 2b). Expression of Npas4-RNAi, but not a scrambled RNAi (control-RNAi), significantly reduced the number of inhibitory synapses (Fig. 2c). Analysis of synapse number using antibodies that recognize a second pair of inhibitory synaptic proteins (GAD67 and GABARα2β3) gave a similar result (data not shown). These data suggest that Npas4 positively regulates the number of inhibitory synapses that form on excitatory neurons.

Different classes of inhibitory neurons synapse onto distinct perisomatic or dendritic regions of pyramidal neurons. We found that Npas4-RNAi leads to a reduction in the number of inhibitory synapses formed on both the perisomatic and dendritic regions, suggesting that Npas4 regulates the number of inhibitory synapses formed by multiple classes of inhibitory neurons (Fig. 2c). In both regions, Npas4-RNAi significantly reduced the density of postsynaptic GABARα2β2 puncta but had less effect on presynaptic GAD65 puncta, compared with the control-RNAi (Fig. 2d). These findings suggest that Npas4 regulates inhibitory synapse number by controlling the number of postsynaptic specializations, resulting in subsequent remodelling or retraction of the presynaptic terminals.

To test whether Npas4 is important for the development of functional inhibitory synapses in a more intact neural circuit, rat organotypic hippocampal cultures were biolistically co-transfected with GFP and either a control vector or Npas4-RNAi, and whole-cell recordings were performed on GFP-positive CA1 pyramidal neurons to measure spontaneous miniature inhibitory postsynaptic currents (mIPSCs, Fig. 3a). Knocking down Npas4 expression in organotypic cultures significantly increased the inter-event interval and decreased the amplitude of mIPSCs (Fig. 3b, c). This effect of Npas4-RNAi in organotypic slices may be indicative of a decrease in inhibitory synapse number, consistent with our observation that Npas4-RNAi reduces inhibitory synapse number in dissociated cultures. Together, these findings indicate that Npas4 plays an important role in regulating the number of functional inhibitory synapses received by an excitatory neuron.

Consistent with a role for Npas4 in inhibitory synapse development, Npas4 knockout mice (Npas4−/−, Supplementary Fig. 2) appear anxious and hyperactive, are prone to seizures and have a shortened lifespan compared with their wild-type littermates, phenotypically resembling other knockout mice lacking genes that control the formation or function of inhibitory circuits. However, we found that the frequencies of mIPSCs in acute hippocampal slices prepared from wild-type and Npas4−/− mice were similar (Supplementary Fig. 3), in contrast to the clear change in mIPSC frequency caused by disrupting Npas4 expression acutely in organotypic slices. This difference may
Npas4 regulates the number of GABAergic synapses in cultured hippocampal neurons. a, Npas4-RNAi, but not control-RNAi, reduces the expression of Npas4 in primary hippocampal neurons. Cultures were transfected at 6 DIV and stimulated with bicuculline (50 μM, 2 h) at 14 DIV. b, The number of GABAergic synapses is significantly reduced by Npas4-RNAi, as illustrated by two representative rat hippocampal neurons. Cultures were co-transfected (6 DIV) with GFP and either Npas4-RNAi (top) or control-RNAi (bottom). Cultures were subsequently immunostained with antibodies against GABA (blue) and GABA$_\gamma$2 (red). c, Quantification of the normalized density of co-localized GABA$_\gamma$2 and GAD65 puncta in 14 DIV rat hippocampal neurons transfected with vector control, Npas4-RNAi or control-RNAi. d, Separate quantification of perisomatic and dendritic GABA$_\gamma$2 and GAD65 puncta measured in e. Npas4-minigene increases the density of co-localized GABA$_\gamma$2 and GAD65 puncta measured in f. Separate quantification of perisomatic and dendritic GABA$_\gamma$2 and GAD65 puncta measured in g. See Methods for details of data normalization and error propagation. Significance was determined using multifactorial analysis of variance. *P < 0.05; **P < 0.005; ***P < 0.0005. Data are presented as mean ± s.e.m. from three (c, d) or four (e, f) independent experiments; total numbers of neurons analysed (n) are indicated.

Npas4-minigene cassette consisting of all Npas4 introns and exons as well as 5 kilobases (kb) of genomic sequence 5′- and 3′- to the coding region. We verified that the Npas4-minigene drives ectopic expression of Npas4 that is activity regulated and functions similarly to the endogenous gene (Supplementary Fig. 6). We found that significantly more inhibitory synapses were formed onto cultured hippocampal neurons expressing the Npas4-minigene than onto neurons transfected with a control vector (Fig. 2e), largely because of an increase in the number of postsynaptic GABA$_\gamma$2 and GAD65 puncta. Furthermore, expression of the Npas4-minigene in CA1 pyramidal neurons of hippocampal slices significantly decreases the changes in excitatory synapses in the number or strength of inhibitory synapses are often coupled to the endogenous gene (Supplementary Fig. 6). We found that significantly more inhibitory synapses were formed onto cultured hippocampal neurons expressing the Npas4-minigene than onto neurons transfected with a control vector (Fig. 2e), largely because of an increase in the number of postsynaptic GABA$_\gamma$2 and GAD65 puncta. Furthermore, expression of the Npas4-minigene in CA1 pyramidal neurons of hippocampal slices significantly decreases the effect on mIPSCs in wild-type neurons (Supplementary Fig. 5). a-c, Significant increases in the number of inhibitory synapses on neurons expressing higher levels of Npas4. Npas4 should lead to an increase in the number of inhibitory synapses formed onto a cell is controlled by the amount of Npas4 expressed in response to excitatory stimuli. If this is the case, increasing the level of Npas4 should lead to a significant increase in the number of inhibitory synaptic inputs.

We next investigated whether the number of inhibitory synapses forming onto a cell is controlled by the amount of Npas4 expressed in response to excitatory stimuli. If this is the case, increasing the level of Npas4 should lead to a significant increase in the number of inhibitory synapses formed onto a cell is controlled by the amount of Npas4 expressed in response to excitatory stimuli.
before synaptogenesis was underway and thus before the balance between excitation and inhibition was established. Cultures were immunostained for the presynaptic marker synapsin1 and the excitatory postsynaptic marker PSD95, and the numbers of co-localized synapsin1 and PSD95 puncta on transfected glutamatergic neurons were quantified. Neither the total numbers of excitatory synapses nor the individual numbers of pre- or postsynaptic markers changed significantly upon expression of Npas4-RNAi (Fig. 4a, b), under conditions that significantly decreased the number of inhibitory synapses. Likewise, expression of the Npas4-minigene had no effect on excitatory synapse number (Fig. 4c, d). Because perturbation of the level of Npas4 expression occurred before synaptic connections were established, these experiments suggest that Npas4 is not a major contributor to excitatory synaptogenesis.

We next examined whether Npas4 affects excitatory synapse function in organotypic hippocampal slices, where homeostatic mechanisms are known to control excitatory/inhibitory balance within neural circuits. Deletion of the Npas4 gene from neurons in Npas4flx/flx organotypic hippocampal slices by Cre recombination significantly decreased the inter-event interval of spontaneous miniature excitatory postsynaptic currents (mEPSCs, Fig. 4e, f), whereas elevating the level of Npas4 with the Npas4-minigene significantly increased the inter-event interval and decreased the amplitude of mEPSCs (Fig. 4g, h), compared with control-transfected neurons. Thus, abolishing Npas4 expression in a pyramidal neuron increases, whereas enhancing the level of Npas4 decreases, the number and/or presynaptic release probability of excitatory synapses that form on the neuron. Therefore, the net result of Npas4 activation in an intact neural circuit is an increase in synaptic inhibition and a decrease in excitation of a neuron. We conclude that Npas4 induction in response to increased excitatory input acts to reduce the level of activity, and therefore may function as a negative feedback mechanism to maintain the homeostatic balance between excitation and inhibition.

**Npas4 regulates genes that control inhibitory synapse development**

To uncover the program of gene expression controlled by Npas4, we acutely knocked down Npas4 expression in a high percentage of wild-type neurons using a lentivirus expressing Npas4-RNAi (Supplementary Fig. 7) and performed a second DNA microarray experiment in each condition (for vector control and Cre, respectively. Total numbers of neurons analysed in each condition (n) are indicated. a, Three independent experiments; c, d, four independent experiments. *P < 0.05; **P < 0.001.

**Figure 4 | Npas4 has no effect on excitatory synaptogenesis but affects excitatory/inhibitory balance in neural circuits.** a. The number of excitatory synapses is not affected by Npas4-RNAi. Quantification of the normalized density of co-localized PSD95 and synapsin1 puncta in 14 DIV rat hippocampal neurons transfected with vector control, Npas4-RNAi or control-RNAi. b. Separate quantification of PSD95 and synapsin1 puncta measured in a. c. Npas4-minigene has no effect on the density of excitatory synapses. Quantification of co-localized synapsin1 and PSD95 puncta is shown. d. Separate quantification of PSD95 and synapsin1 puncta measured in c. In a–d, data are presented as mean ± s.e.m. e, Cumulative distribution of mEPSC inter-event intervals and amplitudes recorded from Npas4flx/flx neurons co-transfected with GFP and either vector control or Cre recombinase. f, Mean ± s.e.m. of data from e. mEPSC inter-event intervals: 2581.4 ± 104.1 ms and 2140.5 ± 79.7 ms; amplitudes: 12.1 ± 0.4 and 12.4 ± 0.3 pA; for vector control and Cre, respectively. g, Cumulative distribution of mEPSC inter-event intervals and amplitudes recorded from neurons transfected with GFP and either vector control or Npas4-minigene. h, Mean ± s.e.m. of data from g. mEPSC inter-event intervals: 684.2 ± 31.2 and 917.4 ± 37.7 ms; amplitudes: 27.5 ± 0.7 and 27.9 ± 0.6 pA; for vector control and Cre, respectively. Total numbers of neurons analysed in each condition (n) are indicated. a, b, Three independent experiments; c, d, four independent experiments. *P < 0.05; **P < 0.001.

**Figure 3 | Npas4 regulates GABAergic synapse development in organotypic hippocampal slices.** a. Representative mIPSCs recorded from CA1 pyramidal neurons in organotypic hippocampal slices biologically co-transfected with GFP and either vector control, Npas4-RNAi or Npas4-minigene. b, Cumulative distributions of mIPSC inter-event intervals and amplitudes recorded from neurons transfected with vector control, Npas4-RNAi or Npas4-minigene. c, Mean ± s.e.m. of data from b. mIPSC inter-event intervals: 2986.3 ± 105.7, 3803.0 ± 136.9 and 1776.9 ± 75.1 ms; amplitudes: 31.5 ± 1.1, 23.7 ± 0.8 and 34.1 ± 1.3 pA; for vector control, Npas4-RNAi and Npas4-minigene, respectively. d, Cumulative distributions of mIPSC inter-event intervals and amplitudes recorded from Npas4flx/flx neurons co-transfected with GFP and either vector control or Cre recombinase. e, Mean ± s.e.m. of data from d. mIPSC inter-event intervals: 684.2 ± 31.2 and 917.4 ± 37.7 ms; amplitudes: 27.5 ± 0.7 and 27.9 ± 0.6 pA; for vector control and Cre, respectively. Total numbers of neurons analysed in each condition (n) are indicated in c and e. 1 and e. *P < 0.05; **P < 0.001.
Figure 5 | Npas4 controls a program of gene expression that regulates GABAAergic synapses. a, Hierarchical clustering of 327 probe sets (270 putative Npas4 target genes) based on their expression profiles using dChip. The expression level of each probe set is normalized to a mean of 0 and a standard deviation of 1. Expression values are displayed within the range [−3, 3] with levels above, equal to or below the mean displayed in red, white and blue, respectively. Dark red represents 3 or higher, and dark blue 3 or lower. b, Biological functions of 270 putative Npas4 target genes based on Gene Ontology information provided by Affymetrix (http://www.affymetrix.com). c, BDNF expression is reduced by Npas4-RNAi (1.9 ± 0.11-fold reduction, P < 0.01, one-tailed paired t test). Mean ± s.e.m. from three independent experiments is shown, and each data point was derived with BDNF promoters I and IV, but not with the coding region or 3′-untranslated region (Fig. 5e), suggesting that Npas4 may directly regulate activity-dependent expression of BDNF.

To determine whether BDNF contributes to the effects of Npas4 on GABAergic synapse development, we tested whether the ability of the Npas4-minigene to increase the number of inhibitory synapses is attenuated by knockdown of BDNF expression, using a previously validated small hairpin RNA that targets the BDNF coding region (BDNF-RNAi)46. Confirming that BDNF increases inhibitory synapse number44, BDNF-RNAi increased mIPSC inter-event intervals compared with control values (2986.3 ± 109.7 and 4086.1 ± 140.7 ms, control and BDNF-RNAi, P < 0.01). Expression of the Npas4-minigene alone led to an approximately 40% decrease in the inter-event interval of mIPSCs recorded from CA1 neurons (Fig. 5b, c), as previously observed, but this was partly attenuated to approximately 20% by the presence of BDNF-RNAi (Fig. 6a). In addition, in the presence of BDNF-RNAi, the effect of the Npas4-minigene on the amplitude of mIPSCs was completely reversed (Fig. 6b). These findings suggest that BDNF mediates a portion of the effect of Npas4 on inhibitory synapse number, but that additional Npas4 targets may also be involved.

Discussion
We have identified the activity-regulated transcription factor Npas4 as a key regulator of GABAergic synapse development. Excitatory synaptic activity induces Npas4 in a Ca2+-dependent manner, and the level of Npas4 determines the number of functional GABAergic synapses by controlling a program of activity-dependent gene expression. Future characterization of Npas4 target genes will help to determine whether Npas4 acts by initiating inhibitory synapse formation, stabilizing nascent inhibitory synapses or promoting the maturation of weak inhibitory synapses. It is possible that different subsets of Npas4 targets control the development of GABAergic synapses formed by distinct classes of interneurons, providing a mechanism for the independent regulation of inhibition received in the absence of Npas4. The expression levels of 327 microarray probe sets representing 270 unique genes were significantly different in cultures expressing Npas4-RNAi compared with those expressing the control virus (Gene Expression Omnibus accession number GSE11258, Supplementary List). The expression of 182 of these genes was also acutely regulated by membrane depolarization in the absence of Npas4-RNAi, indicating that many putative Npas4-regulated genes are activity regulated (Fig. 5a). Although Npas4 has been shown to function as a transcriptional activator (Supplementary Fig. 6b)47, we found that many genes are negatively regulated by Npas4 (Fig. 5a). This may reflect the fact that Npas4 functions as a transcriptional repressor as well as an activator, and/or that Npas4 indirectly affects gene expression by altering neuronal excitation.

Npas4 appears to regulate a wide variety of genes, such as activity-regulated immediate early genes, various classes of transcription factors, channel proteins, G-protein signalling molecules, kinases and phosphatases, and genes involved in pathways that modulate synaptic functions, such as ubiquitination, trafficking and receptor endocytosis (Fig. 5b and Supplementary List). Interestingly, the functions of 94 of the 270 putative Npas4-regulated genes are uncharacterized (Fig. 5b), suggesting that Npas4 regulates many genes that could affect inhibitory synapses in novel ways.

As a first step towards understanding the genetic program regulated by Npas4, we focused on targets that might be directly involved in the development of GABAergic synapses. Brain-derived neurotrophic factor (BDNF) stood out because it had previously been shown to regulate GABAergic synapse maturation and function44,45,46,47. BDNF expression is consistently reduced by almost two-fold in cultures expressing Npas4-RNAi compared with control cultures (Fig. 5c). Primary cultures from Npas4−/− mice showed a similar decrease in depolarization-induced BDNF expression compared with their wild-type littermates (Fig. 5d).
by subregions of a neuron. Another intriguing possibility is that Npas4 controls experience-dependent developmental processes, such as critical period plasticity in the visual cortex, which depend on the function and maturation of GABAergic synapses.

Although Npas4 is not required for the initial formation of excitatory synapses, activation of Npas4 in excitatory neurons within a neural circuit appears to diminish the excitatory synaptic input they receive. It is not known whether this effect is mediated directly by Npas4 or is an indirect consequence of changes in inhibitory input. In either case, our findings suggest that Npas4 functions as part of the homeostatic mechanism that stabilizes the activity of a neuron in the face of changing glutamatergic input. Further investigation of the function of Npas4 and other activity-dependent regulators of inhibitory synapses will provide insight into the mechanism by which neuronal activity controls the balance between excitation and inhibition in the brain, and how the disruption of this balance leads to neurological disorders such as autism, epilepsy and schizophrenia.

METHODS SUMMARY

Dissociated neuron culture and transfection. Dissociated cortical and hippocampal neurons were prepared from E18 rat or E16 mouse embryos as previously described[6]. Cultures were maintained in Neurobasal Medium supplemented with 2% (Invitrogen), penicillin-streptomycin and glutamine. Neurons were plated at 100,000–150,000 per well in a 24-well plate or 15,000,000 per 10-cm plate. For synapse assay, hippocampal neurons (60,000 per well, 24-well plate) were plated on a monolayer of astrocytes[9]. Neurons were transfected at 3–5 days in vitro (DIV) using the calcium phosphate method[6].

Organotypic slice culture and transfection. Organotypic hippocampal slice cultures were prepared from P6 rats or mice as previously described[6]. Slices were biologically transfected with a Helios Gene Gun (BioRad) after 2 days. Bullets for the gene gun were 1.6-μm gold particles coated with 15 μg eGFP and either 5 or 10 μg RNAi construct, 45 μg Npas4-miRNA or 30 μg Cre. Empty plasmid was added to bring the total DNA to 60 μg in each case.

Synapse density assay. Hippocampal neurons (14–18 DIV) were immunostained for synaptic markers and imaged on a Zeiss LSM5 Pascal microscope using a ×63 objective lens. Image acquisition and synapse quantification were performed in a blinded manner. Glutamatergic neurons were identified based on morphology and the absence of cytosolic GAD65 staining. Synapse density was measured using Metamorph software as previously described[27]. Within each experiment, density of puncta was normalized against the control, and the error of the control propagated into each experimental condition. Statistical analysis was performed on the raw data (without normalization) using multifactorial analysis of variance in StatView 4.5 (Abacus Concepts).

Electrophysiology. Whole-cell patch clamp recordings were made at room temperature from CA1 pyramidal neurons 7–10 days after transfection. Data were analysed in IgorPro (Wavemetrics) using custom-written macros. Statistical significance was determined by Kolmogorov-Smirnov test and Monte Carlo simulation.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions Y.L. and M.E.G. conceived and designed the experiments and wrote the manuscript. Y.L. performed or participated in each of the experiments described in the manuscript. B.L.B. performed the electrophysiological recordings and contributed to the writing of the manuscript. J.L.H. quantified Npas4 mRNA levels for the light stimulation experiment, generated the Npas4-mingene construct and performed the luciferase assay to characterize it, managed the Npas4 animal colony and provided extensive technical support. A.D.L. performed immunocytochemistry for the light stimulation experiment and confocal imaging of neurons in the synapse assay with Npas4-RNAi. A.C.K. provided technical support during the early phase of the study and helped generate many reagents used in this study including the Npas4 antibody, Npas4-knockout construct and Npas4-RNAi lentivirus. T.-K.K. performed the chromatain immunoprecipitation experiments. L.S.H. helped generate the Npas4 antibody. A.N.M. performed the initial chromatain immunoprecipitation experiments.

Author Information Data have been placed in the GEO database under accession numbers GSE12526 and GSE12528. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to M.E.G. (meg@hms.harvard.edu).
METHODS
DNA constructs. Npas4-RNAi, 5'-GTTGACTACCTGATAATTTA-3', and control-RNAi with a scrambled sequence (underlined), 5'-GTTGAACTCCTGATAATTTA-3', were cloned into the pSuper expression vector (OligoEngine). The same Npas4-RNAi was used to generate the lentivirus construct (Collegenetics, Inc). The control lentivirus construct, pLenti-shGL3 (Collegenetics, Inc), targets the luciferase gene, which is not expressed by mouse hippocampal neurons.

The Npas4-minigene was generated by subcloning the mouse genomic region from CTGGTTTATTTTCTCTCTGTC to GCTATGTCGGCGTGGCTGATC into a pH1lescriptR+ vector between the SaII and KpnI digestion sites. The sequence targeted by Npas4-RNAi was mutated to AgtCgaTccCgataattta, preserving the amino-acid sequence, to generate an RNAi-resistant Npas4-minigene.

The Npas4 luciferase reporter was constructed by replacing the tandem MEF2 responsive elements with three copies of the Npas4 responsive element in the 3' UTR luciferase plasmid.

Immunocytochemistry and immunohistochemistry. Cultured neurons were fixed and immunostained as previously described. For immunohistochemistry, mice were perfused intracardially with cold PBS followed by 4% paraformaldehyde in PBS. Brains were then post-fixed in 4% paraformaldehyde in PBS at 4°C overnight followed by incubation in 30% sucrose in PBS at 4°C for 24 h. Cryo-protected brains were immediately sectioned on a cryostat (Leica) or stored in Tissue-Tec O.C.T. at -80°C for later sectioning. Confocal image acquisition and synapse density measurement were performed as previously described. AATGGAAT-3', forward: 5'-GACCCACATCGTTGATGAT-3', reverse: 5'-GCAAAAGGGCCAAATGGAAAT-3', Npas4, forward: 5'-CAGATGACCTACGTCGACTGATT-3', reverse: 5'-GCGGAGGAAGAGCTATTTATATCACCAG-3'.

Quantitative reverse transcriptase PCR. The mRNA level of the gene of interest was normalized against the mRNA level of 18S. Because synapse density and immunostaining vary significantly between experiments, it is necessary to normalize each experiment before combining them. Normalization and error propagation were performed as previously described.

Electrophysiology. Slices were perfused with artificial cerebrospinal fluid containing (in mM) 127 NaCl, 25 NaHCO3, 1.25 NaH2PO4, 2.5 KCl, 2 CaCl2, 1 MgCl2, 25 glucose, and saturated with 95% O2, 5% CO2. The internal solution for mIPSCs contained (in mM) 147 GCl, 5 Na2-phosphocreatine, 10 HEPEs, 2 MgATP, 0.5 Na3GTP, and 1 EGTA. The internal solution for mEPSCs contained (in mM) 120 cesium methane sulfonate, 10 HEPES, 4 MgCl2, 0.4 Na2ATP, 10 sodium phosphocreatine and 1 EGTA. Osmolarity and pH were adjusted to 300 Osm/m and 7.3 with Millipore water and CO2, respectively. mIPSCs were pharmacologically isolated by bath application of (in mM) 0.5 tetrodotoxin, 10 (R)-CPP and 10 NRXS disodium salt (all from Tocris Bioscience); mEPSCs were isolated with 0.5 tetrodotoxin and 50 picotrotxin (Tocris Bioscience), and augmented with 10 cyclothiazide. Cells with series resistance larger than 25 MO2 during the recordings were discarded.

Data were analyzed in IgorPro (Wavemetrics) using custom-written macros. For each trace, the event threshold was set at 1.5 times the root-mean-square current. Currents were counted as events if they crossed the event threshold, had a rapid rise time (1.5 pA/ms) and had an exponential decay (2 < τ < 200 ms, 1 < τ < 50 ms for mIPSC and mEPSC, respectively).

Statistical significance was determined by two methods. First, 50 random points selected from each cell were concatenated to describe the cumulative distributions of events in each condition and then compared by a Kolmogorov-Smirnov test. Second, a Monte Carlo simulation was performed in which points were randomly sampled from each condition and the mean of these samples compared at least 1,000 times. P < 0.05 from both tests was considered significant.

DNA microarrays and data analysis. For all microarray experiments, total RNA was purified using RNaseasy mini kits (Qiagen) and biotin-label cRNA was generated following Affymetrix standard protocols. Ten micrograms of the labelled cRNA was hybridized to Affymetrix mouse MOE430 arrays.

To identify activity-regulated genes, P0 mouse cortical neurons were cultured for 7 days and then depolarized with 50 mM KCl for 1 and 6 h. To identify Npas4-regulated genes, E16 mouse hippocampal neurons were infected (3 DIV) with Npas4-RNAi or control lentivirus, depolarized (8 DIV) with 50 mM KCl, and total RNA was collected 0, 1, 3 and 6 h later. Drebin, an Npas4 target previously identified in a neuroblastoma cell line was not affected by Npas4-RNAi in post-mitotic neurons.

The DNA-Chip (dChip) software package was used to analyze the microarray data. Genes were considered candidates if they met the following criteria: (1) there was at least a 1.5-fold difference between the experimental and control conditions (depolarized versus non-depolarized, or Npas4-RNAi versus control virus) at any of the time points; and (2) there was an absolute difference of more than 100 normalized hybridization intensity units in the expression level between the experimental and control samples. Chromatin immunoprecipitation. Rat cortical neurons were treated with 1 μM tetrodotoxin and 100 μM AP5 overnight and then stimulated with 55 mM KCl for 2 h at 8 DIV. Chromatin immunoprecipitation was performed as previously described. The following primers were used for quantitative PCR: Chr3, forward: 5'-GACCCACATCGTTGATGAT-3', reverse: 5'-GCAAAAGGGCCAAATGGAAAT-3'; Npas4, forward: 5'-CAGATGACCTACGTCGACTGATT-3', reverse: 5'-GCGGAGGAAGAGCTATTTATATCACCAG-3'.

For each trace, the event threshold was set at 1.5 times the root-mean-square current. Currents were counted as events if they crossed the event threshold, had a rapid rise time (1.5 pA/ms) and had an exponential decay (2 < τ < 200 ms, 1 < τ < 50 ms for mIPSC and mEPSC, respectively).

For all microarray experiments, total RNA was hybridized to Affymetrix mouse MOE430 arrays. To identify activity-regulated genes, P0 mouse cortical neurons were cultured for 7 days and then depolarized with 50 mM KCl for 1 and 6 h. To identify Npas4-regulated genes, E16 mouse hippocampal neurons were infected (3 DIV) with Npas4-RNAi or control lentivirus, depolarized (8 DIV) with 50 mM KCl, and total RNA was collected 0, 1, 3 and 6 h later. Drebin, an Npas4 target previously identified in a neuroblastoma cell line was not affected by Npas4-RNAi in post-mitotic neurons.

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CHMP1A encodes an essential regulator of BMI1-INK4A in cerebellar development

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Charged multivesicular body protein 1A (CHMP1A; also known as chromatin-modifying protein 1A) is a member of the ESCRT-III (endosomal sorting complex required for transport-III) complex1,2 but is also suggested to localize to the nuclear matrix and regulate chromatin structure1. Here, we show that loss-of-function mutations in human CHMP1A cause reduced cerebellar size (pontocerebellar hypoplasia) and reduced cerebral cortical size (microcephaly). CHMP1A-mutant cells show impaired proliferation, with increased expression of INK4A, a negative regulator of stem cell proliferation. Chromatin immunoprecipitation suggests loss of the normal INK4A repression by BMI in these cells. Morpholino-based knockdown of zebrafish chmp1a resulted in brain defects resembling those seen after bmi1a and bmi1b knockdown, which were partially rescued by INK4A ortholog knockdown, further supporting links between CHMP1A and BMI1-mediated regulation of INK4A. Our results suggest that CHMP1A serves as a critical link between cytoplasmic signals and BMI1-mediated chromatin modifications that regulate proliferation of central nervous system progenitor cells.

As part of ongoing studies of human disorders affecting neural progenitor proliferation, we identified three families characterized by under-development of the cerebellum,pons and cerebral cortex (Fig. 1a–d). In a consanguineous pedigree of Peruvian origin, three children in two branches were affected (Fig. 1e, family 1). Two additional pedigrees from Puerto Rico showed similar pontocerebellar hypoplasia and microcephaly (Fig. 1e, families 2 and 3). Brain magnetic resonance imaging (MRI) of affected individuals from all families showed severe reduction of the cerebellar vermis and hemispheres relative to normal individuals. Notably, the cerebellar folds (folia) were relatively preserved, despite the extremely small cerebellar size (Fig. 1a–d and Supplementary Videos 1 and 2). All affected individuals had severe pontocerebellar hypoplasia, although affected individuals in family 1 showed better motor and cognitive function than those in families 2 and 3 (Supplementary Note).

Genome-wide linkage analysis of families 1 and 2 using SNP microarrays implicated only one region on chromosome 16q as linked and homozygous in all six affected individuals (Fig. 1e and Supplementary Fig. 1), with a maximum multipoint logarithm of odds (LOD) score of 3.68 (Fig. 1e). Although families 2 and 3 are not highly informative for linkage analysis, their shared homozygosity provides additional support for the involvement of this locus. Furthermore, families 2 and 3 shared the same haplotype (Supplementary Fig. 1), suggesting a founder effect. Sequencing of 42 genes within the candidate interval at 16q24.3 revealed homozygous variants predicted to be deleterious only in the CHMP1A gene. CHMP1A (NM_002786) consists of seven exons encoding a 190-amino-acid protein (Supplementary Note). Affected individuals in families 2 and 3 had a homozygous nonsense variant in exon 3 predicted to prematurely terminate translation (c.88C>T, p.Gln30*). Family 1 showed a homozygous variant in intron 2 of CHMP1A (c.28–13G>A; Fig. 2a,b) predicted to create an aberrant splice acceptor site leading to an 11-bp insertion in the spliced mRNA product (Supplementary Fig. 2a). The two mutations were absent from dbSNP, 281 neurologically normal European control DNA samples (562 chromosomes), the 1000 Genomes Project database4 and approximately 5,000 control exomes from the National Heart, Lung, and Blood Institute (NHGRI) Exome Sequencing Project.

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We sequenced CHMP1A in 64 individuals with other cerebellar anomalies without finding additional mutations, but none of these affected individuals shared the rare and distinctive pattern of hypoplasia seen in the individuals with CHMP1A mutations.

RT-PCR analysis of CHMP1A in lymphoblastoid cells from affected individuals from family 1 (CH3101 and CH3105) identified the predicted aberrant transcript with the 11-bp insertion and a second aberrant transcript with a 21-bp insertion but no normal CHMP1A transcript. (Supplementary Fig. 1). In the parents of affected children from family 1 and in unaffected control samples, only the normal transcript was detected, suggesting that the abnormal splice products are unstable. Protein blot analysis revealed a single 24-kDa band in a normal control individual, but no corresponding band was detected in affected individuals from families 1 and 2 (CH3101 and CH2401, respectively; Fig. 2e). In the parent (CH3103), the amount of CHMP1A was 50% relative to the amount detected in control lysate. Hence, this genetic study establishes CHMP1A null mutations as the cause of pontocerebellar hypoplasia and microcephaly in these pedigrees.

CHMP1A has been assigned two distinct putative functions as both a chromatin-modifying protein and a charged multivesicular

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**Figure 1** Brain MRI and linkage mapping of pontocerebellar hypoplasia with microcephaly. (a–d) T1-weighted sagittal brain MRIs of a neurologically normal individual (a) and affected individuals CH3102 (b), CH2402 (c) and CH2701 (d). Compared to control, affected individuals show mild reduction in cortical volume, thinning of the corpus callosum and severe hypoplasia of the pons, cerebellar vermis and cerebellar hemispheres. (e) Family 1 is a consanguineous pedigree from Peru in which three children from two branches are affected. Families 2 and 3 are both from Puerto Rico. Affymetrix 250K Sty SNP data for each child in families 1 and 2 are shown below (red or blue, homozygous SNP call; yellow, heterozygous SNP call), showing a region of homozygosity (dashed box) shared by all affected individuals in distal chromosome 16q. The graph aligned with the box) shared by all affected individuals in distal chromosome 16q. The graph aligned with the box.)

**Figure 2** Loss-of-function mutations in CHMP1A and dysregulation of INK4A in cell lines from affected individuals. (a) Chromatograms showing homozygous mutations (red, indicated by arrow) in intron 2 (CH3101; c.328-132G>A, white background) and in exon 3 (CH2401; c.380+T–T, black background) of CHMP1A. (b) Schematic of full-length wild-type (WT) CHMP1A. The mutation in CH2401 leads to premature termination of translation. The intronic mutation in CH3101 creates a novel splice acceptor site, and usage of this novel acceptor site causes a frameshift after exon 2, resulting in termination of translation after 36 amino acids. (c) Protein blotting of lysates from lymphoblastoid cell lines from CH2401 and CH3101 showing a complete loss of the 24-kDa band detected by antibody to CHMP1A in control lysate. Lysate from a cell line generated from CH3103 (the mother of CH3101) show 50% of the protein amount relative to the control. Protein amounts were normalized to the 40-kDa β-actin loading control bands. (d) Lymphoblastoid cell lines from CH2401 and CH3101 proliferate at a much lower rate than eight control cell lines. (e) qPCR analysis of CDKN2A-derived cDNA levels in human lymphoblastoid cell lines from CH2401 and CH3101 (normalized to GAPDH levels) shows nearly twofold higher expression of INK4A in these cells relative to four unrelated, neurologically normal control cell lines. The other transcribed isoforms at the locus, ARF, shows no significant difference in expression in cells from affected individuals and control cells. (f) ChIP-qPCR in lymphoblastoid cell lines using an antibody to BMI1 shows an approximately eightfold enrichment of INK4A promoter DNA relative to a probe targeted 7-kb upstream of the locus in a control cell line. Enrichment is nearly half of this in cell lines derived from CH2401 with a homozygous mutation in CHMP1A. Enrichment at the ARF promoter is not significantly different from that observed in the control cell line. Error bars in d, f, s.e.m.
body protein. CHMP1A was originally identified as a binding partner of the Polycomb group protein Pcl (Polyc comblike). In the nucleus, it has been suggested to recruit the Polycomb group transcriptional repressor BMI1 to heterochromatin, and overexpressed CHMP1A has been shown to arrest cells in S phase. CHMP1A has been shown to arrest cells in S phase by analysis of cell lines from two affected individuals harboring different CHMP1A mutations (CH13101 from family 1 and CH2401 from family 2), which show severely impaired doubling times compared to control cell lines, suggesting essential roles for CHMP1A in regulating cell proliferation (Fig. 2d). To examine BMI1 function in these cells, we performed quantitative PCR (qPCR) analysis of expression of the BMI1 target locus CDKN2A, which encodes alternative transcripts INK4A (also known as p16INK4A; NM_000077) and ARF (also known as p14ARF; NM_058195) in humans. This analysis revealed abnormally high expression of INK4A, the isoform implicated in cerebellar development, but not of ARF (Fig. 2e), suggesting derepression of INK4A. Chromatin immunoprecipitation (ChIP) with an antibody to BMI1 in control cell lines showed an approximately eightfold enrichment of BMI1 binding at INK4A promoter DNA relative to a control region 7 kb upstream, whereas cells from an affected individual (CH2401) showed only approximately half this enrichment in BMI1 binding (Fig. 2f). Enrichment of BMI1 at the ARF promoter was not substantial in this assay and was similar in both control cells and cell lines from affected individuals, consistent with the specificity of regulation of the INK4A isoform by BMI1 (Fig. 2f). BMI1 has been shown to suppress the Cdkn2a locus and be required for neural stem cell self-renewal. Our evidence suggests a role for CHMP1A in mediating BMI1-directed epigenetic silencing at the INK4A promoter but not at the ARF promoter.

We further explored the relationship between CHMP1A and BMI1 using morpholino-based knockdown experiments in zebrafish. Knockdown of the zebrafish CHMP1A ortholog (chmp1a; NM_200563) resulted in reduced cerebellum and forebrain volume compared to control, un.injected zebrafish, similar to the effects of human CHMP1A mutations and knockdown in zebrafish of BMI1 orthologs (bmi1a, NM_194366, and bmi1b, NM_001080751; Fig. 3a–e and Supplementary Figs. 3 and 4). A second morpholino targeting chmp1a led to a similar phenotype, and both morpholinos were partially rescued by the introduction of human CHMP1A mRNA, confirming morpholino specificity (Supplementary Fig. 4). The cerebellum consists of five major cell types, with the principal cell, known as the Purkinje cell, deriving from the ventricular epithelium, whereas granule cells derive from a separate progenitor pool known as the rhombic lip. Granule cell precursors then migrate over the outer surface of the cerebellum and form the external germinal layer (EGL) before migrating radially past the Purkinje cells to settle in the internal granule layer (IGL). Within the chmp1a-morphant cerebellum, the internal granule and molecular layers were severely affected (Fig. 3a,b), which is consistent with the relatively preserved folia pattern of the human cerebellum (thought to primarily be established by Purkinje cells) and severely reduced volume (which is determined mainly by granule cell quantity).

We then tested genetic interactions between chmp1a and the zebrafish ortholog of INK4A (cdkn2a; XM_002660468). Knockdown of cdkn2a alone did not result in noticeable abnormalities, and double knockdown of chmp1a and cdkn2a resulted in partial rescue of the brain morphology defects seen with chmp1a knockdown (Fig. 3f,g). This rescue was analogous to the rescue of the Bmi1-knockout mouse cerebellar phenotype in Bmi1- and Cdkn2a-double knockout mice. Of note, there were also parallels in brain morphology between individuals with CHMP1A mutations and Bmi1-deficient mice, which show cerebellar hypoplasia. In Bmi1-null mice, the cerebellar architecture was generally preserved, but the thickness of the granular and molecular layers was markedly reduced, and Bmi1-deficient
mice show a modest reduction in cerebral volume\(^{10,12}\), similar to individuals with CHMP1A mutations (Supplementary Note).

Subcellular localization of CHMP1A seems to vary depending on the cell type. Confocal images of NIH 3T3 cells showed prominent exclusion of Chmp1a from the nucleus (mouse Chmp1a, NM_145606), where Bmi1 was detected (Fig. 4a). In contrast, confocal images of HEK 293T cells, although also showing predominantly cytoplasmic localization of CHMP1A, showed some nuclear immunoreactivity as well (Fig. 4b). Primary cultures of cerebellar granule cells from mice also showed predominantly cytoplasmic localization of Chmp1a, along with a speckled nuclear pattern (Fig. 4c). Overexpression of HA-tagged mouse Chmp1a in cultured granule cells resulted in abundant nuclear Chmp1a with a punctate expression pattern, confirming the speckled nuclear localization of endogenous Chmp1a (Fig. 4d) and consistent with earlier reports that CHMP1A can be present in the nucleus\(^3\). Even with Chmp1a overexpression, Chmp1a and Bmi1 do not prominently colocalize within the nucleus, which is also in agreement with previous data\(^3\).

Immunohistochemical studies of the developing cerebellum and cerebral cortex in mice revealed widespread expression of Chmp1a in dividing and postmitotic cells. Chmp1a immunoreactivity was seen in the nucleus and cytoplasm of EGL, Purkinje and IGL cells at postnatal day (P) 2 (Fig. 4e, f and Supplementary Fig. 5). In the nucleus of these cells, Chmp1a immunoreactivity was seen in a speckled pattern. These speckles may be seen adjacent to Bmi1 signals, but they usually did not colocalize (Fig. 4f and Supplementary Fig. 5). At later stages of cerebellar development (P4, P10 and P29), Chmp1a expression persisted in Purkinje and granule cells (Supplementary Fig. 6). Embryonic day (E) 13.5 cerebral cortex showed widespread Chmp1a expression in the neuroepithelial cells (Fig. 4g). In the postnatal cerebral cortex (at P4, P10 and P29), Chmp1a expression in postmitotic neurons of the cortical plate gradually decreased and became almost undetectable by P29 (Supplementary Fig. 6). These expression studies confirm that Bmi1 and Chmp1a are often expressed in the same cells. However, the absence of widespread subcellular colocalization of Bmi1 and Chmp1a suggests that the regulation of Bmi1 by Chmp1a is perhaps not mediated by direct physical interaction.

Our data implicate CHMP1A as an essential central nervous system regulator of Bmi1, which in turn is a key regulator of stem cell self-renewal. The dual cytoplasmic and nuclear localization of CHMP1A and its connection to the ESCRT-III complex position CHMP1A as a potentially crucial link between cytoplasmic signals and the global regulation of stem cells via the Polycomb complex.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
G.H.M. designed the study, interpreted clinical information and brain MRIs, identified the disease locus, helped sequence candidate genes, analyzed the sequencing data to identify CHMP1A mutations, helped analyze the functional data and wrote the manuscript. V.S.G. performed RT-PCR, protein blots, mouse histology and immunohistochemistry, qPCR, ChIP and zebrafish morpholino experiments and wrote the manuscript. M.H.D.M. and H.D. ascertained family 1 and provided clinical information. K.D.A. performed zebrafish protein blots and mouse experiments and wrote the manuscript. M.I.d.M. and H.D. ascertained family 1 and provided clinical information. W.B.D. ascertained family 2 and provided clinical information. J.N.P. organized clinical information and human samples. W.H.-T.H. and L.I.Z. assisted with the morpholino experiments. R.S.H. helped organize genetic data and calculate LOD scores. J.M.F. and D.G. organized genetic data and calculate LOD scores. J.M.F. and D.G. organized genetic data and calculate LOD scores. T.H. and L.I.Z. assisted with the morpholino experiments. R.S.H. helped organize genetic data and calculate LOD scores. J.M.F. and D.G. organized human samples and helped perform sequencing experiments. D.R. organized human samples and helped perform microsatellite analysis. A.D.H. assisted in immunohistochemical studies and imaging. A.N.M. assisted in ChIP. B.J.B. and J.N.P. organized clinical information and human samples. W.H.-T.H. and L.I.Z. provided clinical information for family 3. A.I.B. interpreted the brain MRIs of the affected individuals. W.B.D. ascertained family 2 and provided clinical information. C.A.W. directed the overall research and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Genetic screening. The genetic study was approved by the Institutional Review Boards of Boston Children’s Hospital and the University of Chicago. Appropriate informed consent was obtained from all involved human subjects.

The affected individuals and their parents from family 1 and the affected individuals from family 2 were subjected to genome-wide SNP screening with the Affymetrix GeneChip Human Mapping 250k Array performed at the Microrna Core of the Dana-Farber Cancer Institute. Microsatellite markers for fine mapping were identified using the UCSC Human Genome Browser13 and were synthesized with fluorescent labels (Sigma-Genosys). Two-point and multipoint LOD scores were calculated using Allegro24, assuming recessive inheritance with full penetrance and a disease allele frequency of 0.001. Sequencing primers were designed using Primer3 (ref. 15), and genomic DNA was sequenced using standard Sanger technology. Control DNA samples from neurologically normal individuals of European descent were obtained from the Coriell Cell Repositories (Coriell Institute for Medical Research). All nucleotide sequences were verified.

For the rescue experiment, morphants were screened at 28 h.p.f. and scored for the presence of a defect in the angle of the head to the tail (measured at the otic vesicle) or a deviation in the straightness of the tail15. Human CHMP1A cDNA was PCR amplified from control human lymphoblastoid cell total RNA. Primer sequences are listed in Supplementary Table 1. The PCR product was subcloned into the pcDNA3 vector, and 5’-capped mRNA was synthesized in vitro using the mMESSAGE kit (Ambion). mRNA was diluted in 0.1 M KCl and was titrated for the rescue experiment.

For histological preparation, morphants were grown at 28 °C for 5 d, fixed overnight at 4 °C in paraformaldehyde (PFA) and embedded in 3% low-melt agarose blocks (in PBS), which were fixed again in 4% PFA in PBS overnight. The fixed agarose blocks were embedded in paraffin and sectioned at 5-μm thickness in the sagittal plane. Sections were stained by standard techniques with hematoxylin and eosin and were visualized using a bright field microscope (Nikon).

For protein blotting, zebrafish embryos were harvested at 48 h.p.f. They were dechorionated and deyolled as described16 and treated with lysin buffer (10% SDS and 0.5 M EDTA in 1× PBS) containing Complete Mini Protease Inhibitor Cocktail (Roche). Lysates were mixed with 2× Laemmli sample buffer, loaded onto a NuPage 4–12% Bis-Tris gel (Invitrogen) and run at 100 V for 2 h. Proteins were transferred onto Immobilon-P transfer membrane (Millipore) at 300 mA for 1.5 h at 4 °C. The membrane was blocked with Odyssey Blocking Buffer (LI-COR) and then incubated first with primary antibodies against CHMP1a (1:110 dilution, Abcam, ab104103) and β-actin (1:10,000 dilution, Abcam, ab6276) and then with IRDye secondary antibodies (LI-COR, 926-32212 and 926-68032). The LI-COR Imaging System was used for imaging and quantification.

Immunocytochemistry and immunohistochemistry. NIH 3T3 and HEK 293T cells were grown in DMEM with 10% FBS and 1% penicillin/streptomycin and were fixed and stained with antibodies against CHMP1a (1:200 dilution, Abcam, ab36679) and Bmi1 (1:250 dilution; Abcam, ab104103) using standard techniques. Staining was visualized with a confocal microscope (Nikon).

All animal work was approved by the Beth Israel Deaconess Medical School Institutional Animal Care and Use Committees.

Cerebral granule neuron cultures from euthanized, P5 mouse pups were prepared as described33. After dissociation, cell density was measured using a hemocytometer, and 1 × 10⁴ cells were plated on each poly-L-ornithine-coated coverslip with 500 μl of plating medium in a 24-well plate. After 1 d in vitro (d.i.v.) in a 37 °C incubator, 20 μl of 250 μM AraC (cytosine-1-β-D-arabinofuranoside) was added to each well to arrest mitosis of non-neurons. At 2 d.i.v., conditioned medium was collected from each well, and the wells were washed with DMEM. Cells were then transfected with the HA-Chmp1a mammalian expression construct (GeneCopoeia, EX-Min15085-M06). Transfection solution (87.6 μl of EBSS and 4.4 μl of 2.5 M calcium chloride with 1.5 μg of plasmid DNA) was prepared at room temperature, and 35 μl of the transfection solution was added to a total of 400 μl of conditioned medium and then added to each well. After an additional 36 h (4 d.i.v.), cells were fixed with 4% PFA for 20 min at room temperature, washed with PBS and stained with antibodies against HA (1:1000 dilution, Abcam, ab136679) and Bmi1 (1:250 dilution, Abcam, ab14389). Untransfected cells were processed similarly and were stained with antibodies against CHMP1a (1:200 dilution, Abcam, ab36679) and Bmi1.

Tissues were perfused with 4% PFA, dissected and fixed overnight in 4% PFA and were then embedded in paraffin and sectioned at 5- or 8-μm thickness. After dehydration of the slides in serial washes with xylene, 50% xylene in ethanol, 100% ethanol, 70% ethanol, 50% ethanol, 30% ethanol and finally in PBS, the slides were boiled in antigen-retrieval solution (Retrievagen A, BD Biosciences) for 8 min in the autoclave. Slides were blocked with PBS with 0.1% Triton X-100 supplemented with 1% donkey serum for 1 h at room temperature, and antibodies against Bmi1 (1:400 dilution, Millipore, clone F6), CHMP1a (1:300 dilution, Abcam, ab36679 and ab104103) or calbindin (Swant, CB300) were added in the blocking solution for overnight incubation at 4 °C. Slides were washed three times for 5 min per wash in PBS and were developed with secondary antibodies conjugated to Alexa Fluor dyes (Invitrogen) for 1.5 h at room temperature. Slides were again washed three times for 5 min per wash in PBS and were mounted with Fluoromount-G.
(Southern Biotech) containing DAPI (1:1,000 dilution) and visualized on a confocal microscope (Nikon) or fluorescence microscope (Zeiss). For E13.5 and P2 cerebral cortex, frozen section specimens were used. For frozen sections, heads of E13.5 mouse embryos were directly fixed in 4% PFA, and P2 pups were perfused with 2 ml of 1× PBS and then with 4 ml of 4% PFA in PBS, followed by overnight fixation in 4% PFA. They were then placed in gradually increasing sucrose solutions (10%, 15% and 30%), each overnight, for cryopreservation and were then embedded in optimum cutting temperature (OCT) compound (Sakura Finetek) and sectioned at 20-μm thickness. The same antigen retrieval and staining procedure was used as for the paraffin-embedded sections.


A chemical genetic approach reveals distinct EphB signaling mechanisms during brain development

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EphB receptor tyrosine kinases control multiple steps in nervous system development. However, it remains unclear whether EphBs regulate these different developmental processes directly or indirectly. In addition, given that EphBs signal through multiple mechanisms, it has been challenging to define which signaling functions of EphBs regulate particular developmental events. To address these issues, we engineered triple knock-in mice in which the kinase activity of three neurally expressed EphBs can be rapidly, reversibly and specifically blocked. We found that the tyrosine kinase activity of EphBs was required for axon guidance in vivo. In contrast, EphB-mediated synaptogenesis occurred normally when the kinase activity of EphBs was inhibited, suggesting that EphBs mediate synapse development by an EphB tyrosine kinase–independent mechanism. Taken together, our data indicate that EphBs control axon guidance and synaptogenesis by distinct mechanisms and provide a new mouse model for dissecting EphB function in development and disease.

The EphB family of receptor tyrosine kinases (RTKs) are critical regulators of cell-cell contacts in the developing nervous system, mediating processes as diverse as axon guidance, topographic mapping, neuronal migration and synapse formation1–3. In addition to these developmental roles, EphB dysfunction in the mature organism contributes to pathologies such as cancer, Alzheimer’s disease and, possibly, autism4–6. The signaling mechanisms underlying EphB-mediated development and disease are largely unknown.

As the EphB family of receptors has been shown to regulate a large number of developmental processes, it has been particularly difficult to determine the specific functions of EphBs at defined times during brain development. The presence of at least three partially redundant EphB family members in the nervous system further complicates investigation into the biological functions of EphB proteins. For example, Ephb1, Ephb2 and Ephb3 single and compound mutant mice have defects in a number of processes, including stem cell proliferation, axon guidance, filopodial motility, dendritic spine formation, synapse development and long-term potentiation, but it is unclear which of these interdependent phenotypes are direct and which are secondary to the disruption of EphB signaling at an earlier developmental step7–13.

Another major hurdle in understanding the function of EphBs is the complex nature of their signaling capabilities. EphBs can engage in bidirectional signaling with their transmembrane ligands, the ephrins. In the forward direction of signaling, the interaction of clustered ephrin-B ligands on one cell with EphB receptors on another leads to EphB oligomerization and auto-phosphorylation, the induction of EphB kinase activity, and the recruitment of cytoplasmic proteins via SH2-binding and PDZ-binding motifs of EphBs14. In addition, the extracellular region of EphBs, which contains fibronectin repeat domains, can recruit binding partners such as subunits of the NMDA subtype of glutamate receptor15,16. In the reverse direction of EphB and ephrin-B signaling, phosphorylation of the cytoplasmic tail of ephrins results in the recruitment of SH2 domain–containing proteins and initiation of downstream signal transduction17. Thus, through a complex array of potential signaling pathways, EphBs are able to mediate a wide range of processes during nervous system development.

For the most part, it remains unknown which cellular processes require EphB RTK activity and which cellular responses are mediated by EphB tyrosine kinase–independent signaling events. Cytoplasmic deletions of EphBs have been used to assess the requirement of the intracellular domain in mediating specific EphB-regulated processes, but this approach fails to distinguish kinase activity from other modes of cytoplasmic signaling18. In particular, given that ephrin-B binding to EphBs induces the formation of EphB oligomers in the plasma membrane, it remains a likely possibility that EphB oligomerization and scaffolding, in the absence of induction of EphB tyrosine kinase activity, mediates some of the biological effects of EphBs14. Thus, new ways of selectively inhibiting specific functions of EphBs are critically needed to clarify the kinase–dependent and kinase–independent mechanisms by which EphBs control specific developmental events, such as axon guidance and synapse formation.

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Mach of what is currently known about the role of EphB signaling during axon guidance in vivo comes from studies of retinal and cortical axon tracts. Notably, genetic deletion of individual or combinations of EphB family members cause marked axon guidance defects that result in the abnormal formation of several axon tracts, including the ipsilateral retinocollicular projection and axonal tracts in the corpus callosum and the anterior commissure. However, it remained to be determined whether EphB-dependent axon guidance decisions are mediated by the kinase activity of EphBs or by other modes of EphB signaling, such as PDZ-domain interactions, cytoplasmic domain oligomerization, reverse signaling through ephrin-Bs or EphB extracellular domain interactions.

In general, the mechanisms by which the cytoplasmic domains of axon guidance receptors signal growth cone attraction or repulsion have been difficult to identify. Most axon guidance receptors that have been studied to date, such as Robo, DCC, plexins and neuropilins, do not possess intrinsic kinase activity, suggesting that the predominant mode of axon guidance signaling may be kinase independent. With regard to EphBs, studies in the visual system have suggested a role for the kinase activity, whereas studies in the cortex have suggested kinase-independent roles of EphBs. However, none of these experiments were able to directly address the requirement of the kinase activity of EphBs when they are expressed at endogenous levels in vivo.

In addition to their function in axon guidance, EphBs have been shown to be important for cortical and hippocampal synapse formation. Ephb knockout mice or knock-in mice with cytoplasmic domain deletions display defects in dendritic spine and synapse development in dissociated neuronal cultures and in hippocampal slices. Overexpression of kinase-defective dominant-negative mutants also results in abnormal spine and synapse development, suggesting that the kinase activity of EphBs is involved in these processes. However, these studies did not directly test the requirement of the kinase activity of EphBs in the regulation of synapse formation under conditions in which ephrin-Bs and EphBs are expressed at physiological levels, thereby complicating the interpretation of the findings and leaving open the possibility that aspects of EphB-dependent synapse development may be kinase independent.

To address the importance of the kinase activity of EphBs for axon guidance and synapse development, we combined chemical biology with mouse genetic engineering to reversibly inhibit EphB tyrosine kinase signaling in cultured neurons, brain slices and live animals. By mutating a bulky gatekeeper residue in the ATP-binding pocket of wild-type kinases to a smaller alanine or glycine, we rendered the enzymatic catalytic function of the kinase domain. However, this gatekeeper residue, when mutated to an alanine or a glycine, can render the kinase sensitive to inhibition by PP1 analogs that cannot effectively enter the catalytic function of the kinase domain. However, this gatekeeper residue, when mutated to an alanine or a glycine, can render the kinase sensitive to inhibition by PP1 analogs that cannot effectively enter the catalytic function of the kinase domain.

RESULTS

A chemical genetic approach to studying EphB signaling

To selectively inhibit the tyrosine kinase activity of EphBs, we employed a strategy that combines the advantages of both pharmacology and genetics and in which drug sensitivity can be engineered into a protein. The ATP-binding pocket of all kinases contains a bulky hydrophobic gatekeeper residue that is not essential for the catalytic function of the kinase domain. However, this gatekeeper residue, when mutated to an alanine or a glycine, can render the kinase sensitive to inhibition by PP1 analogs that cannot effectively enter the catalytic function of the kinase domain. However, this gatekeeper residue, when mutated to an alanine or a glycine, can render the kinase sensitive to inhibition by PP1 analogs that cannot effectively enter the catalytic function of the kinase domain.

To design AS-EphB mutants, we compared the amino acid sequence of kinase domains of EphBs with those of related tyrosine kinases for which analog-sensitive versions had been successfully made. This analysis revealed a gatekeeper threonine residue in the ATP-binding pocket of mouse EphB1, EphB2 and EphB3 (Fig. 1b). We substituted these residues with either alanine or glycine to generate Ephb1T697G, Ephb2T696A and Ephb3T706A. To verify that the mutations introduced into the EphB ATP-binding pockets did not affect the kinase activity of EphBs in the absence of the PP1 analogs, we assessed the activity of these kinases using a heterologous cell culture system. Activation of EphBs results in receptor auto-phosphorylation on several juxtamembrane tyrosine residues. We previously generated an antibody that specifically recognizes the phosphorylated form of these juxtamembrane tyrosine residues for all EphBs, and used the juxtamembrane tyrosine phosphorylation detected by this antibody as a readout for receptor kinase activation.

We overexpressed EphBs in HEK 293 cells in which EphBs cluster spontaneously and become activated in a ligand-independent manner. We probed lysates from cells overexpressing AS-EphBs or wild-type EphBs with antibody to phosphorylated EphB and found that wild-type EphBs were phosphorylated at their juxtamembrane tyrosines to a similar extent (Fig. 1c). This result indicates that the analog-sensitive mutation does not affect the ability of EphBs to activate their kinase domains in the absence of PP1 analogs.

To test the ability of PP1 analogs to inhibit AS-EphBs, we treated HEK 293 cells overexpressing AS-EphBs or wild-type EphBs with either of two bulky PP1 analogs, 4-amino-1-tert-butyl-3-(1H-naphthyl)pyrazole[3,4-d]pyrimidine (1-NA-PP1) or 1-(tert-butyl)-3-(3-methylbenzyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (3-MB-PP1) (Fig. 1a) and assessed EphB tyrosine phosphorylation. We found that incubation with 1-NA-PP1 (250 nM) or 3-MB-PP1 (1 µM) blocks the phosphorylation of AS-EphBs, but not the phosphorylation of wild-type EphBs (Fig. 1c). The drug vehicle DMSO alone did not have any effect on either wild-type EphBs or AS-EphBs. These results indicate that the kinase activity of AS-EphBs was selectively inhibited by PP1 analogs. Inhibition of kinase activity of AS-EphBs was rapid, occurring in minutes (Fig. 1d). Given that PP1 analogs act competitively, inhibition was readily reversible following removal of 1-NA-PP1 (Fig. 1e).

The specificity, rapidity and reversibility of the kinase inhibition make this chemical genetic strategy an ideal platform for studying dynamic biological processes in cells and animals.

To quantify the potency and specificity of 1-NA-PP1 and 3-MB-PP1 with respect to inhibition of EphB1, EphB2 and EphB3, we conducted
dose-response analyses. We determined the half maximal inhibitory concentrations of kinase inhibition, which revealed a preference of the PP1 analogs for inhibiting analog-sensitive kinases (9–48 nM) over wild-type kinases (0.6–8.9 μM) by two orders of magnitude (Supplementary Fig. 1a,b). These results indicate that the kinase activity of AS-EphBs was selectively inhibited by PP1 analogs.

To confirm that binding of PP1 analogs to AS-EphBs does not affect kinase-independent aspects of EphB function, we performed in vitro binding assays to compare the effect of 1-NA-PP1 on tyrosine kinase–dependent and tyrosine kinase–independent protein-protein interactions. The SH2 and SH3 domain–containing adaptor protein Grb2 is a classic example of a signaling molecule that interacts with EphBs to recruit Grb2 through its SH2 domain. In contrast, binding of the PDZ domain–containing protein Pick1 to EphBs occurs through the C-terminal PDZ domain–binding motif of EphBs and does not require the tyrosine kinase activity of EphBs. In the binding experiments, we found that EphB1 bound strongly to both GST-Grb2 and GST-Pick1, but not to the negative control, GST alone (Fig. If). Notably, although 1-NA-PP1 (1 μM) completely abolished the kinase-dependent interaction between AS-EphB1 and GST-Grb2, the inhibitor treatment had no effect on the kinase-independent interaction between AS-EphB1 and GST-Pick1 (Fig. f). Thus, these observations provide evidence that 1-NA-PP1 specifically targets kinase-dependent functions of EphB proteins.

Generation and validation of AS-EphB TKI mice

Encouraged by these initial experiments, we generated knock-in mice harboring the gatekeeper mutations in EphB1 (T697G), EphB2 (T699A) and EphB3 (T706A), the three catalytically active EphB RTKs expressed in the developing brain. Ephb1<sup>T697G</sup>, Ephb2<sup>T699A</sup> and Ephb3<sup>T706A</sup> and single mutant mice were generated individually by homologous recombination in mouse embryonic stem (ES) cells (Supplementary Fig. 2a–c). ES cell clones were confirmed by sequencing the targeted alleles in ES cells (Fig. 2a).

Previous studies have found substantial functional redundancy of EphBs in several contexts<sup>4,13,22,30</sup>. To overcome potential compensation by different EphB family members, we intercrossed Ephb<sup>T697G</sup>, Ephb<sup>T699A</sup> and Ephb<sup>T706A</sup> single mutants to generate a line that is triply homozygous for each of the targeted EphB alleles, which we refer to as EphB triple knock-in (AS-EphB TKI) mice.

Although Ephb1, Ephb2 and Ephb3 triple knockout mice suffer from marked developmental defects, including morphological abnormalities of the palate and the anogenital region<sup>4,13,22,30</sup>, AS-EphB TKI mice developed normally into healthy, fertile adults, indistinguishable from wild-type mice. Notably, AS-EphB TKI brains exhibited normal expression, trafficking and ligand-mediated receptor activation in the developing brain. In contrast, AS-EphB TKI neurons develop normally in the absence of PP1 analogs. Critical to the interpretation of experiments comparing wild-type and AS-EphB TKI mice is evidence that EphB mRNA expression, trafficking and ligand-mediated receptor activation occur normally in AS-EphB TKI neurons in the absence of PP1 analogs. First, we performed quantitative PCR (qPCR) to measure expression of the Ephb1, Ephb2 and Ephb3 mRNAs in neurons from wild-type and AS-EphB TKI mice. We found that mRNAs were expressed at the same levels as in wild-type mice (Fig. 2b). We conclude that the gene targeting did not affect the expression of Ephb1, Ephb2 and Ephb3 mRNA in AS-EphB TKI neurons.
We next asked whether neurons from AS-EphB TKI mice could engage in ephrin-B–induced signaling. We cultured dissociated cortical neurons from embryonic day 16.5 to 18.5 (E16.5–18.5) AS-EphB TKI or wild-type mice and stimulated the neurons with clustered ephrin-B1 for 30 min. Western blotting of AS-EphB TKI or wild-type lysates with the antibody to phosphorylated EphB revealed that both wild-type and AS-EphB TKI neurons exhibited robust EphB activation at similar levels, indicating that AS-EphBs are fully competent to engage in ephrin-B–induced kinase signaling in the absence of PPI analogs (Fig. 3a).

To test whether the kinase function of endogenously expressed EphBs from AS-EphB TKI mice can be effectively and selectively inhibited by PPI analogs, we pre-incubated cultured E16.5–18.5 neurons with vehicle or PPI analogs for 1 h before ephrin-B1 stimulation. Treatment with 250 nM 1-NA-PP1 or 1 μM 3-MB-PP1 completely abolished ephrin-B1–induced EphB activation in the AS-EphB TKI, but not wild-type, cells, thereby demonstrating the efficacy and selectivity of these PPI analogs for AS-EphBs expressed at endogenous levels in neurons (Fig. 3a).

To more rigorously test the selectivity of PPI analogs, we assessed the effect of these inhibitors on the kinase activity of EphA4, one of the closest relatives of EphBs. We stimulated wild-type cortical neurons that had been pre-incubated with 250 nM 1-NA-PP1 or 1 μM 3-MB-PP1 completely abolished ephrin-B1–induced EphB activation in the AS-EphB TKI, but not wild-type, cells, thereby demonstrating the efficacy and selectivity of these PPI analogs for AS-EphBs expressed at endogenous levels in neurons (Fig. 3a).

To begin to address whether the kinase activity of EphBs is required for repulsive axon guidance, we asked whether the kinase activity of EphBs is required for stereovision activity that can be recapitulated in vitro. Others have suggested a role for the kinase activity of EphBs in axon guidance, others have suggested kinase-independent modes of EphB signaling4,21,24. To assess the role of EphB kinase activity in axon guidance, we initially chose the visual system because the importance of EphBs in this system is well established. During development of the visual system, most retinal ganglion cell (RGC) axons emigrating from the retina enter and cross the optic chiasm at the midline to form the contralateral retinal projections in the lateral geniculate nucleus and superior colliculus25,40. However, RGC axons from the ventrotemporal region of the retina, which express EphB1, become repelled by the ephrin-B2–expressing glia of the optic chiasm and form the ipsilateral projection that is required for stereovision42. Ephrin-B2 is thought to repel EphB1-expressing axons by inducing the collapse of their growth cones, an activity that can be recapitulated in vitro.

To block the kinase signaling of EphBs and the phosphorylation of their substrates in AS-EphB TKI cells.

**EphB RTK signaling is required for growth cone collapse**

EphBs have classically been studied in the context of axon guidance, others have suggested kinase-independent modes of EphB signaling4,21,24. Although some studies have suggested a role for the kinase activity of EphBs in axon guidance, others have suggested kinase-independent modes of EphB signaling4,21,24. To assess the role of EphB kinase activity in axon guidance, we initially chose the visual system because the importance of EphBs in this system is well established. During development of the visual system, most retinal ganglion cell (RGC) axons emigrating from the retina enter and cross the optic chiasm at the midline to form the contralateral retinal projections in the lateral geniculate nucleus and superior colliculus25,40. However, RGC axons from the ventrotemporal region of the retina, which express EphB1, become repelled by the ephrin-B2–expressing glia of the optic chiasm and form the ipsilateral projection that is required for stereovision42. Ephrin-B2 is thought to repel EphB1-expressing axons by inducing the collapse of their growth cones, an activity that can be recapitulated in vitro.
a slight reduction in explants exposed to 1-NA-PP1 or 3-MB-PP1 (Fig. 4c). This inhibitor-dependent blockade of growth cone collapse was not seen in wild-type explants; wild-type cells showed a robust collapse regardless of the presence of inhibitors, as well as a substantial reduction in average growth cone width after stimulation (Fig. 4a-c).

To verify that treatment of explants with 1-NA-PP1 or 3-MB-PP1 led to inhibition of ephrin-B-dependent EphB tyrosine phosphorylation in RGC axons, we stained explants with antibody to phosphorylated EphB. In the absence of stimulation, minimal phosphorylated EphB staining was observed in growth cones from wild-type or AS-EphB TKI explants (Fig. 4a). Following ephrin-B2 stimulation, punctate patterns of phosphorylated EphB staining were seen in the growth cone and along the axon (Fig. 4a). This staining was blocked in AS-EphB TKI explants treated with 1-NA-PP1 (250 nM) or 3-MB-PP1 (1 μM), but not in wild-type explants, indicating that PP1 analogs selectively inhibit the kinase activity of EphBs in AS-EphB TKI RGC explants (Fig. 4a).

A crucial feature of this chemical genetic approach is the reversibility of kinase inhibition. To determine whether the inhibitory effect of 1-NA-PP1 on growth cone collapse is indeed reversible, we removed 1-NA-PP1 midway through a 30-min ephrin-B2 stimulation in AS-EphB TKI explants. In the presence of 1-NA-PP1, ephrin-B–stimulated growth cones did not collapse, but did collapse rapidly after washout (Fig. 4d). This finding demonstrates the reversibility of PP1 analog inhibition of growth cone collapse in retinal explants from AS-EphB TKI mice and rules out a general health issue as a cause of reduced growth cone collapse.

Figure 3 Selective inhibition of the kinase function of EphBs in AS-EphB TKI mice. (a) Inhibition of the EphB kinase activity in AS-EphB TKI neurons. We pre-incubated 15 DIV dissociated AS-EphB TKI embryonic cortical neurons with vehicle, 250 nM 1-NA-PP1 or 1 μM 3-MB-PP1 for 1 h before a 30-min ephrin-B1 stimulation. Cell lysates were then analyzed by western blotting for phospho-EphB or β-actin. (b) Effect of PP1 analogs on the kinase activity of EphA4. We pre-incubated 4 DIV cortical neurons with vehicle, 250 nM 1-NA-PP1 or 1 μM 3-MB-PP1 for 1 h before a 30-min ephrin-A1 stimulation. Lysates were immunoprecipitated with an antibody to EphA4 and blotted for phospho-EphA4 and EphA4.

(c) Effect of PP1 analogs on ephrin-B1–induced Vav2 phosphorylation. We stimulated 4 DIV AS-EphB TKI or wild-type cortical neurons with ephrin-B1, immunoprecipitated them with an antibody to Vav2, and blotted them with either a pan-phospho-tyrosine (pY99) or Vav2 antibody. Cells were pre-incubated with vehicle, 250 nM 1-NA-PP1 or 1 μM 3-MB-PP1 for 1 h before a 30-min ephrin-B1 stimulation. 

Figure 4 The kinase function of EphBs is required for growth cone collapse in ventrotemporal (VT) retinal ganglion cells. (a) Effect of PP1 analog on RGC growth cone collapse. E14 ventrotemporal retinal explants were treated with vehicle, 250 nM 1-NA-PP1 or 1 μM 3-MB-PP1 before ephrin-B2 stimulation. Explants were stained for neurofilament (red), phospho-EphB (white) and labeled with phalloidin to visualize F-actin (green). White arrows denote clusters of phospho-EphB staining. Scale bar represents 10 μm. (b) Quantification of the percentage of collapsed growth cones from a. A two-way ANOVA revealed a significant interaction between genotype and inhibitor treatment in each condition, indicating that the effects of inhibitors were significantly greater in AS-EphB TKI neurons than in wild-type neurons (vehicle versus 1-NA-PP1: F1,32 = 6.66, P = 0.018; vehicle versus 3-MB-PP1: F1,32 = 7.41, P = 0.0026). (c) Quantification of the average growth cone width from a. A two-way ANOVA revealed a significant interaction between genotype and inhibitor treatment in each of the conditions (vehicle versus 1-NA-PP1: F1,32 = 11.75, P = 0.0027; vehicle versus 3-MB-PP1: F1,32 = 11.49, P = 0.0026). (d) Quantification of the percentage of collapsed growth cones after 1-NA-PP1 washout. Retinal explants were treated with 250 nM 1-NA-PP1 as in a, but media were removed 15 min into a 30-min ephrin-B2 stimulation and replaced with fresh media containing vehicle. The percentage of collapsed growth cones was then quantified as in b. All data are presented as mean ± s.e.m. N = 4–8 explants (biological replicates) per condition for each experiment.
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Figure 5 The kinase function of EphBs is required for the formation of the ipsilateral retinal projection in vivo. (a) Schedule of in vivo 1-NA-PP1 administration. Twice-daily subcutaneous injections of 80 mg per kg of body weight 1-NA-PP1 were administered to pregnant females from E13.5 to E16.5. (b) Representative image demonstrating the orientation of the ipsilateral and contralateral retinal projections (short red arrows) of the optic tract with respect to the optic chiasm. Scale bar represents 100 μm. (c) Representative images of DiI-filled retinal projections at E16.5. Pregnant AS-EphB and C57BL/6 wild-type mice were treated as described in a. Red arrows denote the ipsilateral projection. (d) Quantification of the ipsilateral phenotype shown in c. The ipsilateral index is defined as ipsilateral/(ipsilateral + contralateral) fluorescence intensity and normalized for each genotype (untreated AS-EphB TKI (n = 30 embryos), 1-NA-PP1–treated AS-EphB TKI (n = 30, embryos), untreated wild-type (n = 15, embryos), 1-NA-PP1–treated wild-type (n = 17, embryos)). ***P < 0.001; n.s., not significant by Student’s t test. A two-way ANOVA revealed a significant interaction between genotype and inhibitor treatment, indicating that 1-NA-PP1 treatment effects were significantly greater in AS-EphB TKI embryos than in wild-type embryos (F<sub>r<sub>6,88</sub>= 6.5, P = 0.0125). Data are presented as mean ± s.e.m. Samples are biological replicates.

EphB RTK signaling mediates retinal axon guidance in vivo

Having established the requirement of the tyrosine kinase activity of EphBs for growth cone collapse in vitro, we next asked whether this requirement is relevant for repulsive guidance in vivo, where growth cones exhibit saltatory motions rather than simple extension and collapse. In addition, growth cone collapse in vivo is mediated by a number of cues, including membrane-bound ephrin-B rather than ectopically added aggregated soluble ephrin-Bs. To determine whether the kinase activity of EphBs is required for the repulsive guidance of axons under physiological conditions of ephrin-B and EphB signaling in vivo, we examined the effect of inhibiting the kinase activity of EphBs on axon repulsion at the optic chiasm. We treated pregnant mice with 1-NA-PP1 from E13.5 to E16.5, the time at which RGC axons encounter the optic chiasm (Fig. 5a), and analyzed the retinal projections at E16.5 by DiI labeling.

To assess the degree of ipsilateral versus contralateral retinal projection, we measured the fluorescence intensity of a rectangular region in the ipsilateral projection and divided this by the total fluorescence intensity in the combined ipsilateral and contralateral projections to derive the ipsilateral index (Fig. 5b). In 1-NA-PP1–treated AS-EphB TKI embryos, we found that the ipsilateral retinal projection was strongly reduced (by 42%) compared with untreated AS-EphB TKI embryos (Fig. 5c,d). In many of the AS-EphB TKI embryos treated with 1-NA-PP1, the ipsilateral projection was absent. In contrast, 1-NA-PP1 treatment had no effect on the ipsilateral retinal projection in wild-type embryos, indicating that the observed guidance deficit was a result of specific inhibition of EphBs (Fig. 5c,d). These findings suggest that the tyrosine kinase activity of EphBs is required for axon repulsion at the optic chiasm. They also illustrate the utility of AS-EphB mice for examining the importance of the tyrosine kinase activity of EphBs in both in vitro and in vivo settings. Our results demonstrate that PP1 analogs are capable of entering the brain tissue of an intact organism and then effectively and potently inhibiting EphB tyrosine kinase activity. These analogs also completely inhibited EphB tyrosine kinase activity in neuronal cultures, making it possible to use the AS-EphB TKI neurons to investigate the role of the kinase activity of EphBs in a diverse array of neuronal functions.

EphB RTK signaling mediates corpus callosum formation

Based on the finding that EphB tyrosine kinase activity mediates axon guidance at the optic chiasm, we next asked whether a similar mechanism might be used during other axon guidance decisions. We focused on the role of the tyrosine kinase activity of EphBs in the formation of the corpus callosum. Different EphB family members have been suggested to mediate this process...
through both forward and reverse signaling, but the requirement of the kinase activity has not been tested.\textsuperscript{12,45}

To address this question, we administered 1-NA-PP1 to pregnant AS-EphB TKI mice from E12.5 to E19.0 and visualized major axon tracts by L1-CAM staining (Fig. 6a). As expected, untreated AS-EphB TKI mice had normal corpus callosum (0 of 6 mice with agenesis; Fig. 6b). However, AS-EphB TKI mice treated with 1-NA-PP1 had partial corpus callosum agenesis with a gap in the dorsal midline region (11 of 11 mice with agenesis; Fig. 6b). Wild-type mice treated with 1-NA-PP1 had a normal corpus callosum (0 of 6 mice with agenesis), indicating that the corpus callosum agenesis phenotype was specific to inhibition of AS-EphBs (Fig. 6b). These data indicate that the tyrosine kinase activity of EphBs is essential for proper formation of the corpus callosum in vivo. Taken together with our findings on retinal axon guidance, these observations suggest that tyrosine kinase-dependent signaling is likely a general mechanism by which EphB forward signaling mediates repulsive axon guidance in vivo.

EphB RTK signaling is not required for synaptogenesis

In addition to their role in axon guidance, EphBs have been shown in numerous studies to control synapse development and function\textsuperscript{2,4,13,15,16,18,25,44,45}. Experiments involving overexpression of kinase-defective EphB mutants and the use of knock-in mice containing cytoplasmic truncations of EphBs have suggested a role for kinase signaling in synaptogenesis\textsuperscript{2,4,13,15,18,25}. However, the relevance of tyrosine kinase activity of EphBs for synaptogenesis has not been examined under conditions in which ephrin-Bs and EphBs are expressed at endogenous levels and engage in physiological signaling.

The most notable synaptic defect observed after perturbation of EphB expression is the loss of dendritic spines, the sites on dendrites where most excitatory synapses form. In neurons from EphB1, EphB2 and EphB3 triple knockout mice, dendritic spine development is severely compromised\textsuperscript{13,15}. To assess the importance of EphB tyrosine kinase activity for spine development, we cultured cortical neurons from E15–17 AS-EphB TKI or wild-type embryos and treated them with 1-NA-PP1 (1 μM) between 10–21 DIV, the peak of spineogenesis. To ensure exposure to the full dose of the inhibitor during this long period, we changed the culture medium completely every 3–4 d. To assess spine density, we counted spines per unit dendritic length over multiple segments of dendrite totaling more than 50 μm. Notably, we found that 1-NA-PP1 treatment had no detectable effect on spine number and length in AS-EphB TKI or wild-type neurons (Fig. 7a,b). This result suggests that the tyrosine kinase activity of EphBs is not required for dendritic spine development in dissociated cortical neurons.

To functionally test the effect of 1-NA-PP1 on synaptogenesis, we measured miniature excitatory postsynaptic currents (mEPSCs) in dissociated cortical neurons from AS-EphB TKI mice in the presence or absence of 1-NA-PP1. These cultures were treated with...
The kinase activity of EphBs is dispensable for the formation of dendritic spines and functional excitatory synapses in organotypic hippocampal slices. Representative images of apical spines from AS-EphB TKI slices. Slices from postnatal day 5–7 (P5–7) mice were treated with vehicle or 1-NA-PP1 from 0–8 DIV. Scale bar represents 5 µm.

(a) Representative traces from recordings of mEPSCs from P5–7 AS-EphB TKI slices at 12–14 DIV. (b) Quantification of basal spine density and spine length from a wild-type (WT) and 1-NA-PP1 treatment group. All data are presented as mean ± S.E.M. Samples are independent biological replicates.

DISCUSSION

Employing a chemical genetic strategy, we engineered mice in which the kinase activity of EphBs in vivo is possible to acutely, reversibly and specifically inhibit the kinase signaling of EphBs in vitro and in vivo. We found that synaptogenesis, a process that requires EphB proteins, did not depend on the tyrosine kinase activity of EphBs. In contrast, we found a clear requirement for the tyrosine kinase activity of EphBs in the formation or maintenance of functional synapses.

1 µM 1-NA-PP1 from 3 to 10–12 DIV, and mEPSCs were measured by whole-cell electrophysiological recordings. Consistent with the spine analysis, we found no difference in mEPSC frequency or amplitude between AS-EphB TKI neurons treated with either vehicle or 1-NA-PP1 (Fig. 7c,d), which again strongly suggests that the tyrosine kinase activity of EphBs is not required for synaptogenesis, at least in dissociated neurons. To confirm that chronic 1-NA-PP1 treatment inhibits EphB tyrosine phosphorylation in these cultures, we performed western analysis on concurrent cultures and found that exposure of AS-EphB TKI neurons to 1-NA-PP1 (1 µM) resulted in a complete inhibition of ephrin-B–induced EphB auto-phosphorylation (Fig. 7e).

To determine whether the tyrosine kinase activity of EphBs might control excitatory synapse development or function in the context of a more intact neural circuit, we also examined the effect of 1-NA-PP1 inhibition on dendritic spine development, mEPSCs and evoked excitatory postsynaptic currents (eEPSCs) in organotypic hippocampal slice cultures, indicating that EphBs are important for excitatory synapse development under these conditions. However, in sharp contrast with these findings, the addition of 1-NA-PP1 to slices from AS-EphB TKI mice had no effect on dendritic spine growth (Fig. 8a–d), mEPSC characteristics (Fig. 8e,f) or eEPSC events (Fig. 8g,h). Taken together, these findings suggest that, at least under the conditions tested, EphB tyrosine kinase activity is not required for the formation or maintenance of functional synapses.
that EphB tyrosine kinase activity is required for retinal guidance is consistent with data from a previous study that found that overexpression of EphB1 is sufficient to drive retinal axons to ectopically project ipsilaterally and that this function requires an intact EphB1 kinase domain. These findings are also consistent with those of a recent study in which a knock-in mouse was generated with the intracellular region of EphB1 replaced with lacZ. This mouse displays a loss of the ipsilateral retinal projection, which indicates that EphB forward signaling is necessary for the formation of the ipsilateral retinal projection. Using AS-EphB TKI mice, we found that EphB tyrosine kinase activity is directly involved in the formation of the ipsilateral retinal projection.

Mutations of the cytoplasmic domains of EphBs or ephrin-Bs suggest that the formation of the corpus callosum is more complex than the ipsilateral retinal projection and could involve both EphB forward and reverse signaling. However, our finding that inhibition of the kinase activity of EphBs in vivo results in a highly penetrant corpus callosum agenesis phenotype provides clear evidence for the requirement of EphB kinase signaling in the development of the corpus callosum.

On the basis of our observations, we favor a model in which EphB tyrosine kinase activity is required for repulsive interactions, such as in axon guidance, but may not be required for adhesive interactions, such as axon fasciculation and synapse formation. This model is consistent with previous theories that have suggested that the amount of kinase activation predicts the strength of repulsion. Furthermore, inhibiting kinase activity in a normally repulsive context (such as axon guidance) may lead to unnatural adhesion. Thus, it will be interesting to study the fate of the misprojected axons that we observed in the optic tract and corpus callosum. It will also be important to search for any counterexamples to our model, such as an adhesive interaction that is EphB tyrosine kinase dependent. The delicate balance between the opposing functionalities of the ephrin-B and EphB signaling system underscores the importance of studying these interactions under physiological conditions.

Understanding the downstream mechanism by which the tyrosine kinase activity of EphBs controls axon guidance represents an important direction for future studies. One crucial mediator of this signaling might be the Vav family of guanine nucleotide exchange factors for the small GTPase Rac. Vav2 is known to control growth cone collapse, and Vav2 and Vav3 double mutant mice display defects in the development of the ipsilateral retinocollicular projection. We found that the tyrosine kinase activity of EphBs was required for ephrin-B-induced Vav2 tyrosine phosphorylation. A thorough investigation of how EphBs control repulsive axon guidance will require knowledge of the full complement of tyrosine kinase substrates of EphBs. As analog-sensitive kinases accept orthologous ATP analogs that can directly label the targets of the kinase, it should be possible to use AS-EphB TKI mice to identify direct kinase substrates of EphBs. Given the general limited knowledge of axon guidance mechanisms, identification of EphB substrates in the relevant neurons could be a powerful approach for uncovering these mechanisms.

Our chemical genetic approach offers several advantages over conventional genetic loss-of-function studies. Given that we were able to block the kinase activity of EphBs while leaving their scaffolding and reverse signaling capabilities intact, it was possible to dissect the role of the kinase activity of EphBs in vivo under conditions in which EphBs were expressed at physiological levels. As there are no Cre/loxP-based conditional EphB mice of any kind available, AS-EphB TKI mice represent an alternative for many experiments that require conditional regulation of EphB signaling. In addition, the reversible nature and the fine temporal control afforded by the chemical genetic approach should permit investigations into the functions of EphBs in the mature animal, such as in adult neurogenesis and synaptic plasticity, and in pathologies such as Alzheimer’s disease, autism and cancer. This new window into EphB signaling should also provide insights that are crucial for therapeutic drug development for the treatment of EphB-mediated diseases.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS


COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.
ONLINE METHODS

Animals. EphB3^T349M, EphB3^T349M and EphB3^T349M single mutants were generated individually by homologous recombination in mouse ES cells. Mice harboring each of the knock-in mutations were intercrossed to obtain the triple homozygous A5-EphB1 TKI mice.

To generate the targeting constructs, the 5′ and 3′ arms were PCR amplified from J1 ES cell DNA using primers listed in Supplementary Table 1 and subcloned into a modified pKSNeoDTA vector (originally constructed in the laboratory of P. Soriano, Mount Sinai Hospital) containing a LoxP-puro-LoxP cassette for positive selection and a diapher toxin A Negative-selection cassette (DTA). The analog-sensitive mutations were introduced by site-directed mutagenesis. The targeting constructs were confirmed by sequencing. Linearized targeting construct was electroporated into J1 ES cells, which were subsequently selected with G418. Correct targeting of ES cells was initially screened by PCR and then confirmed by Southern analysis and direct sequencing of PCR products amplified from the mutated alleles. The positive clones were karyotyped and the neomycin resistance cassette was removed by electroporating targeted ES cells with a Cre expression plasmid. ES cell clones were microinjected into C57BL6/J blastocysts to generate chimeric mice. Male chimeric animals were mated to C57BL6/J wild-type females for germline transmission of the targeted allele.

The ES cell targeting efficiencies were as follows: EphB1 knock in (17 of 192, 9%), EphB2 knock in (3 of 136, 2%) and EphB3 knock in (12 of 96, 13%). Mice were maintained as homozygotes in a mixed 129 and C57BL6/J background. Unless noted, wild-type mice were F1 offspring of a C57BL/6 × 129 mouse cross. Animals were housed under a 12-16 h light/dark cycle. No more than five animals were housed in each cage. Mice and embryos were chosen at random, regardless of sex, for treatment condition. All experiments with mice were approved by the Animal Care and Use Committee of Harvard Medical School. Ed was defined as midnight preceding the morning a vaginal plug was found.

HEK 293 cell culture and transfection. HEK 293 cells were maintained in DMEM supplemented with 10% fetal bovine serum (vol/vol, Gibco), 2 mM L-glutamine (Gibco) and penicillin/streptomycin (100 U ml⁻¹, respectively; Gibco). HEK 293 cells were transfected using the calcium phosphate method, as previously described. Antibodies and western blotting. The following antibodies were purchased commercially: pan-phospho-tyrosine pY99 mouse monoclonal (Santa Cruz), EphB1 (H-80) and EphB3 (H-85) rabbit polyclonal (Santa Cruz), β-actin mouse monoclonal (Abcam), P53-95 mouse monoclonal (Pierce), and synapsin rabbit monoclonal (Cell Signaling).

The following antibodies were purchased from Cell Signaling Technology: anti-CTB (ab105077), anti-MAP2 (ab13110) and anti-Nestin (ab17183). The following antibodies were purchased from Abcam: synapsin (ab26060), PSD-95 (ab9977). The following antibodies were purchased from Santa Cruz: EphB1 (H-80) and EphB3 (H-85), and all were used at 1:1,000 dilution. The following antibodies were purchased from Dynal: anti-MAG (ab18205), anti-MAG (ab21883) and anti-MAG (ab32041), and all were used at 1:2,000 dilution. The following antibodies were purchased from Invitrogen: anti-Nestin (ab961), anti-MAG (ab18205) and anti-MAG (ab21883), and all were used at 1:1,000 dilution.

Protein binding assays. Full-length human Grb2 and rat Pick1 were PCR amplified from plasmids #26085 and #31613 (Addgene, respectively). Grb2 and Pick1 were fused to GST at their N termini by cloning them into a pGEX vector (Pharmacia), expressed in E. coli, and affinity purified on glutathione sepharose beads. GST, used as a negative control, was expressed from the empty pGEX vector. AS-EphB1 protein was expressed in HEK 293 cells by transient transfection in DMEM supplemented with 10% fetal bovine serum (vol/vol, Gibco), 2 mM L-glutamine, 0.0012% ascorbic acid (wt/vol), 1 mg ml⁻¹ insulin, 1 mM CaCl₂, 2 mM MgCl₂, 2.3 mg ml⁻¹ glucose, 0.44 mg ml⁻¹ NaHCO₃ and 7.16 mg ml⁻¹ HEPES in MEM.

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Pharmacology. 1-NA-PP1 was synthesized as described previously and dissolved in DMSO. 3-MB-PP1 was synthesized using a similar procedure and dissolved in DMSO. Dose-response curves using 1-NA-PP1 and 3-MB-PP1 were calculated on GraphPad Prism using the least-squares method. The vehicle dose was calculated as two orders of magnitude below the lowest dose (0.05 mM). For wild-type EphBs, 100% inhibition was defined at 1 mM.

Neuronal cell culture. Cortical and hippocampal neurons were prepared from E15–17 mouse embryos as previously described. Cultured neurons were maintained in Neurobasal medium (Invitrogen) supplemented with 1× B27 (Invitrogen), penicillin/streptomycin (100 U ml⁻¹ and 100 μg ml⁻¹, respectively) and 2 mM L-glutamine. For biochemical analysis of neurons, neurons were seeded at a density of 7.5 × 10⁶ neurons per well on a six-well plate coated with polyornithine and laminin (Invitrogen).

Organotypic slices. Hippocampal organotypic slices were prepared in ice-cold dissection media (1 mM CaCl₂, 5 mM MgCl₂, 10 mM N-glutamine, 4 mM KCl, 26 mM NaHCO₃, 218 mM sucrose, 1.3 mM sodium phosphate and 30 mM HEPES, pH 7.4). Brains were isolated from P5–7 pups, and hippocampi were excised and chopped into 400-μm sections. Slices were cultured on Millicell cell culture inserts (Millipore) in media containing 20% horse serum, 1 mM L-glutamine, 0.0012% ascorbic acid (wt/vol), 1 μg ml⁻¹ insulin, 1 mM CaCl₂, 2 mM MgCl₂, 2.3 mg ml⁻¹ glucose, 0.44 mg ml⁻¹ NaHCO₃ and 7.16 mg ml⁻¹ HEPES in MEM.

Ephrin stimulation. For ephrin stimulations in dissociated cultured neurons and retinal explants, mouse ephrin-B1–Fc or ephrin-B2–Fc (R&D Systems) was pre-stimulated for 50 min with goat antibody to human IgG Fc (Jackson ImmunoResearch, 109-001-008) at 22–25 °C in phosphate-buffered saline (PBS) at a molar ratio of 1:1 before stimulation. Pre-stimulated ephrin-B1–Fc or ephrin-B2–Fc was added to the appropriate medium at a final concentration of 2.5 μg ml⁻¹. As a control, clustered human Fc in media was applied to neurons where specified.

Cell lysis and immunoprecipitation. Cultured cells were collected and homogenized in RIPA buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholanic, 0.1% SDS (wt/vol), 10 mM NaF, complete protease inhibitor cocktail tablet (Roche), 1 mM sodium orthovanadate, and phosphatase inhibitor cocktails 1 and 2 (1×, Sigma)). After clearing lysates, supernatants were incubated with the appropriate antibody for 1 h at 4 °C, followed by addition of Protein A Fastflow agarose beads (Sigma) for 1 h. Beads were washed in lysis buffer or PBS three times and eluted in 2× SDS sample buffer followed by boiling.

Surface labeling. Labeling of surface proteins was performed using the Pierce Cell Surface Protein Isolation Kit (Thermo Scientific). After chronic treatment with vehicle or 1 μM 1-NA-PP1, cultured cortical neurons were incubated with EZ-link biotin for 30 min at 22–25 °C, washed with PBS, and lysed in RIPA buffer. Lysates were immunoprecipitated with an antibody to EphB2 or a control antibody, and probed with either antibody to EphB2 or fluorescently labeled streptavidin (Invitrogen).

Retinal explants. Ventromedial segments of retina were microdissected from E14.5 mouse embryos and cultured as previously described. Embryos were removed from the uterus and decapitated, and heads were placed in ice-cold DMEM/F12 (Gibco). Ventromedial segments of the retina were excised and placed on glass coverslips coated with polyornithine and laminin. Explants were maintained in serum free medium (10 mg ml⁻¹ BSA (Sigma), 1% ITS supplement (vol/vol) (Sigma), penicillin/streptomycin (20 U ml⁻¹ and 20 μg ml⁻¹, respectively, Gibco) in DMEM/F12 supplemented with 0.2% methyl cellulose (wt/vol, Sigma) to increase media viscosity and minimize explant movement. All experiments were conducted 18–24 h after initial plating.

Immunocytochemistry and growth cone collapse assay. For experiments with inhibit the treatment, explants were pre-incubated with vehicle or PP1 analogs for 1 h, followed by a 30-min ephrin-B2 stimulation, with vehicle or PP1 analogs described in the text. Following stimulation, retinal explants were fixed for 20 min at 25 °C with 4% paraformaldehyde (PFA)/2% sucrose (wt/vol) in PBS. Explants were
then blocked in 10% goat serum (vol/vol), 0.2% Tween-20 (vol/vol) in PBS for 1 h, followed by incubation with antibody to either neurotransmitter-phospho-EphB in 50% blocking solution overnight. After PBS washes, explants were incubated in Alexa Fluor-conjugated secondary antibody (Invitrogen) and Alexa Fluor 488-conjugated phalloidin (Invitrogen). Explants on coverglasses were mounted on glass slides using Fluoromount-G (Southern Biotech). Neurons were imaged using a laser-scanning Zeiss Pascal microscope using a 40x objective with sequential acquisition settings at 1,024 × 1,024 pixel resolution. All imaging and image analysis were performed blind to the genotype and treatment condition of the samples. At least ten growth cones were analyzed per explant.

In vivo 1-NA-PP1 delivery. Pregnant wild-type or AS-EphB TKI mice were injected subcutaneously twice daily with 80 μg per kg of body weight 1-NA-PP1 dissolved in 10% DMSO, 20% Cremophor-EL and 70% saline (vol/vol) from E13.5–16.5 for optic tract experiments or from E12.5–19 for cortical tract experiments. All experiments included data from at least two separate litters of embryos per condition. Animals used for in vivo 1-NA-PP1 treatment had no prior exposure to 1-NA-PP1 or other drugs.

Dil labeling. Dil labeling was performed as previously described47. At E16.5, embryo heads were fixed in 4%PFA/2% sucrose in PBS overnight and then washed with PBS. The lens and retina were removed from the left eye and a small crystal of Dil (Invitrogen) was placed in the optic disc. The retina was then replaced securely and the heads were stored in PBS + 0.1% azide (wt/vol) at 22–25 °C for 12-24 h. After labeling, brains were removed and fluorescent optic tracts were imaged on a Leica MZ16F fluorescent stereomicroscope. Images were captured using Spot Advanced software. Labeling was quantified using Metamorph software by drawing rectangular regions of interest around the ipsilateral and contralateral tracts, subtracting background, and calculating the ipsilateral index on the basis of the integrated intensity of fluorescence: ipsilateral index = ipsilateral / (ipsilateral + contralateral). To compare wild-type and AS-EphB TKI responses with respect to 1-NA-PP1 treatment, each genotype was normalized to its untreated condition, producing a normalized ipsilateral index.

Analysis of corpus callosum phenotypes. E19.0 embryo brains were fixed in 4% PFA/2% sucrose in PBS overnight and then washed with PBS. The lens and retina were removed from the left eye and a small crystal of Dil (Invitrogen) was placed in the optic disc. The retina was then replaced securely and the heads were stored in PBS + 0.1% azide (wt/vol) at 22–25 °C for 12 days. After labeling, brains were removed and fluorescent optic tracts were imaged on a Leica MZ16F fluorescent stereomicroscope. Images were captured using Spot Advanced software. Labeling was quantified using Metamorph software by drawing rectangular regions of interest around the ipsilateral and contralateral tracts, subtracting background, and calculating the ipsilateral index on the basis of the integrated intensity of fluorescence: ipsilateral index = ipsilateral / (ipsilateral + contralateral). To compare wild-type and AS-EphB TKI responses with respect to 1-NA-PP1 treatment, each genotype was normalized to its untreated condition, producing a normalized ipsilateral index.

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qPCR. Total RNA was isolated from mouse 7 DIV cortical cultures using Trizol (Invitrogen) according to the manufacturer’s instructions. Isolated RNA was treated with DNaseI Amplification Grade (Invitrogen) and a cDNA library was synthesized by cDNA High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA was the source of input for qPCR, using a Step One Plus Real-Time PCR Instrument and SYBR Green reagents (Applied Biosystems). The relative expression plot was generated using concentration values that were normalized to corresponding actin concentrations. The following qPCR primer pairs were used: EphB1 forward, ACTGCAGAGTTGGGATGGAC; EphB1 reverse, CATATGACACTGACTTCCTCCG; EphB2 forward, TTCATGGACATCATGCT; EphB2 reverse, C2GACTGACTTCTCTTCACCG; EphB3 forward, CCGCAGGACTCTCTTTACG; EphB3 reverse, GCAATGCCGCTGTAACATGC.

Analysis of dendritic spines. For dissociated neuron experiments, cortical neurons were cultured as described above and grown on a monolayer of astrocytes on glass coverslips. Neurons were treated with vehicle or PP1 analogs at 10 DIV and media was changed entirely every 3–4 days (using neuronally pre-conditioned media). Neurons were transfected with GFP at 10 DIV using Lipofectamine 2000 and fixed with 4% PFA/2% sucrose in PBS at 21 DIV. Cells on coverslips were stained for GAP-43 and mounted on glass slides using Fluoromount-G (Southern Biotech). Neurons were imaged on a Zeiss Pascal confocal microscope, using a 63x objective, and maximal z projections were analyzed using Metamorph software. Multiple sections of dendrite totalizing >50 μm were counted for each neuron. For experiments in slice, hippocampal slices were treated with vehicle or PP1 derivatives at 2 DIV and media/drain were fully replaced every 2–3 d. At 2–3 DIV, plasmids encoding GFP (or GFP and EphB shRNAs) were biolistically transfected using a Helios gene gun. DNA bullets were prepared from 1.6-μm gold microcarrier particles (Biorad). After 8–9 DIV, slices were fixed in 3% PFA/5% sucrose in PBS for 1 h and stained with chicken antibody to GFP (Aves Labs) and rabbit antibody to NeuN (Millipore, clone A60, 1:1,000 dilution) to visualize the structure of hippocampal fields. Basal and apical dendrites were analyzed separately and sections of dendrite totalizing >50 μm were counted for each neuron.

Electrophysiology. For experiments in dissociated neurons, whole-cell voltage-clamp recordings were obtained using an Axopatch 200B amplifier at 25 °C. During recordings, neurons were perfused with artificial cerebrospinal fluid containing 127 mM NaCl, 25 mM NaHCO₃, 1.25 mM Na₂HPO₄, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 25 mM glucose, and saturated with 95% O₂, 5% CO₂. Vehicle or 1-NA-PP1 treatment was initiated at 3 DIV and continued throughout recordings. The internal solution used in all electrophysiological experiments contained 120 mM cesium methane sulfonate, 10 mM HEPES, 4 mM MgCl₂, 4 mM Na₂ATP, 0.4 mM Na₂GTP, 10 mM sodium phosphate and 1 mM EGTA. Osmolarity and pH were adjusted to 310 mOsm and 7.3 with Millipore water and CO₃H, respectively.

mEPSCs were isolated by exposing neurons to 0.5 μM tetrodotoxin, 50 μM picrotoxin, and 100 μM cytoplodihydine (all from Tocris Bioscience). Cells with series resistance larger than 25 MΩ during the recordings were discarded. Data were analyzed in IgorPro (Wavemetrics) using custom-written macros. For each trace, the event threshold was set at 1.5-fold greater than the root-mean-square current. Currents were counted as events if they crossed the event threshold, had a rapid rise time (1.5 μA ms⁻¹) and had an exponential decay (τ < 50 ms for mEPSC).

As a control for inhibition of EphB1, concurrent plates of neurons were treated with inhibitor and at the time of recording were stimulated with ephrin-B1 for 30 min. Neurons were bathed in 1×SDS-sample buffer, run on western blot and probed with rabbit antibody to phospho-Eph and mouse antibody to J-actin (Abcam).

For mEPSC experiments in organotypic hippocampal slices, whole-cell voltage-clamp recordings were made from visibly identified CA1 pyramidal neurons and the mEPSC amplitude and frequency measured. Slices were treated with vehicle or 1-NA-PP1 from 2 DIV until the time of recording. To evaluate evoked synaptic transmission, the Schäfer collaterals were depolarized with an extracellular stimulating electrode and the post synaptic eEPSC response measured from CA1 neurons. In these experiments, the artificial cerebrospinal fluid contained 4 mM Sr²⁺ instead of CaCl₂ and 4 mM MgCl₂ so that the extracellular stimulation resulted in asynchronous presynaptic vesicle fusion. The stimulus-strength was set so that the initial post synaptic response was 50-100 pA and the current amplitude and frequency of the asynchronous eEPSCs occurring 400–900 ms post-stimulation was measured. Slices were treated with vehicle or 1-NA-PP1 from 2 DIV until the time of recording. Analysis of eEPSCs was performed using custom-written macros in IgorPro.

Statistical analysis. All animal experiments contained pups from multiple litters. All imaging analyses were done blind to condition. No data points were excluded in any experiment. Unpaired t-tests and two-way ANOVA (for comparing effect of drug on AS EphB TKI versus wild-type neurons) analyses were conducted using GraphPad Prism software. All tests were two-sided (standard).

Using Whole-Exome Sequencing to Identify Inherited Causes of Autism

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SUMMARY

Despite significant heritability of autism spectrum disorders (ASDs), the extreme genetic heterogeneity has proven challenging for gene discovery. Studies of primarily simplex families have implicated de novo copy number changes and point mutations, but are not optimally designed to identify inherited risk alleles. We apply whole-exome sequencing (WES) to ASD families enriched for inherited causes due to consanguinity and find familial ASD associated...
with biallelic mutations in disease genes (AMT, PEX7, SYNE1, VPS13B, PAH, and POMMNT1). At least some of these genes show biallelic mutations in nonconsanguineous families as well. These mutations are often only partially disabling or present atypically, with patients lacking diagnostic features of the Mendelian disorders with which these genes are classically associated. Our study shows the utility of WES for identifying specific genetic conditions not clinically suspected and the importance of partial loss of gene function in ASDs.

INTRODUCTION

Despite studies suggesting that autism spectrum disorders (ASDs) are significantly heritable, the basis of this heritability remains largely unexplained (Devlin and Scherer, 2012). Autism is characterized by the triad of communication deficits, abnormal social interests, and restricted and repetitive behaviors. Genome-wide association studies (GWAS) have so far detected no strong contribution of common alleles (State, 2010), motivating renewed interest in rare variants (Malhotra and Sebat, 2012). Transmitted, rare copy number variants (CNVs), such as 16p11.2 microdeletion/duplication and 15q11.2–q13 duplication, have been found to contribute, although the total number of cases accounted for by these conditions is small (Levy et al., 2011; Pinto et al., 2010; Weiss et al., 2008). Significant roles have also been demonstrated for diverse, de novo CNVs (Levy et al., 2011; Sanders et al., 2011; Sebat et al., 2007) and more recently, de novo, protein-altering point mutations (Iossifov et al., 2012; Neate et al., 2012; O’Roak et al., 2011; O’Roak et al., 2012; Sanders et al., 2012). In the cohorts examined, de novo events may be projected to account for up to 15%–20% of ASD cases. Despite the high total rate of de novo point mutations, estimates of the number of contributing loci to autism susceptibility are in the several hundreds, so that validating specific causative genes is a significant challenge, since recurrent mutation in any given gene is so uncommon. Nonetheless, these studies have been successful at elucidating gene dosage-sensitive ASD molecular pathways, since the typical mutations observed are loss/disruption, or sometimes gain, of one functional copy of a gene or contiguous genes, rather than biallelic mutations of both copies of a gene. However, despite the importance of de novo mutations, much of the heritability of ASDs remains unaccounted for (Devlin and Scherer, 2012).

We hypothesized that at least some cases of autism reflect rare, inherited point mutations that existing study designs, often involving families with one or two affected individuals, are not designed to capture. Consanguineous and multiplex pedigrees have been extremely useful for identifying inherited mutations responsible for rare heritable conditions in the setting of extreme genetic heterogeneity, because single families can provide substantial genetic linkage evidence (Lander and Botstein, 1987; Woods et al., 2006). Applying high-throughput sequencing to such families has been extremely useful for identifying recessive causes of intellectual disability (Najmabadi et al., 2011). The potential role of biallelic mutations in ASDs is strongly supported by a number of syndromic recessive conditions that have already been associated with autistic symptoms (Betancur, 2011). Additional evidence supporting a role of biallelic mutations comes from studies that have implicated homozygous CNVs (Levy et al., 2011; Morrow et al., 2008) and long homozygous intervals as significantly associated with ASDs (Casey et al., 2012). Finally, a recent whole-exome sequencing (WES) study has suggested a role for biallelic point mutations in a subset of patients with ASDs that show long runs of homozygosity (Chahrour et al., 2012).

In this study, we apply WES to a cohort of consanguineous and/or multiplex families with ASD that also show shared ancestry between the parents, typically as cousins. We find several families where mapping and sequence analysis allow the identification of specific causative mutations and show that many of these mutations represent partial loss of function in genes where null mutations cause distinctive Mendelian disorders. These hypomorph mutations confirm the complex and heterogeneous nature of ASDs, but also highlight the importance of WES in identifying specific genetic causes underlying this heterogeneity.

RESULTS

Identifying Inherited Mutations in Three ASD Families

We studied an ASD cohort recruited by the Homozygosity Mapping Collaborative for Autism (HMCA), an international multicenter effort to identify genetically informative ASD families with consanguinity and/or multiple affected individuals (Morrow et al., 2008). We first performed genome-wide linkage analysis on the most informative families, using high-resolution single nucleotide polymorphism (SNP) arrays, reasoning that some families would show homozygous, biallelic mutations embedded within larger blocks of homozygosity inherited from the ancestor common to both parents. Families were prescreened to exclude those harboring autism-associated CNVs or other known diagnoses (Supplemental Experimental Procedures). Three families provided particularly strong genetic power to localize potential disease loci.

The first family had three children affected with ASD and two unaffected children, born to parents who were first cousins (Figure 1A; Table 1B; see Supplemental Text available online). Mapping under a single locus, biallelic model (i.e., allowing for both homozygous and compound heterozygous mutations) excluded 99.3% of the genome and revealed a single linkage peak centered at 3p21.31, in a large homozygous interval, reaching the maximum LOD score obtainable in the pedigree, 2.96 (Figure 1B), suggesting a >900:1 likelihood that the responsible mutation was contained within this homozygous interval. WES of a single affected child was performed. The linked interval contained only a single rare, nonsynonymous change that was absent from known databases and population-matched controls: a homozygous single base substitution in the aminomethyltransferase (AMT) gene, encoding an enzyme essential for the degradation of glycine. The mutation resulted in p.I108F, altering an ile residue that is highly conserved in all AMT orthologs (Figure 1C) and is packed tightly into a hydrophobic pocket.
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Inherited Causes of Autism (Figure 1D; Okamura-Ikedo et al., 2005), Sanger validation confirmed that the mutation was heterozygous in both parents, homozygous in all affected children, and absent or heterozygous in the unaffected controls.

Mutations in AMT classically cause nonketotic hyperglycinemia (NKH) (Applegarth and Toone, 2004), a neonatal syndrome leading to progressive lethargy, hypotonia, severe seizures, and death within the first year of life (Hamosh and Johnston, 2001). Patients with neonatal NKH have impaired activity of the glycine cleavage system, leading to abnormal elevation of glycine levels in serum or cerebrospinal fluid (CSF). Rarer, atypical forms of NKH have been described in association with hypomorphic mutations (Motley et al., 1997; Motley et al., 2007). These children exhibited a range of neurologic symptoms that in aggregate were strongly suggestive of NKH (Supplemental Text).

The eldest child was twelve years old and had, in addition to a diagnosis of ASD, a history of severe epilepsy, with first seizures presenting by age 10 months, very consistent with NKH. The second child was nine years old and also suffered from a combination of autism and epilepsy, though her seizures were milder. The third child was two years old, suffered from language and motor delays, and carried a presumptive PDD diagnosis. He had had only a single febrile seizure. Though the two older children had had plasma amino acid screening that disclosed no abnormalities, milder forms of NKH typically have no abnormalities on serum biochemical analyses (Applegarth and Toone, 2001; Dinopoulos et al., 2005).

Direct biochemical analysis of the p.I308F mutation confirmed that it has reduced activity. While wild-type AMT is fully soluble at 30°C when expressed in bacteria, mutant AMT p.I308F was very poorly soluble (Figure 1E), indicating a protein folding defect, similar to that observed with NKH-associated AMT mutations (Figure S1; Table S1). This defect could be rescued by co-expressing GroES and GroEL heat-shock proteins at 22°C (Figure 1E). AMT p.I308F, even after solubilization, retained only 45% (SD 4.1%) and 1.8% (SD 0.5%) of wild-type glycine cleavage and glycine synthesis specific activity, respectively, that it has reduced activity. While wild-type AMT is fully soluble (PhyH), and alkylglycerone phosphate synthase (AGPS) are imported into the peroxisome and proteolytically processed into smaller, mature forms (Figure 2F). Peroxisomal uptake is thus reflected in the ratio of the mature protein to the preprotein.

In RCDP cells, these three proteins all remain in the preprotein state, reflecting failure of peroxisomal import. Transfection of wild-type PEX7 fully restores peroxisomal import (Figure 2E). In contrast, transfection with PEX7 p.W75C failed to rescue (Figure 2E): the majority of cells showed cytosolic PTS2-mCherry, although a fraction showed partial rescue. To characterize this effect, we utilized a semiquantitative assay of peroxisomal import. The PTS2 proteins thiolase, phytanoyl-CoA hydroxylase (PhyH), and alkylglycerone phosphate synthase (AGPS) are imported into the peroxisome and proteolytically processed into smaller, mature forms (Figure 2F). Peroxisomal uptake is thus reflected in the ratio of the mature protein to the preprotein.

In RCDP cells, these three proteins all remain in the preprotein state, reflecting failure of peroxisomal import. Transfection of wild-type PEX7 fully restores peroxisomal import (Figure 2E). These results demonstrate that this allele is pathogenic, but partial loss of function, consistent with these individuals not exhibiting full features of the RCDP syndrome.

To our knowledge, a link between mid-RCDP and ASDs has not been described previously. However, two previously reported patients with biochemical evidence of RCDP and cata-

racts, but lacking the dysmorphic features of RCDP, were found to be compound heterozygous for partial loss-of-function PEX7 mutations (Braverman et al., 2002; Dinopoulos et al., 2005). One was originally described as intellectually disabled and the second as neurotypical. We recontacted these patients. A review of clinical records and reex-
amination of the first child revealed that she had subsequently been diagnosed with ASD, and the second child was diagnosed with severe ADHD, providing additional examples of the range of clinical expressivity of mild mutations in PEX7. Partial loss of
Figure 1. Identification of Mutations in AMT in a Family with ASD
(A) AU-1700, a Saudi family with three children affected by autism. Shaded symbols indicate affected individuals. The triangle represents a miscarriage. WES was performed on samples from individuals indicated with a star. Genotyping by Sanger sequencing in additional family members was performed where indicated (+, reference allele; /, alternate allele).

(B) Mapping to a locus on chromosome 3. Genome-wide linkage plot (top) and maximum obtainable LOD score in the family across the interval (bottom).

Legend continued on next page
function for one of the alleles in these patients, S25F, was verified in fibroblast assays (Figures 2E and 2F).

Analysis of a third large family pointed to a candidate autism gene potentially implicated in synaptic plasticity, SYNE1. In this family, five children were born to parents who were double first cousins. Four were affected with autism and the fifth child was unaffected (Figure 3A; Supplemental Text). The family showed linkage to two loci on chromosome 6q25 and 7q33 (LOD 2.83, maximal obtainable in the pedigree, indicating a >670:1 chance that the disease-causing gene lies in one of these intervals) (Figure 3B). WES was performed for the entire nuclear family. No rare, protein-altering variants were found in the 7q33 linkage interval, whereas 6q25 harbored only one protein-altering variant, absent from known databases and population-matched controls, that segregated with disease: a homozygous missense change in population-matched controls, that segregated with disease: a homozygous missense change in the 7q33 linkage interval, whereas 6q25 harbored only one protein-altering variant, absent from known databases and population-matched controls, that segregated with disease: a homozygous missense change in SYNE1 (p.L3206M) (Table 1). SYNE1 has previously been implicated as an ASD gene candidate by the presence of a de novo single nucleotide variant in a patient with ASD (O’Roak et al., 2011) and has been implicated in bipolar disorder in a GWAS study (Sklar et al., 2011; Figure 3C).

Truncating, presumably null, mutations in SYNE1 cause cerebellar ataxia (Gros-Louis et al., 2007) and a recessive form of arthrogryposis multiplex congenita (Attali et al., 2009; Figure 3C), again suggesting that the ASD-associated allele may be hypomorphic, since the phenotype is milder. SYNE1 p.L3206M alters a highly conserved residue that lies within a spectrum repeat (Figure 3D; SIFT score 0.01).

Full-length SYNE1 encodes a large 8,797 amino acid protein with two N-terminal actin-binding domains, multiple spectrin repeats, a transmembrane domain, and a C-terminal KASH domain. The SYNE1 mutation identified here is predicted to map to exon 61 of the full-length transcript (RefSeq NM_182961), although the SYNE1 locus is complex, with many predicted alternative splice forms (Simpson and Roberts, 2008).

To identify what human transcript(s) might be affected by the p.L3206M mutation, we mapped transcriptonial start sites in human neurons using ChIPseq (Figure 3E). ChIPseq using antibodies to H3K4Me3, a mark associated with active promoter sites (Ernst et al., 2011), and to H3K27Ac, a mark associated with active promoter sites (Heintzman et al., 2009), demonstrated mapped read peaks corresponding to at least four major transcriptional start sites within the SYNE1 locus (P1–P4), one of which (P3) lies immediately upstream of the p.L3206M mutation (Figure 3E). S’ and 3’ RACE (data not shown) confirmed the existence of at least one polyadenylated transcript emanating from this promoter, corresponding to GenBank mRNA clone BC039121, encompassing exons 57–63 of the predicted full-length SYNE1 mRNA. This is the minimal confirmed transcript that overlaps the p.L3206M mutation, although contributions of additional or even full-length transcripts cannot be excluded.

SYNE1 has been shown to have roles in cellular nuclear migration in C. elegans and Drosophila (Starr and Han, 2002; Zhang et al., 2002), anchoring of synaptic nuclei with postsynaptic membranes at the vertebrate neuromuscular junction (Grady et al., 2005), although based upon patients with SYNE1-associated cerebellar ataxia, it has been suggested that vertebrates may have compensatory mechanisms for these two processes and that SYNE1 may have adapted to perform a specialized function in the brain (Gros-Louis et al., 2007). In rodents, a spectrin-rich splice form of SYNE1 called CPG2 has been shown to control dendritic spine shape and gluta- mate receptor turnover in response to neuronal activity (Cottrell et al., 2004). To test whether SYNE1 might be responsive to neuronal activity, we performed RNAseq on cultured human primary neurons, before and after depolarization. Transcription of full-length SYNE1 was induced 1.27-fold (n = 5, SE 0.06, p = 0.0203, t test, one-tailed) by neuronal activity, and transcription of BC039121 was induced by 1.50-fold (n = 5, SE 0.11, p = 0.0225, t test, one-tailed) across five biological replicates (Figures 3E and S2). This suggests that both full-length SYNE1 and the shorter BC039121 isoform may have neuronal activity-dependent roles in regulating synaptic strength, like other synaptic genes implicated in autism.

**WES for Known Disease Genes**

Our findings of inherited, biallelic, hypomorphic ASD mutations in larger families prompted us to ask whether additional cases of ASD might be explained by either unsuspected or atypical presentations of known diseases. Over 450 genes have been identified that, when mutated, have neurocognitive impact (van Bokhoven, 2011). To increase the specificity of our analysis, we chose to analyze a limited subset of 70 of these genes, each associated with a monogenic, autosomal recessive or X-linked neurodevelopmental syndrome in which autistic features have been previously described (Table S2; Betancur, 2011). We also screened for additional alleles of AMT, PEX7, and SYNE1. We used WES to screen for mutations in these genes in a total of 163 consanguineous and/or multiplex families using established heuristic filtering for rare, high penetrance disease (Bamshad et al., 2011; Siztel et al., 2011) to identify homozygous, compound heterozygous, or hemizygous variants.

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(C) Ile508 residue is highly conserved across species.

(D) Mapping of the 103BF missense mutation onto the human AMT crystal structure (PDB accession 1WSV). (Left) Overview showing 103BF in domain 3 of AMT. (Right) Detail illustrating the hydrophobic pocket in which Ile508 resides. Neighboring hydrophobic residues are shown in white. The white brackets indicate the different domains: AMT domain 1 (folding); AMT domain 2 (catalytic); AMT domain 3 (caping).

(E) 103BF results in protein misfolding and aggregation. C-terminal 6xHis-tagged human AMT, AMT 103BF, and AMT 103BA were expressed in E. coli (Left). When overexpressed at 30°C by induction with 25 μM IPTG, wild-type AMT was fully soluble, but both 103BF and 103BA segregated to inclusion bodies despite overall similar expression levels. W, whole-cell extract; S, supernatant; P, pellet. Right panel, slower induction at 22°C for 44 hr resulted in partially soluble mutants. Near wild-type solubility could be achieved by coexpression with GroEL and GroES.

(F) AMT 103BF results in partial loss-of-function of glycine cleavage and synthesis activity. Wild-type and mutant 6xHis-human AMT were expressed in E. coli, purified, and assayed for glycine cleavage and glycine synthesis activity. Relative to wild-type (blue traces), AMT 103BF (red traces) demonstrates significant reduction of activity in glycine cleavage (left) and glycine synthesis (right) assays. R320H, N145I, and G269D are previously reported NKH-associated alleles (Okamura-Ikeda et al., 2005).

See also Figures S1, S2, and S5 and Table S1.
### Severe Mutations

<table>
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<tr>
<th>Mutation</th>
<th>Known Disease Association</th>
<th>Family</th>
<th>Structure</th>
<th>Consanguinity</th>
<th># Affected</th>
<th># Unaffected</th>
<th>Linkage</th>
<th>Primary Phenotype</th>
<th>Additional Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MECP2 p.E483X</td>
<td>Rett syndrome, ASD</td>
<td>AU-5400</td>
<td>Multiplex</td>
<td>No</td>
<td>2 (2M)</td>
<td>—</td>
<td>—</td>
<td>Yes</td>
<td>Autism</td>
</tr>
<tr>
<td>NLGN4X p.Q329X</td>
<td>Non-syndromic X-linked ID and/or ASD</td>
<td>AU-5700</td>
<td>Simplex</td>
<td>Yes</td>
<td>1 (M)</td>
<td>1</td>
<td>—</td>
<td>Yes</td>
<td>Autism</td>
</tr>
<tr>
<td>PAH p.198_205del</td>
<td>Phenylketonuria</td>
<td>AU-13100</td>
<td>Simplex</td>
<td>Yes</td>
<td>1 (M)</td>
<td>2</td>
<td>Yes</td>
<td>Autism</td>
<td>ID</td>
</tr>
<tr>
<td>PAH p.Q235X</td>
<td>Phenylketonuria</td>
<td>AU-1100</td>
<td>Simplex</td>
<td>Yes</td>
<td>2 (2F)</td>
<td>—</td>
<td>—</td>
<td>Yes</td>
<td>Autism</td>
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<tr>
<td>VPS13B p.A3943fs</td>
<td>Cohen syndrome</td>
<td>AU-21100</td>
<td>Simplex</td>
<td>Yes</td>
<td>1 (M)</td>
<td>3</td>
<td>Yes</td>
<td>Autism</td>
<td>ID, dysmorphic features, hyperextensible joints</td>
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</table>

### Hypomorphic Mutations

<table>
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<tr>
<th>Mutation</th>
<th>Known Disease Association</th>
<th>Family</th>
<th>Structure</th>
<th>Consanguinity</th>
<th># Affected</th>
<th># Unaffected</th>
<th>Linkage</th>
<th>Primary Phenotype</th>
<th>Additional Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMT p.I308F</td>
<td>Nonketotic hyperglycinemia</td>
<td>AU-1700</td>
<td>Multiplex</td>
<td>Yes</td>
<td>3 (2M, 1F)</td>
<td>2</td>
<td>Yes</td>
<td>Autism</td>
<td>ID, seizures</td>
</tr>
<tr>
<td>AMT p.D198G</td>
<td>Nonketotic hyperglycinemia</td>
<td>AU-11800</td>
<td>Simplex</td>
<td>Yes</td>
<td>1 (M)</td>
<td>1</td>
<td>Yes</td>
<td>Autism</td>
<td>ID, seizures</td>
</tr>
<tr>
<td>PEX7 p.W75C</td>
<td>Rhizomelic chondrodysplasia punctata</td>
<td>AU-3500</td>
<td>Multiplex</td>
<td>Yes</td>
<td>3 (2M, 1F)</td>
<td>3</td>
<td>Yes</td>
<td>PDD-NOS</td>
<td>ID, seizures, cataracts</td>
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<tr>
<td>POMGNT1 p.P367H</td>
<td>Muscle-eye-brain disease</td>
<td>AU-13300</td>
<td>Simplex</td>
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<td>1 (M)</td>
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<td>—</td>
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<td>Autism</td>
</tr>
<tr>
<td>SYNE1 p.L3036M</td>
<td>Autosomal recessive cerebellar ataxia type 1, arthrogryposis congenita, ASD, bipolar disease</td>
<td>AU-1600</td>
<td>Multiplex</td>
<td>Yes</td>
<td>4 (1M, 3F)</td>
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<td>Yes</td>
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<td>ID</td>
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<tr>
<td>VPS13B p.S824A</td>
<td>Cohen syndrome</td>
<td>AU-17800</td>
<td>Simplex</td>
<td>Yes</td>
<td>1 (M)</td>
<td>1</td>
<td>—</td>
<td>Yes</td>
<td>ID, dysmorphic features, hyperextensible joints</td>
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</table>

Severe (nonsense, frameshift) (A) and hypomorphic (missense) (B) mutations in known disease genes were identified in 11 ASD families. M, male; F, female; ID, intellectual disability. See also Tables S3 and S4.
with allele frequencies of less than 1% (dbSNP132, 1000 Genomes Project, NHLBI Exome Sequencing Project, and population-matched controls consisting of 831 exomes from the Middle Eastern population; see Experimental Procedures for details) and which were predicted to be protein altering (missense, nonsense, splice site, or frameshift). Candidate mutations were confirmed by Sanger sequencing in the entire family and were required to segregate with disease status within the family (i.e., homozygous or hemizygous in the affected individuals, inherited in the heterozygous state from parents, and heterozygous or absent from unaffected siblings). An overview of the analytic strategy is shown in Figure 4.

In five families (Tables 1A and S4), our screen revealed molecular genetic diagnoses due to severe loss of function (nonsense or frameshift) hemizygous or homozygous mutations in known genes. One of these was a nonsense mutation in NLGN4X (p.Q329X), found in a single affected male child. NLGN4X is an X-linked gene encoding a neuronal synaptic adhesion molecule, and mutations in NLGN4X have been described in individuals with autism, Asperger syndrome, and intellectual disability (Jamain et al., 2003). This mutation was inherited from an unaffected mother, consistent with prior observations that carrier females may be asymptomatic (Südhof, 2008; Table 1A; Figure S4).

In another family, two male children affected with autism carried a nonsense mutation in the X-linked gene MECP2 (p.E483X), the gene responsible for Rett syndrome (Table 1A). This mutation was also inherited from the unaffected mother, who was heterozygous (Figure S4). The finding of MECP2 nonsense mutations in this family was unusual since these are typically lethal in males (Chahrour and Zoghbi, 2007), suggesting that this allele is likely hypomorphic. Consistent with this idea, p.E483X is a late truncation predicted to remove only the last four amino acids of the full-length protein.

Two consanguineous families had homozygous nonsense or frameshift mutations in PAH (Table 1A; Figure S4), the cause of phenylketonuria and one of the earliest neurometabolic syndromes described as a cause of ASD (Zacavati and Spence, 2009). These families were confirmed to have phenylketonuria by clinical laboratory testing.

An additional ASD family implicated a syndrome associated with dysmorphic features and microcephaly. We found a homozygous frameshift alteration in VPS13B/COH1 in the proband in a consanguineous family who had ASD and mild dysmorphic features (Figure 5A; Table 1A). The mutation (p.A3943fs) causes truncation of the C-terminal 54 amino acids of VPS13B/COH1. Reccessive mutations in VPS13B/COH1 cause Cohen syndrome, characterized by a constellation of intellectual disability, facial dysmorphism, retinal dystrophy, truncal obesity, joint laxity, intermittent neutropenia, and postnatal microcephaly (Hennies et al., 2004) that has previously been associated with autistic symptoms in some cases (Douou and Petersen, 2011). However, significant variability in the features associated with Cohen syndrome makes clinical diagnosis challenging (Mochida et al., 2004; Seifert et al., 2006). The affected child in our cohort had several features that suggest a diagnosis of Cohen syndrome, including microcephaly (head circumference of 49 cm at age 4, <3rd percentile) and the characteristic facial dysmorphism typically seen in Cohen syndrome (Figures 5B and 5C; Supplemental Text).

In addition to severe loss-of-function mutations, a significant proportion of rare missense variants are also expected to be significantly deleterious (Kryukov et al., 2007), as underscored by our AMT and PEX7 findings. Eleven families were found to have rare, segregating, homozygous or hemizygous missense changes in known genes (Tables 1B, 53, and S4). While some of these may be expected to be functionally silent, we found clinical and/or biochemical evidence supporting their pathogenicity in at least four instances (Table 1B).

In one consanguineous ASD family, we identified a linked homozygous missense change in AMT (p.D198G) in a single affected child with ASD and intellectual disability (Table 1B). This variant was heterozygous in both parents and an unaffected sibling, and disrupts a highly conserved residue of AMT (SIFT score 0.01). Functional assays of AMT p.D198G demonstrated that, like p.I308F and other mutations, p.D198G is poorly soluble (Figure S5). AMT p.D198G also exhibited a temperature-sensitive protein stability defect (Figure S5). Enzyme specific activity was preserved (Figure S5), suggesting that pathogenicity may be due to protein misfolding/stability and not catalytic dysfunction, similar to what is observed for p.G47R, a known NKH-associated AMT mutation (Figure S1; Table S1). These findings suggest that this child may have also suffered from undiagnosed, atypical NKH.

A child affected with ASD and moderate intellectual disability was found to have a homozygous missense change (p.R367H) in POMGNT1 (Table 1B; Figure S4). POMGNT1 is responsible for an inherited dystroglycanopathy characterized by brain malformation, intellectual disability, developmental delay, hypotonia, and myopia; interestingly, rare patients have been reported with severe autistic features (Halleloju et al., 2004; Hehr et al., 2007). The p.R367H missense variant in this patient disrupts a highly conserved residue, and this exact allele has been reported as causative in a patient with relatively mild clinical disease, as a compound heterozygous mutation in combination with a splice site mutation (Godfrey et al., 2007).

Finally, in another consanguineous family, the single affected child was homozygous for a rare missense variant (p.S824A) in VPS13B (Table 1B; Figure S4). The proband had in retrospect some but not all features of Cohen syndrome (autism with mild facial dysmorphism and joint laxity), consistent with mild versions reported previously (Hennies et al., 2004).

To begin to explore how these results might extend to nonconsanguineous families, we screened for mutations in genes implicated from our cohort (AMT, PEX7, SYNE1, VPS13B, PAH, and POMGNT1) in 612 families from the Simons Simplex Collection (193 trios with parents and affected child, plus 419 quartets with parents, affected child, and unaffected sibling). An analysis of publicly released whole-exome sequence data (Lonsdorf et al., 2012; O’Roak et al., 2012; Sanders et al., 2012) showed a modest trend toward an excess of biallelic, inherited, rare (MAF < 1%), protein-altering variants in cases (8/612) compared to control siblings (2/419) (p = 0.21, Fisher’s exact test, two-tailed) in at least one of the genes we screened (Table S5). As expected for a nonconsanguineous cohort, all but one were found in the compound heterozygous state. Although functional validation
Figure 2. Identification of Mutations in PEX7 in a Family with ASD
(A) AU-3500, a family with three children affected with ASD. Shaded symbols indicate affected individuals. WES was performed on samples from individuals indicated with a star. Genotyping by Sanger sequencing in additional family members was performed where indicated (+, reference allele; –, alternate allele).

[Image: diagram of genetic markers and symbols]

(B) [Graph showing chromosomal analysis]

(C) [Table showing protein sequence comparison across species]

(D) [Graph showing protein expression levels]

(E) [Images showing protein expression under different conditions]

(F) [Images showing protein expression comparison across different conditions]

Legend continued on next page.
of all of these mutations is not available, in at least two cases, phenotype data are supportive of the mutations’ pathogenicity. One affected male child was compound heterozygous for two different mutations in VPS13B (p.W963X/p.G2704R). Gly2704 is a highly conserved residue, while p.W963X leads to early truncation of the protein and has been previously reported in Cohen syndrome (Koelhammer et al., 2004). Review of the clinical phenotype of this individual confirmed that he manifested, in addition to autism, features of Cohen syndrome including prominent microcephaly (<3 SD) and somatic dysmorphisms (Supplemental Text), making the diagnosis of a Cohen syndrome mutation highly likely. A second, unrelated male child affected with autism and compound heterozygous for two rare point mutations in VPS13B, p.S3303R and p.A3691T, both altering highly conserved residues. In addition to being autistic, this child also manifested dysmorphismis of the face and extremities as well as an abnormal hair growth pattern, known to characterize Cohen syndrome. These data confirm that biallelic mutations are also found in nonconsanguineous autism cohorts, but analysis of much larger numbers of genes and patients will be needed to quantify their prevalence.

### DISCUSSION

Our data combine WES with segregation analysis to demonstrate that biallelic, hypomorphic mutations underlie at least a subset of ASDs (Chahour et al., 2012; Morrow et al., 2008), and together with a report on biallelic null mutations in this same issue of Neuron (Lin et al., 2013), provide strong evidence for a role of inherited recessive mutations in contributing to ASD. We demonstrate the utility of our approach in identifying three new ASD genes from a relatively small sample enriched for recessive inheritance. We present three families that simplify the identification of causative genes by narrowing genetic loci to 1%–3% of the genome and allow identification of single mutations that are rare and likely to be functional. These analyses demonstrate a strategy for dissection of an otherwise highly heterogeneous disorder. We present additional evidence that biallelic mutations occur in other smaller families, as well as in European American families from the Simons Simplex Collection. As high-quality whole-exome sequencing data from additional cohorts becomes available, it will be valuable to quantify the prevalence of these biallelic mutations in ASD in general. A common theme of many of the mutations identified in this cohort is hypomorphic mutations that partially impair gene function, though one or two null mutations are also identified.

The finding of partially disabling mutations in AMT and PEX7 suggests that mild forms of neurometabolic conditions may present with autistic symptoms, although such very mild mutations may be quite rare, especially in genes in which complete knockout causes more severe syndromes, but which present with milder ASD phenotypes when partially inactivated. Exome sequencing will likely improve our ability to recognize these difficult-to-diagnose cases. Metabolic conditions are especially critical to identify since for some neurometabolic conditions, interventions may be available.

In this study, we focused on identifying rare, deleterious, penetrant variants that are causative of ASD in the families in question. Our data does not rule out contributions of common variation to ASD in other cases. While common variants are under less selective pressure than rare variants and are more likely to be benign, the functional impact of most common variants is poorly understood. Some might be expected to lie in autism gene pathways, impact biochemical function, and modify disease risk. For example, a common deletion in TMLHE, encoding the first enzyme in carnitine biosynthesis, has been recently implicated as a risk factor for autism (Celestino-Soper et al., 2012).

Genes implicated in this study include ones known to regulate or be regulated by synaptic activity (MECP2, SYN1) but also genes not traditionally thought of as having synaptic roles (AMT, PEX7, VPS13B/COH1). This could reflect an important role for nonsynaptic genes and suggest the involvement of hitherto unexpected pathways in ASDs. Alternatively, given the strength of genetic evidence implicating genes of the synapse as causative in ASDs, AMT, PEX7, and VPS13B/COH1 may have involvement in synaptic pathways that have yet to be characterized. AMT for example, regulates turnover of glycine, a crucial inhibitory neurotransmitter (Baer, 2009; Keck and White, 2008). PEX7 regulates peroxisomal protein import, and peroxisomes are abundant in dendrites (Kou et al., 2011). Finally, VPS13B/COH1 has essential roles in vesicle trafficking through the Golgi apparatus (Selbert et al., 2011). Hence, while null

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**B** Mapping to a locus on chromosome 6. Genome-wide linkage plot (top) and maximum obtainable LOD score in the family across the interval (bottom). A homozygous missense mutation in the AU-3500 family disrupts a conserved tryptophan (p.W75C) (C) in the first WD40 repeat of PEX7, the receptor responsible for import of PTS2-containing proteins into the peroxisome (D), and an established cause of rhizomelic chondrodysplasia punctata. (E) PEX7 W75C and S25F, a previously characterized mutation from a patient with mild RCDP and ASD, result in partial loss of function in a peroxisomal import assay. In fibroblasts from patients with RCDP, PTS2-tagged mCherry accumulates in the cytoplasm due to lack of PEX7 transport activity (top). Transfection of wild-type PEX7 cDNA causes PTS2-mCherry to redistribute to small puncta, indicative of restoration of peroxisomal import (bottom). Transfection of mutant PEX7 W75C or S25F does not restore complete import, with a minority of cells showing partial import (middle). Quantification of defects in peroxisomal import mediated by ASD-associated PEX7 alleles, scored by visual assay. Error bars represent standard error (right).

**F** Immunoblotting of whole cell lysates illustrates deficient maturation of PTS2-targeted proteins in a RCDP cell line transfected with ASD-associated PEX7 alleles. AGPS, PhyH, and thiolase are imported into the peroxisome and undergo cleavage into a smaller, mature peptide. p, preprotein; m, mature protein; Beta- tubulin, loading control. Quantification of processing defects by densitometry of transfected cell lysates (right).

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Figure 3. Identification of Mutations in SYNE1 in a Family with ASD

(A) AU-1600, a family with four children affected with ASD. Shaded symbols indicate affected individuals. WES was performed on samples from individuals indicated with a star. Genotyping by Sanger sequencing in additional family members was performed where indicated (+, reference allele; –, alternate allele).

(B) Mapping rules out the majority of the genome and points out to only two candidate loci, one on chromosome 6 and the other on chromosome 7. Genome-wide linkage plot (top) and maximum obtainable LOD score in the family across the interval (bottom).

(C) SYNE1 is a complex locus implicated in neuropsychiatric disease. SYNE1 encompasses multiple alternative transcripts (Known Genes, UCSC Genome Browser), and mutations have been associated with a wide range of neurodevelopmental phenotypes. The location of the recessively inherited missense change (legend continued on next page).
Inherited Causes of Autism

Neuron

In this study is definitied by the green star. Late truncation (open triangle) causes autosomal recessive atrophyoparaxis multiplex congenita (Attia et al., 2009), while truncating mutations further upstream (closed triangles) cause autosomal recessive cerebellar ataxia (Gross-Louis et al., 2007). An intronic SNP (red square) is a putative bipolar disorder susceptibility locus (Shkar et al., 2011). A de novo missense change was previously reported in ASD (blue star) (O’Roak et al., 2011).

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Figure 4. Schematic of the Analytical Pipeline

Tiered strategy for examining the cohort of ASD families for inherited mutations in known disease genes. Whole-exome sequencing data were used to systematically survey 70 genes (listed in Table S2) known to be associated with autosomal recessive or X-linked neurodevelopmental illness and autistic features. Variants were filtered based on rarity (MAF < 1%) in known databases and our internal, population-matched data set (see Experimental Procedures for details), and predicted functional effect on the protein. All candidate variants were validated by Sanger sequencing and were required to segregate within the family. XL, X-linked; AR, autosomal recessive; MAF, minor allele frequency; 1000G, 1000 Genomes Project; NHLBI EVS5000, NHLBI Exome Sequencing Project. See also Figure S4.

Exome sequencing

70 candidate XL/AR disease genes

Variant calling

Rarity filtering in known databases (MAF<1% in dbSNP, 1000G, NHLBI EVS5000)

Rarity filtering in population-matched samples (MAF<1% in 831 exomes)

Functional filtering (nonsense, frame shift, splice site, missense)

Familial segregation

Mutations in these genes have effects in many tissues, hypomorphic mutations may cause subtler defects primarily limited to neurons.

Our data support the interpretation that biallelic mutations in the proper setting can cause a spectrum of clinical phenotypes, which at one extreme cause a Mendelian disorder, but at the other extreme represent risk alleles for ASDs. In our multiplex pedigrees, siblings who share homozygosity for the identical biallelic mutation still can show a variety of phenotypes, ranging from ASD to intellectual disability, and including epilepsy and/or other features. This variable expressivity has parallels in known associations of ASD with Mendelian genes like FMR1 or TSC2, which are far more penetrant for syndromic features of Fragile X or Tuberous Sclerosis, respectively, but only partially penetrant for ASD (Hagerman et al., 2010; Watanabe, 2004). Variability of phenotype is also characteristic of recurrent ASD-associated CNVs, such as 16p11.2, which has been linked not only to ASD but also to schizophrenia, epilepsy, ADHD, and obesity (McCarthy et al., 2009; Shih et al., 2010; Walters et al., 2010; Weiss et al., 2009). The common theme of variability of phenotype despite underlying shared genetic susceptibility increasingly suggests that highly penetrant mutations associated strictly with ASD, and never with other conditions, may be extremely rare or nonexistent.

Our data extend the observation that hypomorphic alleles can commonly cause conditions that may be dramatically different from null mutations in the same gene (Walsh and Engle, 2010). Hypomorphic, biallelic mutations, combined with CNVs and heterozygous stop mutations (Neale et al., 2012; O’Roak et al., 2012; Sanders et al., 2012) which completely inactivate one of two alleles, suggest that a common theme for ASD mutations in general might be partial loss of function and/or dosage sensitivity. In other words, ASD, and potentially other neuropsychiatric conditions, may be united by incomplete loss of function of specific synaptic genes. Such incomplete loss might provide a general model for the complex genetic architecture, and genetic heterogeneity, of ASDs. In this respect, neuropsychiatric conditions may increasingly come to be understood as much by their allelic architecture as by the specific causative genes.

EXPERIMENTAL PROCEDURES

Human Studies

All human studies were reviewed and approved by the institutional review board of the Boston Children’s Hospital, Beth Israel Deaconess Medical Center, and local institutions.

Patient Recruitment

The families presented were collected by the Homozygosity Mapping Collaborative for Autism (HMCA) (Morow et al., 2009), with referral centers in Turkey, the Kingdom of Saudi Arabia, Kuwait, United Arab Emirates, Oman, Jordan, and Pakistan. Inclusion criteria included a diagnosis of autism or ASD by a neurologist, child psychiatrist, or psychologist and families with multiple affected children and/or suspected consanguinity. See Supplemental Experimental Procedures for further phenotyping details. Additional clinical information on families described here is provided in Supplemental Text.

Genome-wide Linkage and Homozygosity Scans

Genome-wide SNP screens were performed at the Broad Institute and Dana Farber Cancer Institute. Families were genotyped using Affymetrix 500K (Naspi/Rfly) or Affymetrix 6.0 microarrays. Linkage disequilibrium-based
SNP pruning was performed with PLINK, followed by filtering of loci homozygous in all samples and those with Mendelian inheritance errors. Multipoint LOD scores were calculated using MERLIN, assuming a recessive mode of disease inheritance, full penetrance, and a disease allele frequency of 0.001. Runs of homozygosity were calculated using custom Perl scripts, allowing for no more than two consecutive heterozygous SNPs in a run and three heterozygous calls in every ten consecutive SNPs. Intervals homozygous for the same haplotype and shared by all affected individuals were used to narrow the locus in each family. See Supplemental Experimental Procedures for details.

**Whole-Exome Sequencing and Data Analysis**

DNA samples were sequenced at the Broad Institute. Whole blood DNA was subject to exome capture (SureSelect v2, Agilent Technologies) and whole-exome sequence (Illumina HiSeq) was obtained on a total of 277 affected children and 409 parents, with a mean target coverage of 85.6% at ≥20x and a mean read depth of 158x. For this study, families harboring known autism-associated CNVs were excluded (Supplemental Experimental Procedures). Reads were aligned to NCBI human genome build v37 and variants were called and annotated using GATK. ANNOVAR (Wang et al., 2010) and custom pipelines. All reported variants were confirmed by Sanger sequencing. See Supplemental Experimental Procedures for additional details.

**SBC Exome Reanalysis**

Exome data from 612 families from the Simons Simplex Collection were obtained from dbGAP and NDAR. Raw sequence reads were aligned with BWA and variants were called with Samtools and annotated as previously described (Sanders et al., 2013).

**Sanger Resequencing**

See Supplemental Experimental Procedures for details.

**Data Visualization**

See Supplemental Experimental Procedures for details.

**AMT Expression and Enzymatic Assays**

Wild-type and mutant human AMT proteins with a C-terminal His6-tag were expressed and purified as previously described (Okamura-Ikeda et al., 2005). I308F, I308A, D198G, or D198A substitutions were introduced using site-directed mutagenesis, and enzymatic activities were determined as previously described (Okamura-Ikeda et al., 2010).

For heat stability studies, wild-type and mutant AMTs (about 0.5 mg/ml in 20 mM Tris-HCl [pH 7.5], 1 mM DTT, 20 mM (p-amidinophenyl) methansulfonyl fluoride, and 10% glycerol) were incubated for 1, 2, and 3 hr at 37°C and 42°C. After incubation, the solutions were centrifuged and the protein concentrations in the supernatants were determined using Coomassie Plus (Thermo Scientific, USA) with BSA as standard. The remaining protein concentrations in the supernatant were showed as a percent of the initial concentrations.

**PEX7 Peroxisomal Import Assays**

A peroxisomal import marker was generated by fusing mCherry fluorescent protein to the PTS2 signal isolated in the first 26 amino acids of rat 3-ke- toacyl-CoA thiolase (BWA and variants were called with Samtools and annotated as previously reported (Okamura-Ikeda et al., 2010)). I308F, I308A, D198G, or D198A substitutions were introduced using site-directed mutagenesis, and enzymatic activities were determined as previously described (Okamura-Ikeda et al., 2010).

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these exons. Then fold-change ratios (95% CI) of these levels were calculated for each replicate and isoform, and finally each isoform’s mean and standard error over the replicates’ fold changes.

**ChIPseq of Human Neurons**

The mini-ChIP assays were performed as previously described (Adil and Bernstein, 2011) on human neuronal cells that had been cultured for around two weeks. Briefly, cells were cross-linked, lysed, and the fragmented chromatin was then immunoprecipitated with H3K27ac (Abcam Cat# ab4728) and H3K27me3 (EMD Millipore Cat# 074490) antibodies. The ChIP DNA was recovered and precipitated following standard procedures. The ChIP DNA libraries were then constructed using ChIP-seq Sample Prep Kit (Illumina) and subsequently sequenced using HiSeq 2000 (Illumina) in Biopolymers facility at Harvard Medical School. ChIPseq reads were aligned to the human genome (GRCh37/hg19 assembly). See Supplemental Experimental Procedures for details.

**Data Access**

Whole-exome sequence data is available online (The National Database for Autism Research [NDAR] Collection ID: NDAR0001918).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures, five tables, Supplemental Experimental Procedures, and Supplemental Text and can be found with the article online at http://dx.doi.org/10.1016/j.neuron.2012.11.002.

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