THE ROLE OF IMMEDIATE EARLY GENES IN NEUROPSYCHIATRIC ILLNESS

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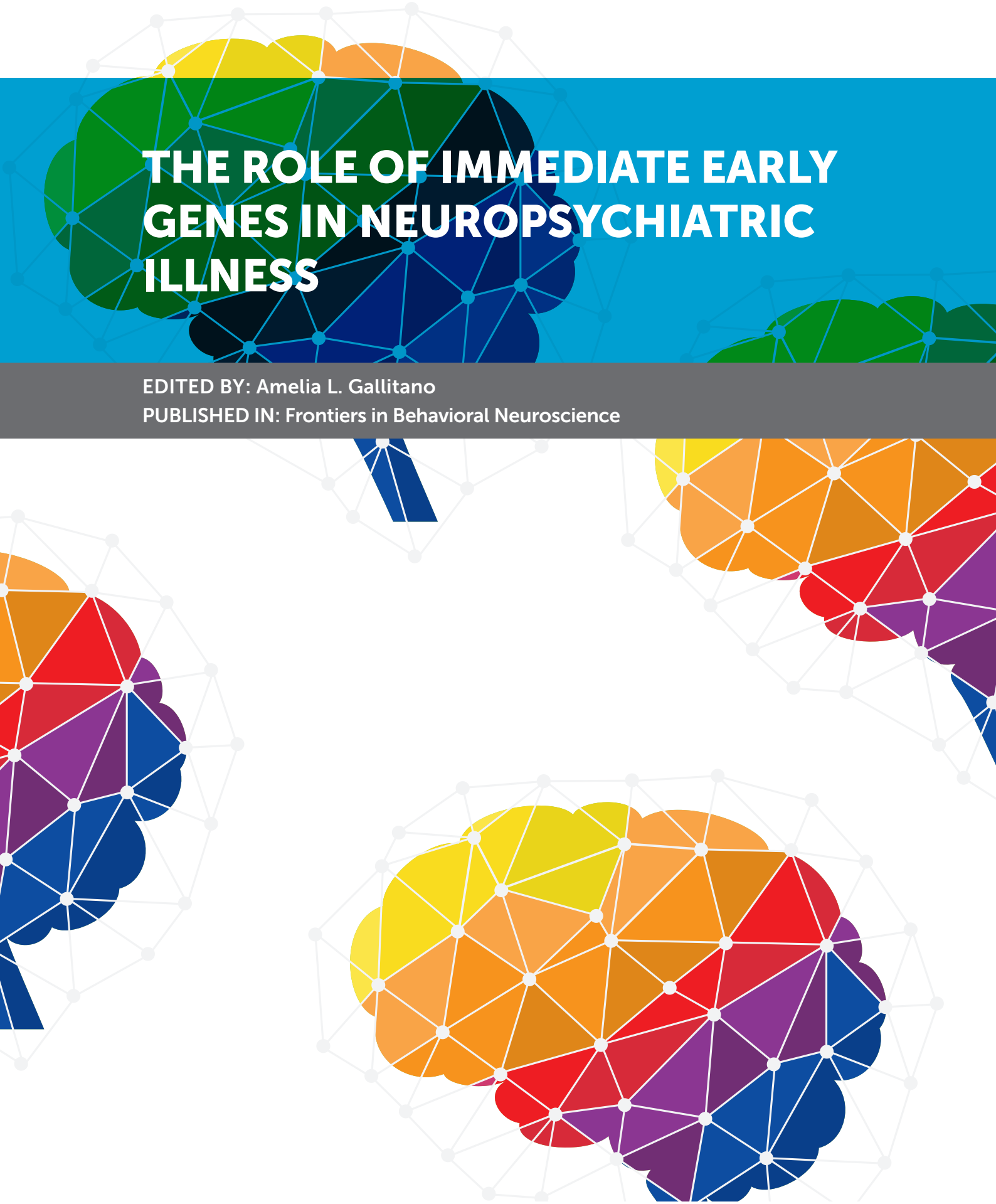

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THE ROLE OF IMMEDIATE EARLY GENES IN NEUROPSYCHIATRIC ILLNESS

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Table of Contents

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EDITORIAL
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Editorial: The Role of Immediate Early Genes in Neuropsychiatric Illness

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Keywords: immediate early gene, mental disorder, environment, stress, memory

Editorial on the Research Topic

The Role of Immediate Early Genes in Neuropsychiatric Illness

The field of psychiatry lags behind other areas of medicine in not yet having identified a single gene that causes a mental illness (excluding neuro-developmental or neurodegenerative disorders such as autism spectrum disorders or dementias). This is due to the "complex genetics" that underlie these disorders. Specifically, *many* genetic variations across the genome influence risk for neuropsychiatric illnesses, no single one of which is responsible for a large percentage of cases, and non-genomic factors play a major role in their development. Together, these form the two major challenges that the field of psychiatry faces to identify the causes of mental health disorders: (1) how so many genes can influence risk for these illnesses, and (2) how environment interacts with predisposing genetic variations to result in neuropsychiatric illness.

A category of genes called immediate early genes (IEGs) are poised to answer both of these questions. IEGs are rapidly activated in the brain in response to neuronal activity which, itself, is triggered by environmental events. Many IEGs encode transcription factors, which regulate an array of target genes that carry out the cellular response to the stimulus. Thus, IEGs are positioned at the nexus between environmental stimuli, and the molecular events that can dictate long-term changes in the brain, including processes such as synaptic plasticity and memory formation. From this position, IEGs may determine both the "gene-environment" and "multiple-gene" influences on risk to develop psychiatric illness. The research, review, and hypothesis articles comprising this Research Topic explore "The Role of Immediate Early Genes in Neuropsychiatric Illness."

One of the leading categories of environmental factors that increase risk for neuropsychiatric illnesses is stress. This term encompasses events ranging from *in utero* exposure to famine or infection, to stressful life events. Stress also activates expression of IEGs. In their Hypothesis/Review, Marballi and Gallitano describe the cascade of proteins activated in neurons in response to stress that culminate in expression of *Egr3*, a member of the Early Growth Response (*Egr*) family of IEG transcription factors. This stress-responsive "biological pathway" is essential for memory formation and synaptic plasticity. They point out that numerous genes encoding the proteins in this pathway have been associated with risk for schizophrenia, and present a model to explain how this pathway may represent a neuroprotective response to stress. Thus, variations that result in an insufficient activation of this protective stress-responsive pathway may result in the neuropathology that gives rise neuropsychiatric illness in individuals exposed to stress. However, an individual carrying the same susceptibility variations who is not exposed to significant stressors may not develop illness.

Once activated, IEGs mediate processes such as growth factor regulation, myelination, vascularization, synaptic plasticity, and memory and cognition. Notably, dysfunction in each of these areas has been implicated in mental illness pathogenesis. Two papers in the Research Topic address interactions between *Egr3* and the critical growth factor brain-derived neurotrophic factor (BDNF). In their review article, Pfaffenseller et al. integrate results from their group and others,

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showing that peripheral BDNF levels are reduced in patients experience mood disorders, with their recent findings indicating that *Egr3* is a master regulator of genes that are reduced in the prefrontal cortex of bipolar disorder patients. Prior studies showing that *Egr3* is activated downstream of BDNF, and suggesting that BDNF may play a beneficial role in bipolar disorder, lead them to propose a "feed-forward" model in which both BDNF and *Egr3* play protective roles, and are reduced in bipolar disorder.

The second of these articles reports the novel research finding that *Egr3* is required for the induction of hippocampal BDNF expression in response to electroconvulsive seizure, a known stimulus of BDNF expression Meyers et al. These results suggest that *Egr3* may regulate activity-dependent expression of BDNF, and support the model of a feed-forward pathway proposed by Pfaffenseller et al. but providing the evidence that *Egr3* is acting upstream, in addition to being activated downstream, of BDNF. The results of Meyers et al. provide a mechanism to suggest how dysfunction of *Egr3* may result in the BDNF deficiency found in bipolar disorder.

Other articles in the Research Topic address the role of IEGs in reward circuitry and addictive disorders. Manning et al. examine the role that IEGs play in stress-dependent remodeling of neural circuits involved in reward. Changes in the circuitry linking brain regions involved in pleasure, motivation, memory, decision-making and behavior is thought to influence mood disorders as well as addictive behaviors. After describing the cortico-basal ganglia reward network, they review the stress-responsive expression, and evidence for potential contributions to mood and addictive disorders, of a range of IEGs: *CREB*, *SRF*, *Egr1*, *Arc*, *NPAS4*, *Homer1a*, and the AP-1 Proteins c-fos, FosB/ΔFosB, and Jun. They conclude by posing key questions about the roles of IEGs that must be answered to advance our understanding of how these important genes may be influencing neuropsychiatric illnesses.

In their review article, Chandra and Lobo make the important point that IEGs are not simply markers of neuronal activity in response to processes but, in fact, play essential roles in the neurobiology underlying them. They focus on findings indicating that specific IEGs expressed in subsets of neurons in the striatum mediate cellular and molecular processes influencing plasticity and behavior that may mediate addiction to psychostimulants. The reviewed studies have relevance to neuropsychiatric disorders characterized by dysfunction in reward-seeking behaviors and motivational states as well as habitual behaviors.

In their research article, Muñiz et al. examine the role that caffeine plays in potentiating the effects of cocaine on reward-related learning in mice. They examined the expression of IEGs and dopamine receptor (DAR) subtypes in animals learning to associate a novel environment with drug exposure in the conditioned place preference paradigm. Their findings show that the combination of cocaine plus caffeine induced a set of IEG and DAR subtypes in the nucleus accumbens and prefrontal cortex that differ from the genes activated in response to cocaine alone. Their finding suggests that caffeine, the most widely used psychostimulant worldwide, may potentiate the effect of cocaine in reward-related memory formation.

Another group of articles address the contribution of IEGs to processes of learning and memory. Many IEGs have essential functions in memory formation and regulation of synaptic plasticity. Thus, defects in the functioning of IEGs could contribute to the cognitive symptoms characteristic of many neuropsychiatric illnesses. Duclot and Kabbaj provide a comprehensive review of the roles of a single IEG, *Egr1*, in the central nervous system. *Egr1* is an IEG transcription factor required for memory reconsolidation and the late phase of long-term potentiation (LTP), a form of synaptic plasticity (Jones et al., 2001). The authors systematically review the upstream processes that activate *Egr1*, as well as the downstream genes, pathways, and biological functions of *Egr1*. They conclude with a discussion of the role of *Egr1* in neuronal physiology, and how its dysfunction may contribute to neurological and psychiatric disorders.

In their review article, Gallo et al. address the critical role of numerous IEGs in learning, memory, and synaptic plasticity, pointing out how deficiencies in these processes may underlie the cognitive deficits characteristic of neuropsychiatric disorders. They expand their focus on *Egr1* to including the additional IEGs *c-fos* and *Arc*, covering the molecular actions of these genes in brain and their roles in learning and memory. With this framework, they then discuss the potential contribution that these IEGs may make in neuropsychiatric disorders ranging from mood disorders, to PTSD, to schizophrenia.

Datko et al. contribute an original research article in which they decipher the separate roles of the constitutively active form of the *Homer1* gene product from the IEG transcript, which encodes the protein *Homer1a*. The authors have previously reported that *Homer1a* is required for the establishment of cocaine-induced behavioral and neurochemical sensitization. In the current study of a mouse that selectively lacks *Homer1a*, they find that, rather unexpectedly, the IEG isoform is not required for spatial learning or conditioning in response to cocaine. This is in contrast to numerous other IEGs which are required for memory formation in some form.

Finally, two articles address the relationship between IEGs and the neurobiology and treatment of the psychotic disorder schizophrenia. Managò and Papaleo review the range of evidence from human and animal research studies suggesting that dysfunction of the IEG *Arc* may play a role in schizophrenia. The initial evidence arose from numerous genome-wide associations studies that identified genes that share the common feature of encoding proteins that interact with ARC. They further summarize the gene expression and association studies examining Arc, as well as findings from a family carrying a deletion that disrupts *ARC*, in addition to other genes, that has psychiatric and cognitive symptoms. The authors then employ the Research Domain Criteria (RDoC) as a framework for reviewing findings from the animal literature demonstrating functions of Arc in systems that are dysfunctional in neuropsychiatric illnesses. The authors complete their review with a summary of the roles that *Arc* plays in neurobiology, including in the dopaminergic and glutamatergic systems.

Finally, a comprehensive review by de Bartolomeis et al. addresses the clinically important question of how chronic use of antipsychotic medications causes long-term changes in the brain. While much of research examining the neurobiological effects of these medication has focused on acute changes, much less is known about the consequences of chronic treatment. IEGs have been used for decades as markers of neuronal activity. The authors review the literature examining brain regional activity following acute vs. chronic treatment with antipsychotic medications to examine effects on cognition, behavior, and the symptoms of psychosis. The authors suggest the intriguing possibility that these medication-induced changes in IEG expression may mediate the long-term neurobiological changes responsible for the therapeutic effects of antipsychotic medications.

Together these findings imply a critical role for IEGs, and the pathway of genes in which they function, in mediating the genetic and environmental influences on risk for mental illnesses. Ongoing genomics and post-mortem gene expression studies continue to reveal important findings suggesting that IEGs play a role in neuropsychiatric disorders. Studies in post-mortem brain tissue from schizophrenia patients indicate deficits in activity dependent IEGs, such as *EGR1*, and the genes that this transcription factor regulates (Ramaker et al., 2017). And studies from the ENCODE project have revealed that the network of genes that are reduced in schizophrenia patients' brains, but which correlate with antipsychotic medication treatment, are predominated by IEGs (Gandal et al., 2018). With the accelerating rate of discoveries being made with the coordinated effort of labs worldwide the field is sure to soon discover whether IEGs do, in fact, mediate the role of environmental stress on the risk to develop neuropsychiatric illnesses, and whether the genes they regulate account for a relevant proportion of the many genomic regions associated with these severe illnesses.

AUTHOR CONTRIBUTIONS

AG conceptualized and wrote the article.

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REVIEW
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The Role of Early Growth Response 1 (EGR1) in Brain Plasticity and Neuropsychiatric Disorders

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It is now clearly established that complex interactions between genes and environment are involved in multiple aspects of neuropsychiatric disorders, from determining an individual's vulnerability to onset, to influencing its response to therapeutic intervention. In this perspective, it appears crucial to better understand how the organism reacts to environmental stimuli and provide a coordinated and adapted response. In the central nervous system, neuronal plasticity and neurotransmission are among the major processes integrating such complex interactions between genes and environmental stimuli. In particular, immediate early genes (IEGs) are critical components of these interactions as they provide the molecular framework for a rapid and dynamic response to neuronal activity while opening the possibility for a lasting and sustained adaptation through regulation of the expression of a wide range of genes. As a result, IEGs have been tightly associated with neuronal activity as well as a variety of higher order processes within the central nervous system such as learning, memory and sensitivity to reward. The immediate early gene and transcription factor early growth response 1 (EGR1) has thus been revealed as a major mediator and regulator of synaptic plasticity and neuronal activity in both physiological and pathological conditions. In this review article, we will focus on the role of EGR1 in the central nervous system. First, we will summarize the different factors influencing its activity. Then, we will analyze the amount of data, including genome-wide, that has emerged in the recent years describing the wide variety of genes, pathways and biological functions regulated directly or indirectly by EGR1. We will thus be able to gain better insights into the mechanisms underlying EGR1's functions in physiological neuronal activity. Finally, we will discuss and illustrate the role of EGR1 in pathological states with a particular interest in cognitive functions and neuropsychiatric disorders.

Keywords: early growth response 1, Zif268, synaptic plasticity, memory, stress, anxiety

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INTRODUCTION

Despite a high level of heritability observed in the most common neuropsychiatric disorders, a clear genetic basis in their etiology has proven difficult to identify (Plomin et al., 1994). Rather, extensive evidence now indicates that genetic variations among the population markedly influence one's vulnerability to develop neuropsychiatric disorders and thus represent major risk factors (Burmeister et al., 2008; Lee et al., 2013). Indeed, such genetic variations can underlie differences in the integration of and response to environmental insults that can transpose into deep and lasting

neuroadaptations responsible for social, emotional and cognitive impairments characteristics of severe neuropsychiatric disorders (Caspi and Moffitt, 2006). In this context, it appears critical to better understand the molecular processes and mechanisms underlying such gene × environment interactions.

In the central nervous system, immediate early genes (IEGs) are critical mediators of gene × environment interactions and thus have been the focus of an extensive research interest in order to elucidate how environmental stimuli trigger a fast response with enduring neuroadaptations on neuronal activity and plasticity (Herdegen and Leah, 1998; Bahrami and Drabløs, 2016). Indeed, the defining characteristic of IEGs is the rapid and transient up-regulation—within minutes—of their mRNA levels independent of protein synthesis. Furthermore, this regulation can be triggered by a wide variety of stimuli through activation of general intracellular signaling pathways such as the mitogen-activated protein kinases (MAPK) or phosphoinositide 3-kinase (PI3K) pathways (Beckmann and Wilce, 1997; Fowler et al., 2011; Bahrami and Drabløs, 2016). Combined with the fact that many IEGs act as transcription factors, these features allow for a rapid and dynamic response to neuronal activity, followed by a second wave of transcriptional regulation likely to encode enduring adaptations at the synaptic and neuronal levels. Unsurprisingly, IEGs involvement in neuronal functions is widespread. In addition to representing key elements in understanding neuronal activity and physiological response to environmental stimuli, deciphering IEGs functions can provide a wealth of information on how these mechanisms are impaired in pathological conditions and thus bring novel insights into the molecular mechanisms underlying severe neuropsychiatric disorders.

Despite their widespread nature and overlap, each IEG differs in activators, upstream regulatory pathways, targets and expression pattern (Beckmann and Wilce, 1997; Herdegen and Leah, 1998; O'Donovan et al., 1999; Poirier et al., 2008; Bahrami and Drabløs, 2016). As such, early growth response 1 (EGR1) represents a particularly interesting IEG in the context of neuropsychiatric disorders due to its involvement in critical processes underlying neuronal activity, from neurotransmission and synaptic plasticity, to higher order processes such as learning and memory, response to emotional stress and reward. In this review aticle, we will thus focus on the role of EGR1 in the central nervous system in both physiological and pathological conditions. We will first briefly summarize the different factors regulating EGR1 expression, and then take advantage of recent genome-wide transcriptomic data to analyze the genes, pathways, and biological functions targeted by EGR1 in the central nervous system. Finally, we will discuss and illustrate the role of EGR1 in pathological states with a particular interest in cognitive functions and neuropsychiatric disorders.

FUNCTIONS AND REGULATIONS OF EGR1

Structure and Expression Pattern

EGR1 was first discovered and cloned almost three decades ago during a screening of genes rapidly up-regulated by nerve growth factor (NGF) in the rat PC12 cells in the presence of the protein synthesis inhibitor cyclohexamide (Milbrandt, 1987), thereby meeting criteria for an IEG. The same protein was cloned and described simultaneously by different groups in multiple cell lines stimulated by various growth factors, which explains the existence of several alternate names: EGR1 (Sukhatme et al., 1988), NGFI-A (Milbrandt, 1987), Krox-24 (Lemaire et al., 1988), TIS8 (Lim et al., 1987, 1989), and Zif268 (Christy et al., 1988). Notably, similar screening strategies led to the identification of EGR2, EGR3 and EGR4, which alongside EGR1 constitute the EGR family of IEGs (Beckmann and Wilce, 1997; O'Donovan et al., 1999).

The structural similarities and differences between all four EGR proteins have been described in details and summarized elsewhere (Beckmann and Wilce, 1997) and thus will not be extensively detailed in the current review article. Nevertheless, it is important to note that all four members of the EGR family are highly homologous both within and between species around a region containing three Cysteine2-Histidine2 (C_2H_2) zinc fingers DNA-binding domains, suggesting similarities in the DNA sequences recognized by each EGR protein and thus the possibility of overlap in their respective targets and functions (**Figure 1**). Similarly, EGR1, EGR2 and EGR3, but not EGR4, exhibit a domain of interaction with the transcriptional co-repressors NGFI-A-1/2 (NAB1 and NAB2) that, in addition to providing a negative control on the transcriptional activity of EGR proteins (Gashler et al., 1993; Russo et al., 1993, 1995; Svaren et al., 1996; Beckmann and Wilce, 1997), suggests that EGR1, EGR2 and EGR3 can lead to transcriptional repression—a role supported in part by experimental evidence *in vivo* (James et al., 2005, 2006; Duclot and Kabbaj, 2015). Interestingly, aligning the amino-acids sequences for all EGR proteins from humans, rats and mice, reveals that differences between EGR proteins are greater within species than between species, suggesting that similarities and specificities of each EGR member are evolutionary conserved. Despite this homology, however, the N-terminal region differs substantially between all four members of the EGR family, indicating specificities in protein-protein interactions and thus differences in regulation, reactivity, transcriptional control, and ultimately neuronal function (O'Donovan et al., 1999; Poirier et al., 2008).

In line with functional differences between members of the EGR family, the constitutive EGR2 knock-out is lethal whereas mice lacking EGR1 are viable despite reduced body size, sterility associated with alterations of the pituitary-gonadal axis, as well as axial myopia (Lee et al., 1995; Beckmann and Wilce, 1997; Topilko et al., 1998; Schippert et al., 2007), which indicates that EGR1 is not critically involved in prenatal development. Accordingly, EGR1 expression is undetectable in the embryonic nervous system (McMahon et al., 1990; Crosby et al., 1992), but slowly rises throughout postnatal development to reach adult expression levels by postnatal day 17 in the rat hippocampus, for instance (Watson and Milbrandt, 1990; Herms et al., 1994; Beckmann and Wilce, 1997). Interestingly, this progressive increase in EGR1 expression parallels the time of synaptic formation in cortical regions, and in the hippocampal CA1 area, corresponds closely to the period of maximal response

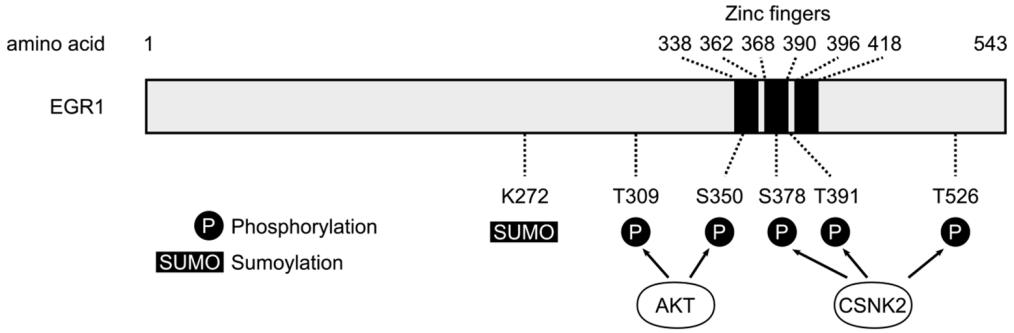

FIGURE 1 | Schematic representation of human early growth response 1 (EGR1) protein. The three zinc fingers domains of the human EGR1 protein (Uniprot #P18146) are depicted with black bars, alongside the main post-translational modification sites identified so far (P, Phosphorylation; SUMO, Sumoylation; S, Serine; T, Threonine). The T309 and S350 sites are phosphorylated by AKT (Yu et al., 2009), whereas S378, T391 and T526 represent the main sites phosphorylated by casein kinase 2 (CSNK2; Jain et al., 1996).

to N-methyl-D-aspartate (NMDA) and long-term potentiation (LTP) inducibility (Herms et al., 1994), which underscores the relationship between EGR1 expression and synaptic plasticity. In adulthood, EGR1 is expressed widely throughout the brain, and thus maintains baseline expression levels in several key areas for control of cognition, emotional response, social behavior and sensitivity to reward such as the medial prefrontal cortex (mPFC), striatum, hippocampus and amygdala (Herdegen et al., 1995; Beckmann and Wilce, 1997; Knapska and Kaczmarek, 2004).

Upstream Regulators

Signaling Pathways and Transcriptional Control

Following the original discovery of EGR1 induction following PC12 cells stimulation by NGF (Milbrandt, 1987), its expression levels were quickly linked to synaptic activity in mature neurons. In particular, *in vivo* electrical stimulations inducing long-term potentiation (LTP) also up-regulate *Egr1* mRNA levels in an NMDA receptor-dependent manner (Cole et al., 1989; Wisden et al., 1990). Similarly, *Egr1* mRNA levels rapidly and transiently increase in the rat forebrain, cerebellum and hippocampus following pharmacological induction of seizures (Saffen et al., 1988). Since then, the range of stimulations able to induce *Egr1* mRNA up-regulation has greatly expanded and includes a variety of factors linked to neurotransmission and synaptic activity. These include neurotransmitters such as glutamate and dopamine, their receptors such as NMDA or dopamine D1 receptors, as well as their respective agonists or cellular depolarization itself (Beckmann and Wilce, 1997; Herdegen and Leah, 1998; Knapska and Kaczmarek, 2004). In line with these extracellular signals, multiple intracellular signaling pathways downstream of these receptors directly regulate EGR1 expression. Similar to other IEGs (Bahrami and Drabløs, 2016), the RhoA-actin (Mullin et al., 2007), extracellular signal-regulated kinase (ERK; Sgambato et al., 1998; Davis et al., 2000) and p38 (Lim et al., 1998; Rolli-Derkinderen et al., 2003) MAPK, or PI3K (Kumahara et al., 1999) have been reported to control EGR1 expression in various systems, including neurons *in vivo* (Beckmann and Wilce, 1997; Herdegen and Leah, 1998; Knapska and Kaczmarek, 2004). Altogether, these observations would indicate that EGR1 expression can be activated upon a wide variety of stimuli, as reflected by its up-regulation following an intracellular calcium increase in hippocampal neurons (Bading et al., 1995), and support the notion that EGR1 is generally activated upon neuronal activity (**Figure 2**). While such a wide range of stimulating factors can represent a challenge in pinpointing the exact role of EGR1 in synaptic activity, this feature can be turned into an advantage by using EGR1 expression as a marker of neuronal activity allowing to map brain activation following a specific behavioral, pharmacological, or environmental event (Farivar et al., 2004; Stack et al., 2010; Okuno, 2011; Hollis et al., 2012; Duclot et al., 2016). Interestingly, EGR1's induction following neuronal activity could also prove useful in tagging neurons activated by specific stimuli which, coupled with optogenetics, for instance, offers interesting methods to study the functions of neuronal ensembles in high order brain functions (Ramirez et al., 2015; Tonegawa et al., 2015). In this context, it is particularly interesting to note that *Egr1* promoter can successfully be used in a reporter construct (Tsai et al., 2000). Combined with the specific roles of EGR1 in regulating neuronal plasticity (see "EGR1 Role in Pathological States" Section), this provides unique opportunities to investigate the neuronal ensembles underlying anxiety, stress response, and stress-related disorders.

Upon activation, these intracellular signaling pathways will engage their respective final effector(s) and transcription factor(s) to directly regulate *Egr1* gene transcription. Induction of the p38 and ERK MAPK pathways, for instance, leads to activation of the Ets-like-1 (Elk1) and cyclic AMP-response element binding protein (CREB) transcription factors, which can bind their respective response elements located in the *Egr1* promoter (Tur et al., 2010). In addition to these serum response elements (SRE) and cAMP response element (CRE), several other binding sites for key transcription factors were identified on the *Egr1* promoter: specificity protein 1 (Sp1), activator protein-1 (AP-1), nuclear factor kappa B (NFκB),

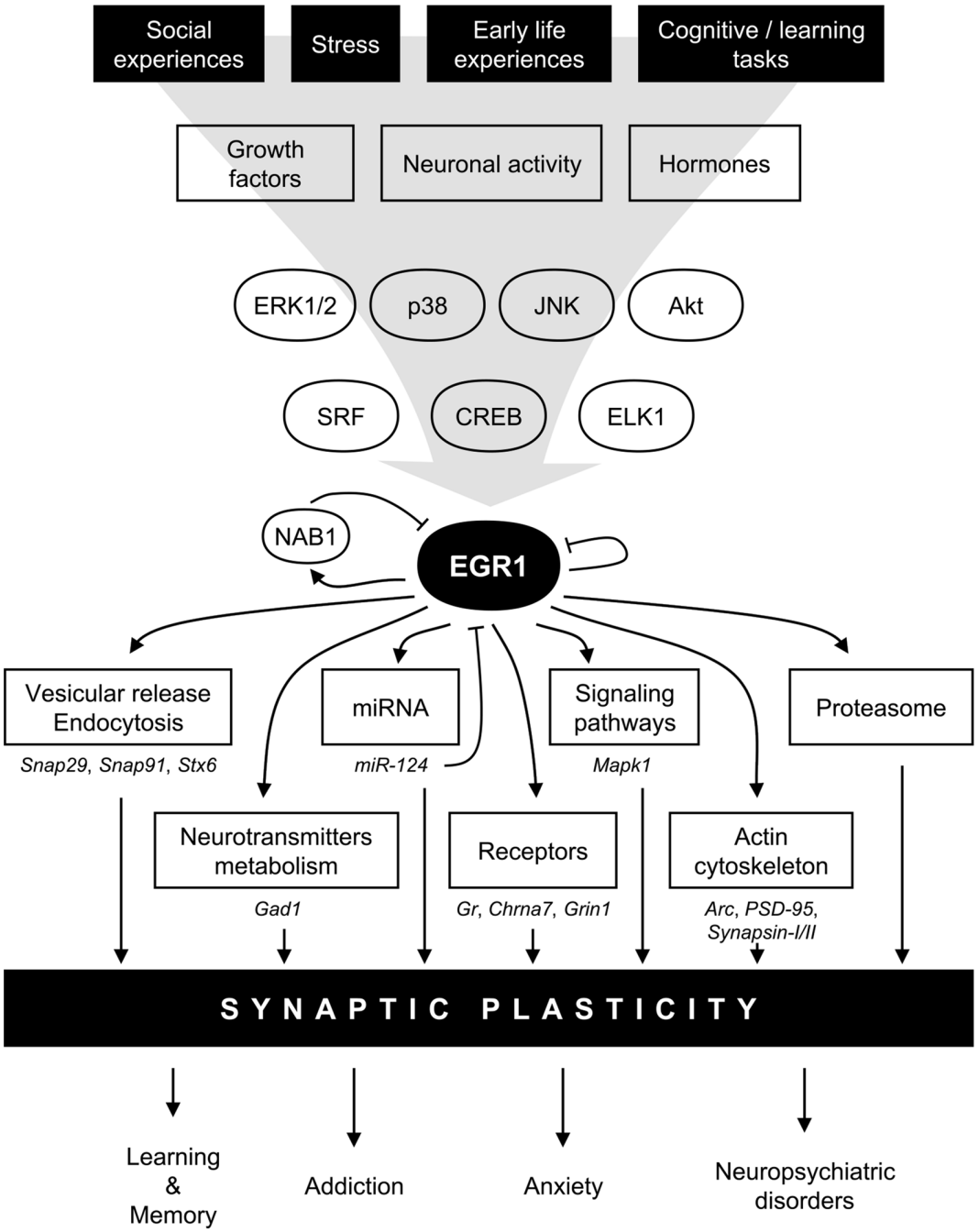

FIGURE 2 | Model for EGR1 regulations and functions in the central nervous system in the context of synaptic plasticity. In response to various stimuli such as stress or learning tasks triggering growth factors release, hormones secretion, or neuronal activity, several intracellular signaling pathway including mitogen-activated protein kinases (MAPK) or AKT are activated. Transcription factors such as serum response factor (SRF), cyclic AMP-response element binding protein (CREB), or Ets-like-1 (ELK1), are thus induced and rapidly regulate *Egr1* transcription. EGR1 can in turn directly regulate a wide array of transcriptional targets related to multiple biological functions related to synaptic plasticity: vesicular release and endocytosis, neurotransmitters metabolism, micro-RNA (miRNA), receptors, signaling pathways, actin cytoskeleton, as well as component of the proteasome complex. A few validated EGR1 targets are depicted under each biological functions. Through such a wide array of direct transcriptional targets, EGR1 can thus regulate multiple aspects of synaptic plasticity, and thus orchestrate the integration of environmental stimuli at the synaptic plasticity level to modulate relevant high order processes such as learning and memory, addiction, anxiety, and neuropsychiatric disorders. Finally, several negative feedback mechanisms are also engaged, either directly through EGR1 itself, or indirectly through its direct targets such as NAB1 or miR-124. *Arc*, activity-regulated cytoskeleton-associated protein; *Chrna7*, cholinergic receptor nicotinic alpha 7 subunit; ERK1/2, extracellular signal-regulated kinase (ERK)s 1/2; *Gad1*, glutamate decarboxylase 1; *Gr*, Glucocorticoids receptor; *Grin1*, glutamate ionotropic receptor N-methyl-D-aspartate (NMDA) type subunit 1, JNK, Jun N-terminal kinase; *Mapk1*, mitogen activated protein kinase 1; NAB1, NGFI-A-1; *PSD-95*, Postsynaptic density protein 95; *Snap29*, synaptosomal-associated protein 29; *Snap91*, synaptosomal-associated protein 91; *Stx6*, syntaxin 6.

or EGR1 itself (Knapska and Kaczmarek, 2004; Tur et al., 2010). While most of these factors are generally considered as positive regulators of transcription, this view is challenged by the bivalent role of Elk1, for instance, either promoting transcription through recruitment of histone acetyltransferases (Li et al., 2003), or repressing transcription through recruitment of histone deacetylases (HDAC; Yang et al., 2001). Similarly, EGR1 binding to its own promoter represses its transcription (Cao et al., 1993).

The complexity of *Egr1* transcriptional regulation can be resolved, however, when accounting for kinetics and interactions between transcription factor binding, cofactors recruitment and chromatin dynamics including histone methylation, acetylation and phosphorylation, as well as nucleosome positioning. Indeed, by focusing on *Egr1* gene transcription in MLP29 mouse progenitor cells, Riffo-Campos et al. (2015) propose a model in which Elk1, CREB and EGR1 interact in a timely manner to allow for a quick and transient activation of *Egr1* transcription. Following application of phorbol esters in this system, EGR1 expression is induced within minutes, peaks at 30 min post-application, and returns to baseline levels by 180 min (Tur et al., 2010; Riffo-Campos et al., 2015). Prior to treatment with phorbol esters, three components of HDAC complexes, mSin3, HDAC3 and N-CoR are present on the *Egr1* promoter (Tur et al., 2010). Interestingly, however, CREB, Elk1, SRF and RNA-PolII are also found at the promoter even prior to its induction, explained in part by a favorable nucleosome positioning (Riffo-Campos et al., 2015), which thus suggests that, similar to other IEGs (Bahrami and Drabløs, 2016), *Egr1* transcription is poised at baseline. Induction by phorbol esters, however, triggers characteristic nucleosome repositioning events with partial eviction of the +1 and −1 nucleosomes, as well as downstream sliding of the −2 nucleosome at the 15 min timepoint. At the same time, *Egr1*-promoter bound CREB and Elk1 are phosphorylated in a p38- and MEK1/2-dependent manner (Tur et al., 2010), resulting in an increase in phosphoacetylation (pS10AcK14) and acetylation (AcK14) of histone H3 at the +1 nucleosome (Riffo-Campos et al., 2015). Such acetylation events are likely mediated by the histone acetyltransferase activity of the transcriptional cofactor CREB-binding protein (CBP) as its binding to the mouse *Egr1* promoter increases in parallel with its transcription (Tur et al., 2010). As a result, RNA-PolII recruitment rises and promotes *Egr1* transcription in a rapid manner. Simultaneously, however, the downstream sliding of the −2 nucleosome partly uncovers an EGR1 recognition site located slightly upstream and thus allows EGR1 binding to its own promoter (Riffo-Campos et al., 2015), which in turn leads to the progressive recruitment of the transcriptional repressors NAB1 and NAB2 peaking from 30 min–60 min following induction (Tur et al., 2010). As NAB2 is known to interact with the nucleosome remodeling and deacetylase complex (NuRD; Srinivasan et al., 2006), it is likely that this interaction is responsible for the progressive decline in histone acetylation and phosphoacetylation, as well as the return of nucleosomes to baseline positions leading to reduction in EGR1 expression (Tur et al., 2010; Riffo-Campos et al., 2015). Interestingly, NAB2 is not constitutively expressed but induced by factors such as EGR1, which does provide a negative feedback loop mechanism for EGR1 expression allowing to explain the transient nature of its expression.

Epigenetics, Post-Translational Modifications and Other Regulators

Importantly, such regulations of *Egr1* transcription by histone acetylation and methylation events are also found in neurons *in vivo* as part of neuroadaptations underlying learning and memory, cognitive functions and response to stress (Gräff et al., 2012; Xie et al., 2013; Hendrickx et al., 2014; Rusconi et al., 2016). Furthermore, DNA methylation and hydroxymethylation have also been linked to the control of *Egr1* transcription associated with environmental impact on synaptic transmission upon aging in the rat hippocampus (Penner et al., 2016), or sleep deprivation in the mouse cortex (Massart et al., 2014). Altogether, it is therefore clear that epigenetic mechanisms are not only an essential part of *Egr1* regulation, but also key mediators of neuroadaptations critical to physiological and pathological brain functions.

Furthermore, EGR1 levels can be regulated on another epigenetic layer through micro-RNA (miRNA). Indeed, in peripheral tissues and several cancer cell lines, several studies report direct targeting of EGR1 by miR-543 (Zhu et al., 2016), miR-192 (Wu et al., 2016), miR-146a (Contreras et al., 2015), miR-7578 (Zhang et al., 2013), miR-183 (Sarver et al., 2010), or miR-124 (Liu et al., 2016; Wang et al., 2016). Interestingly, the latter is highly expressed in the brain and is a critical regulator of neuronal function and thus an important mediator of neuroadaptations in response to chronic stress, reward and learning and memory (Sun et al., 2015). In line with the involvement of EGR1 in these processes as well, a regulation of EGR1 levels by miR-124 has also been reported in the central nervous system. Indeed, miR-124 knockdown in the mouse mPFC and hippocampus increase EGR1 mRNA and protein levels, reflected by improvements in spatial learning and social behaviors impaired in exchange protein directly activated by cyclic AMP (EPAC)-knockout (KO) mice (Yang et al., 2012). Interestingly, although no effect on synaptic transmission was observed, this effect was associated with complete restoration of LTP that was previously abolished in EPAC-KO mice (Yang et al., 2012), which thus indicates that EGR1-targeting miRNA are likely to be involved in the numerous functions under control of EGR1.

In addition to such epigenetic mechanisms, EGR1 transcriptional activity or stability can also be dynamically regulated through post-translational modifications (**Figure 1**) including phosphorylation, acetylation, sumoylation and ubiquitination (Beckmann and Wilce, 1997; Veyrac et al., 2014). For instance, while EGR1 phosphorylation levels are very low in unstimulated cells, EGR1 proteins induced by growth factors or UV radiations undergo substantial phosphorylation events—involving in part protein kinase C or tyrosine kinases—resulting in an increase in its DNA binding activity (Cao et al., 1992, 1993; Huang et al., 1998). Similarly, EGR1 can be acetylated by the histone acetyltransferase complex p300/CBP, which reduces its transcriptional activity

(Yu et al., 2004). Interestingly, EGR1 can undergo sumoylation and ubiquitination, and has been reported to interact directly with proteasome component C8, describing a likely mechanism controlling its targeting for proteolysis by the ubiquitin-dependent proteasome pathway (Bae et al., 2002; Li et al., 2015). Notably, such regulation has been observed following stimulation of ECV304 cells by epidermal growth factor, which increases sumoylation and ubiquitination levels of endogenous EGR1 proteins, ultimately leading to higher EGR1 turnover through proteasome-mediated degradation (Manente et al., 2011). Altogether, these observations suggest that post-translational modifications are critical regulators of EGR1 activity and stability. As further illustration, a detailed mechanistic work describes a signaling pathway in which EGR1 is phosphorylated at the T309 and S350 residues by Akt in response to insulin-like growth factor 1, thereby enhancing its interaction with alternate reading frame (ARF) which mediates sumoylation of EGR1 at the K272 residue and activation of the protein phosphatase and tensin homolog (PTEN; Yu et al., 2009). As such modifications can be observed in the brain following cocaine exposure, for instance (Xu and Kang, 2014), post-translational modifications thus represent a critical level in the regulation of EGR1 functions in the central nervous system.

Finally, it is important to note that EGR1 expression differs between strains (Pollak et al., 2005) and sexes in the central nervous system, in a structure-specific manner. Indeed, adult female rats exhibit lower EGR1 mRNA and protein levels than males in the mPFC, but not in the striatum, or hippocampal CA1 area (Stack et al., 2010; Duclot and Kabbaj, 2015; Yagi et al., 2016). Interestingly, the sex bias is opposite in the dorsal CA3 area, where the density of EGR1-expressing cells is higher in female rats than males (Yagi et al., 2016). A possibility to explain such sex differences in EGR1 expression could reside in the ovarian hormone estrogen, as the latter can directly up-regulate EGR1 expression. In the mouse mammary gland, for instance, EGR1 is at the center of a gene regulation network triggered by exposure to estrogen (Lu et al., 2008), while its mRNA levels in the mouse uterus are up-regulated following estrogen treatment (Kim et al., 2014). Surprisingly, although an estrogen response element (ERE) has been identified on the *Egr1* promoter, the induction of *Egr1* transcription by estrogen is mediated by SRF and Elk1 binding to SRE rather than binding of estrogen receptors to their ERE, and is blocked by a MAPK but not PI3K pathway inhibitor in rat cardiomyocytes or MCF-7 human breast cancer cells (Slade and Carter, 2000; Chen et al., 2004), indicating that EGR1 is a downstream target of estrogen's non-genomic effects. Interestingly, treatment with progesterone either doesn't affect *Egr1* mRNA (Lu et al., 2008), or dampens the estrogen-induced up-regulation of *Egr1* mRNA in the mouse uterus (Kim et al., 2014), which suggests that ovarian hormones can interact to regulate EGR1 expression. These interactions are likely to be specific to neurons, however, as Egr1, among other IEGs, is strongly up-regulated in Schwann cells following progesterone treatment (Mercier et al., 2001). Accordingly, we recently found that *Egr1* mRNA levels in the rat mPFC vary across the estrous cycle with lower levels in the early afternoon of proestrus than in diestrus (Duclot and Kabbaj, 2015), which therefore opens the possibility that genes and biological pathways under direct control of EGR1 also differ between sexes in an estrous cycle-dependent manner.

Downstream Targets

Inherent from the characteristic features of an IEG, EGR1 is rapidly up-regulated in neurons following neuronal activity and orchestrates a subsequent wave of gene regulation to allow for the long-term and enduring encoding of the neuronal information. Surprisingly, despite its well-known association with several processes of neuronal and synaptic plasticity, the precise mechanisms by which EGR1 influences these processes remains unclear. In particular, relatively little is known as to its exact transcriptional targets and gene expression profile under its control, especially in a neuronal context.

From its original cloning nearly three decades ago and the description of three zinc fingers binding domains, the 9-nucleotide long sequence GCGG/TGGGCG was defined as the EGR1 recognition sequence (Christy and Nathans, 1989; Pavletich and Pabo, 1991). The presence of this specific EGR response element could thus theoretically be a good indicator of a direct transcriptional control by EGR1. Nevertheless, a more detailed analysis of EGR1 binding sequence revealed variation in this sequence and identified an optimal site of at least 10 nucleotides rather than 9 (Swirnoff and Milbrandt, 1995). Moreover, experimental evidence indicates that EGR1 can also regulate gene expression through interaction with other transcription factors such as c/EBPβ, Fos, or Jun (Levkovitz and Baraban, 2002; Zhang et al., 2003; Knapska and Kaczmarek, 2004; Cheval et al., 2012), which thus further expands the range of potential EGR1 targets and related biological pathways under its control.

The investigation of EGR1 targets was first conducted on a single-gene basis, through the focus on a particular cellular regulation in a given system. Although this approach led to the identification of numerous EGR1 target genes (Beckmann and Wilce, 1997; Herdegen and Leah, 1998; Knapska and Kaczmarek, 2004), the vast majority of EGR1 potential targets remained to be deciphered. In the early 2000s, the popularization of genome-wide techniques opened the possibility to search for EGR1-regulated genes on a large scale. In several prostate carcinoma cell lines, in which EGR1 is found overexpressed, endogenous or adenovirus-mediated overexpression of EGR1 impacts the expression of multiple genes, including several growth factors such as insulin-like growth factor II (Igf2), platelet-derived growth factor-A (PDGF-A), and transforming growth factor-β1 (TGF-β), as well as membrane-associated proteins, transcription factors and cofactors, all strengthening the involvement of EGR1 in response to growth factors, tumor progression, and apoptosis in these systems (Svaren et al., 2000; Virolle et al., 2003; Arora et al., 2008). Notably, the largest gene class identified in the prostate carcinoma cell lines following EGR1 overexpression includes several neuroendocrine-related genes found highly expressed in the central nervous system (Svaren et al., 2000), which pinpoints a

direct control of neuron-specific genes by EGR1. It is important to note, however, that these regulations are in part cell-specific as similar microarray analyses in human endothelial cells overexpressing EGR1 revealed a different gene regulation profile despite common targets such as TGF-β, Igf2, and $p57^{kip2}$ (Fu et al., 2003). Furthermore, a recent investigation of miRNA directly regulated by EGR1 in the human erythroleukemia cell line K562 reported a total of 124 distinct miRNA and 63 pre-miRNA bound by EGR1 following stimulation by phorbol ester—which activates EGR1 expression in this cell line (Wang et al., 2010). One of these miRNA, miR-124, is of particular interest as it is a known regulator of EGR1 levels (Liu et al., 2016; Wang et al., 2016), including in the central nervous system (Yang et al., 2012), and is tightly associated with neuronal function and higher order processes (Sun et al., 2015). Therefore, in addition to represent a likely mediator in EGR1 control of neuronal activity, these observations suggest that miR-124 could be involved in a negative feedback loop controlling EGR1 expression at the post-transcriptional level.

In order to better characterize how EGR1 binds to its target genes to regulate their transcription, and in an effort to better predict the potential direct EGR1 targets, several studies have investigated EGR1 binding through chromatin immunoprecipitation (ChIP) following by microarray profiling in monocytic differentiation of human monoblastoma cells or following UV-induced apoptosis in prostate carcinoma cells (Arora et al., 2008; Kubosaki et al., 2009). While these studies provide rich information regarding their specific systems, a more comprehensive understanding of EGR1 binding can be drawn from the effort of the Encyclopedia of DNA Elements (ENCODE) project. Indeed, as the ENCODE project included EGR1 as part of the tier 1 chromatin immunoprecipitation followed by deep sequencing (ChIP-seq), a wealth of information regarding EGR1 DNA binding characteristics and target genes has been made available (ENCODE Project Consortium, 2012). In particular, we are thus able to analyze and compare the binding pattern of 161 transcription factors across 91 cell types and a total of 4,380,444 genomic regions, among which 44,985 correspond to an EGR1 binding event. Out of the 15,872 genes thus annotated, 8552 (53.9%) contain at least one EGR1 binding region (peak) within 3 kb of their transcription start site (TSS), which indicates that across several human cell types, EGR1 can bind a very large number of genes and thus potentially regulate a very large gene expression profile (see full annotated list in Supplementary Table S1). As previously reported, EGR1 binds in close vicinity to the TSS (Project Kubosaki et al., 2009; ENCODE Project Consortium, 2012), but even though 41.6% of all EGR1 peaks are located within the promoter region, 26.4% are located within intronic regions. Notably, in line with the high GC content in the EGR1 consensus binding sequence, 31% of all annotated EGR1 peaks are located within a known CpG island, as previously reported by ChIP-chip promoter array analysis in human monoblastoma cells under monocytic differentiation (Kubosaki et al., 2009). Pending further analysis of CpG island and DNA methylation, the presence of a CpG island would thus appear to be a useful informative feature refining the prediction of putative EGR1 binding to a given gene across a variety of cell types.

The functional analysis of genes with at least one EGR1 peak from the ENCODE dataset reveals the enrichment of pathways and processes related to growth factors signaling, including neurotrophins, as well as general intracellular signaling cascades such as Ras or MAPK, which also controls EGR1 expression itself (**Figure 3**, and "Upstream Regulators" Section). Interestingly, the molecular functions of EGR1-bound genes range from chromatin and transcription factors activity to guanyl-nucleotide exchange factor activity through serine/threonine kinase activity (**Figure 3D**), which therefore indicates that EGR1 exerts a transcriptional control on every level of signal transduction cascade, from second messenger to transcription factor. Accordingly, the cellular localization of the EGR1-bound genes' products range from the chromatin to the cell membrane (**Figure 3C**). It is important to note, however, that the latter encompasses the top enrichment hits, and reflects an enrichment of a large number of processes and pathways related to cell-cell recognition and interactions, observed across all enrichment domains (**Figure 3**), which suggests that EGR1 is likely to regulate cell-cell communication through a wide number of genes. Although this observation emerges from non-neuronal cell types (ENCODE Project Consortium, 2012), similar observations were made in the mouse brain. Indeed, following EGR1 ChIP-seq in the mouse cortex, a total of 11,103 genes were found bound by EGR1 in close vicinity to their TSS and were enriched for biological processes and pathways related to protein trafficking, synaptic vesicles transport, endocytosis, protein phosphorylation and intracellular signaling cascades (Koldamova et al., 2014). In this context, the relations of EGR1-bound genes with multiple levels of cell-cell communication, from reorganization of the actin cytoskeleton, to transcription factors through intracellular signaling cascades grant EGR1 the ability to control neuronal activity in a widespread manner.

In addition to the biological functions described above, a distinct pattern associated with EGR1-bound genes relates to proteasome-mediated and ubiquitin-dependent protein degradation, found in all annotation domains analyzed (**Figure 3**). Interestingly, while the regulation of growth factors signaling and transcription factors-related processes were observed in genes bound by EGR1 in their promoter or intronic regions, the enrichment of proteasome-mediated degradation processes preferentially involves genes bound by EGR1 in intronic regions (**Figure 3B**). Although its functional significance remains unknown, this may indicate that EGR1 control of proteasomal degradation-related genes is mediated through binding to enhancer regions or alternative TSS. Most importantly, such link between EGR1 and proteasome-mediated protein degradation is also found in neurons, as viral overexpression of EGR1 in cultured neuronal PC12 cells affects the expression of 135 genes, enriched for components of the proteasome and ubiquitin-related factors (James et al., 2005). Similarly, transgenic overexpression of EGR1 in the mouse forebrain results, in the amygdala, in the up-regulation of proteins related to the proteasome-core complex, among other processes such as metabolism,

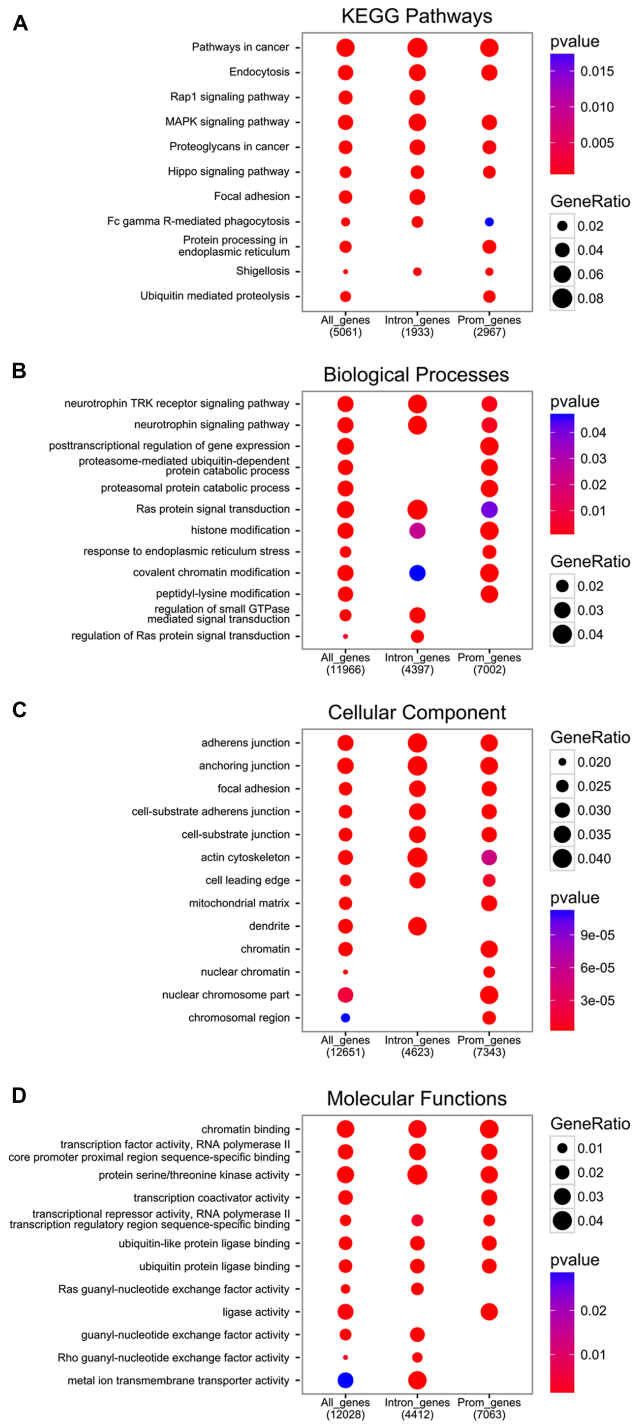

FIGURE 3 | Functional analysis of EGR1 targets from the encyclopedia of dna elements (ENCODE) datasets. All genes annotated near an EGR1 peak ("All_genes"), or those with at least one EGR1 peak called within their promoter region (3 kb around transcription start site (TSS), "Prom_genes"), or within their intronic region ("Intron_genes"), were functionally annotated with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database **(A)** and the gene ontology database distinguishing between the biological processes **(B)** cellular component **(C)** and molecular functions **(D)** domains with the Bioconductor package ChIPSeeker (v1.8.9; Yu et al., 2015).

phosphorylation, or metal ion transport (Baumgärtel et al., 2009). Given that these regulations were also associated with genes involved in intracellular signaling, synapse formation and architecture, as well as neurotransmitter release, it is tempting to conclude that EGR1 is a master regulator of neuronal activity at multiple level of the synaptic and neuronal plasticity processes by orchestrating a widespread gene expression profile (**Figures 2**, **3**).

It is important to keep in mind, however, that although the majority of genes affected by EGR1 overexpression present with one or more predicted EGR response elements (James et al., 2005; Baumgärtel et al., 2009), the absence of direct measurement of EGR1 binding and the possibility of detecting extra-physiological EGR1 transcriptional activity due to exogenous overexpression cannot be ruled out. Recently, however, we took advantage of endogenous differences in EGR1 expression levels in the rat mPFC between males and females, as well as within females across the estrous cycle, to provide additional *in vivo* ChIP-seq information on its direct targets underlying its role in neuronal activity. We thus found that between proestrus and diestrus females, the transcriptomic changes were very large and paralleled by widespread differential binding of EGR1 throughout the genome (Duclot and Kabbaj, 2015). Supporting James et al. (2005) findings in cultured neuronal cells, the EGR1-bound genes were highly enriched for biological processes related to synaptic function—neurotransmitters, signal transduction, presynaptic vesicular trafficking, synapse formation and assembly, and protein translation and degradation (Duclot and Kabbaj, 2015). Notably, this enrichment was even stronger when considering only the genes detected by RNA-seq as differentially expressed, which strongly suggests that EGR1-binding to these genes was transcriptionally effective.

EGR1 ROLE IN SYNAPTIC PLASTICITY

Following the original report of *Egr1* mRNA levels increase by NGF (Milbrandt, 1987), stimulation of neuronal activity was soon identified as a potent trigger for EGR1 induction. In particular, high, but not low, frequency stimulation of the perforant path, which induces LTP, increased *Egr1* mRNA levels in the ipsilateral granule cell neurons (Cole et al., 1989). Importantly, NMDA receptors antagonism or simultaneous synaptic inputs inhibiting LTP were able to block this response, and thereby were the first demonstration that EGR1 expression can be induced by conditions favorable to LTP formation. At the molecular level, this EGR1 regulation requires the MAPK MEK and triggers the ERK1/2, Elk1 and CREB signaling cascade (Davis et al., 2000; Veyrac et al., 2014). Interestingly, early correlations between EGR1 levels and LTP expression pointed towards a link between EGR1 and LTP persistence, rather than its induction (Richardson et al., 1992; Abraham et al., 1993; Knapska and Kaczmarek, 2004). This was confirmed later in EGR1-KO mice in which early hippocampal LTP was intact, but was not present 24–48 h post-tetanic stimulation while, indicating that EGR1 is required specifically for the maintenance of LTP, but not its induction (Jones et al., 2001). Conversely,

EGR1 overexpression in the forebrain enhances LTP in the mouse dentate gyrus (Penke et al., 2014). As hippocampal LTP is considered a molecular hallmark of spatial memory formation (Sweatt, 2016), the role of EGR1 in learning and memory paradigms has been extensively studied and recently reviewed elsewhere (Bozon et al., 2002; Knapska and Kaczmarek, 2004; Veyrac et al., 2014). This will thus not be discussed in detail here.

It is important to note, however, that in line with the important role of EGR1 in late-phase LTP, short-term spatial memory is intact in EGR1-KO mice, while spatial long-term memory is impaired (Jones et al., 2001), suggesting a critical role for EGR1 in memory consolidation. Although EGR1 is up-regulated following a wide range of learning procedures, this effect remains structure-specific and is generally observed in the brain regions relevant to the nature of the learning task (Veyrac et al., 2014), in line with its induction by neuronal activity. Moreover, the functional and behavioral outcome of EGR1 up-regulation in learning in memory is also specific to the nature of the task. For instance, although EGR1 knockdown by RNA interference in the amygdala impairs the consolidation of cued and contextual fear memory, EGR1 knockdown in the hippocampus impairs contextual memory reconsolidation but not consolidation—in line with the known distinction in molecular events recruited under memory consolidation and reconsolidation (Lee et al., 2004; Veyrac et al., 2014). Notably, recent evidence derived from RNA interference experiments in rats suggest that EGR1's role in memory reconsolidation rather reflects suppression of extinction upon short memory recall and thus tilting of the balance between activation of extinction or reconsolidation towards the latter (Trent et al., 2015). Interestingly, EGR1 involvement may not be restricted to memory encoding but is likely to be expanded to neuronal encoding in a more global way. Indeed EGR1 deletion in mice destabilizes the spatial representation of a familiar environment in hippocampal CA1 place cells, and impairs the long-term, but not the short-term stabilization of a novel environment (Renaudineau et al., 2009). In the same cells, EGR1 is up-regulated during a water maze procedure regardless of the memory performance or even in a non-learning version of the task, which suggests that EGR1 up-regulation in place cells is activated each time the animal enters an area related to the given place cells and thus reflects spatial encoding rather than memory encoding (Rapp et al., 1987; Guzowski et al., 2001; Shires and Aggleton, 2008; Laeremans et al., 2015), in line with the functional role played by this cell population (O'Keefe and Dostrovsky, 1971). Similarly, Carter et al. (2015) recently observed that exposure to a water maze task increases EGR1 and c-Fos expression through ERK1/2 activation and histone H3 phosphoacetylation throughout the rat hippocampus regardless of the learning component, although the effects were most pronounced in the dentate gyrus.

Notably, while EGR1 regulation by neuronal activity and plasticity underlying memory processes are well documented, the exact transcriptional targets involved remain unclear. Under this perspective, it is particularly interesting to consider another IEG: Arg3.1 (also known as ARC). Indeed, EGR1 binds to *Arc* promoter *in vivo* following synaptic activation and triggers its transcription (Li et al., 2005). On a functional level, ARC shares a lot of similarities with EGR1. Indeed, ARC is an IEG up-regulated in neurons following synaptic activity, is involved in the maintenance of LTP, and is required for long-term memory consolidation but not short-term memory formation or learning (Minatohara et al., 2015). Contrary to EGR1, however, ARC mRNA and proteins can be found in dendrites and post-synaptic locations (Kobayashi et al., 2005) where it is believed to function by interacting with other post-synaptic proteins. In particular, ARC interacts with endophilin and dynamin to enhance endocytosis of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, but also interacts with the actin cytoskeleton in dendritic spines where it is required for cofilin phosphorylation and local F-actin expansion (Chowdhury et al., 2006; Bramham et al., 2008). As both processes are critical underpinning of major synaptic plasticity events such as LTP, ARC-mediated reorganization of actin cytoskeleton and synaptic architecture represents a very promising candidate in mediating EGR1's critical role in synaptic plasticity and related behavioral outcomes such as memory consolidation. It is important to note, however, that ARC can also be regulated independently of EGR1, as observed following intra-hippocampal brain-derived neurotrophic factor infusion in rats (Ying et al., 2002), which, in addition to illustrating the diversity in ARC regulation, further illustrates the specificity in EGR1 recruitment underlying neuronal and synaptic plasticity.

Interestingly, recent genome-wide investigations of EGR1 transcriptional targets point towards a widespread regulation of genes associated with similar dynamics critical in regulating synaptic plasticity. Indeed, a multitude of genes related to vesicular transport and neurotransmitter release, clathrin-dependent endocytosis (involved in post-synaptic receptor internalization), or actin cytoskeleton, are commonly observed as direct EGR1 targets (ENCODE Project Consortium, 2012; Koldamova et al., 2014; Duclot and Kabbaj, 2015), suggesting that besides ARC, many other EGR1 targets related to these processes may be involved in the regulation of synaptic activity by EGR1. In this context, it is particularly interesting to note that EGR1 was recently described as recruited to the postsynaptic density 95 (PSD-95) gene promoter in response to NMDA receptor activation in hippocampal primary neurons, leading to its repression (Qin et al., 2015). As a result, EGR1 knockdown in rat hippocampal neurons blocks NMDA receptors-induced PSD-95 down-regulation and AMPA receptor endocytosis, while its overexpression has the opposite effects (Qin et al., 2015). Similarly, the observation that EGR1 controls the expression of genes related to protein translation and ubiquitin-dependent degradation (James et al., 2005, 2006; Baumgärtel et al., 2009) indicates that EGR1 can coordinate a complex transcriptional program leading to a synaptic reorganization at multiple levels that promotes stabilization of the synapse, which would be in line with the repeated involvement of this IEG in encoding synaptic information.

EGR1 ROLE IN PATHOLOGICAL STATES

As described above, EGR1 is regulated by a wide variety of environmental stimuli and can regulate a large transcriptional program related to critical processes underlying synaptic plasticity and encoding of information. As a result, EGR1 represents a key factor both in integrating perception of the environment and in shaping an appropriate response. In this context, it is therefore not surprising to find EGR1 associated with neuropsychiatric illnesses in which neuronal plasticity and activity is altered or dysfunctional. In the sub-sections below, we will thus focus on some of the main neuropsychiatric disorders in which EGR1 has been implicated.

Response to Stress

Despite their high prevalence (Kessler et al., 2012), stress-related mood disorders such as anxiety and depression still remain elusive in their exact etiology. Nevertheless, repeated exposure to stressful experiences is now established to represent one of the main risk factors for their development. As a result, a multitude of animal models for depression and anxiety disorders relying on the repeated exposure to stress of different nature have been developed (Czéh et al., 2016). In this context, it is important to first better understand EGR1's regulation and role in response to stress and in such animal models.

In accordance with its activation by neuronal activity, EGR1's regulation following exposure to stress is variable depending on the nature and duration of the stress. An acute physical stressor, such as restraint, immobilization, or forced swim, leads to increase in *Egr1* mRNA levels throughout the brain including neocortical areas, hippocampus, lateral septum, caudate putamen, nucleus accumbens, amygdala, and paraventricular nucleus (PVN) of the hypothalamus (Schreiber et al., 1991; Melia et al., 1994; Watanabe et al., 1994; Cullinan et al., 1995; Olsson et al., 1997; Knapska and Kaczmarek, 2004; Kozlovsky et al., 2009). Despite such a strong response to an acute stress, repetition of the same stress blunts the stress-induced EGR1 response, as observed in the PVN, hippocampus, or cortical regions (Melia et al., 1994; Watanabe et al., 1994; Girotti et al., 2006), and denotes a physiological habituation to homotypic exposures to physical stressors. Despite this habituation, however, exposure to a novel stress (shaking stress vs. restraint stress) still leads to a full increase in *Egr1* mRNA levels in the PVN (Watanabe et al., 1994), which thus indicates that habituation to the stressor is stress-specific. Nevertheless, one study investigating the effects of immobilization stress on IEGs expression in the PVN found that whereas c-Fos and EGR1, among others, are up-regulated upon acute immobilization, repetition of this stress for 6 days suppresses such response for c-Fos, but not for EGR1 (Umemoto et al., 1994, 1997). Furthermore, as chronic treatment with high concentration of glucocorticoids (corticosterone) mimics the effects of repeated stress exposure, the authors concluded that glucocorticoids mediate the habituation of IEGs in the PVN to repeated stress exposure while EGR1 was resistant to such effect (Umemoto et al., 1997). As EGR1 is a reliable marker of neuronal activity, these findings can be seen as illustrations of the importance of the nature and intensity of the stress on the neuronal and transcriptional response and habituation upon repeated exposure. Interestingly, although outside of the central nervous system, a recent genome-wide investigation in the rat adrenal medulla of the evolution of genes whose regulation is correlated with EGR1 following one or six exposures to immobilization stress revealed a distinct profile of interactions across time. Indeed, while EGR1 is up-regulated in the rat medulla following both acute (1×) and repeated (6×) exposure to the immobilization stress (Liu et al., 2008), its gene interaction network differed between the two stress conditions, indicating that EGR1 has different targets and functions between acute and repeated exposure to an immobilization stress (Papanikolaou et al., 2014). Altogether, stress is a major trigger for EGR1 induction in the central nervous system. Nevertheless, this activation mostly reflects the pattern of neuronal activity in response to various stressors and, as a result, varies with the nature, duration and intensity of the stress. This point is particularly well illustrated by the positive correlation observed between the magnitude of HPA axis activity, as measured by plasma adrenocorticotropic hormone (ACTH) levels, and *Egr1* mRNA levels in the PVN—an intrinsic component of the hypothalamic-pituitary-adrenals (HPA) axis—but not in the hippocampus or cortical regions for which EGR1 signal was more related to the exploration of the environment (Pace et al., 2005).

Notably, in addition to being regulated by exposure to stressful experiences, evidence indicates that EGR1 is a critical factor in encoding the behavioral enduring effects of stress. Indeed, acute exposure to forced swim stress or activation of the glucocorticoid receptor (GR) up-regulates EGR1 expression in the rat or mouse hippocampus, which mediates stress-related fear memories (Revest et al., 2005, 2010; Saunderson et al., 2016). Interestingly, such stress-induced EGR1 up-regulation depends on the methylation status of its promoter (Saunderson et al., 2016) and results in an increase in the expression and activation of MAPK pathway-associated proteins (Revest et al., 2005) as well as the synaptic plasticity-associated protein synapsin-I (Revest et al., 2010). Combined with the blockade of stress-related fear memory or GR-induced synapsin-I expression in these paradigms by synapsin-I or EGR1 knockdown, respectively, these data support a model in which EGR1 expression in the rodent hippocampus is highly regulated by stress exposure, and in turn controls synapsin-I expression to influence the synaptic plasticity underlying the consolidation of stress-related memory (Revest et al., 2010).

Stress-Related Mood Disorders and Schizophrenia

Such variability in EGR1 response depending on the nature of the stress is also particularly important in understanding the link between EGR1 and the behavioral outcome of stress. As a result, in postmortem tissue from patients suffering from major depressive disorder, in which stress is a major risk factor (Czéh et al., 2016), EGR1 levels in the prefrontal cortex are lower when compared to healthy controls (Covington et al., 2010). Notably, such reduction was observed in both unmedicated

and medicated subjects not responding to treatment and thus suggests that EGR1 levels in the mPFC are directly associated with a depressive phenotype and could be seen as a marker or mediator of positive response to antidepressant treatment (Covington et al., 2010). In light of the tight link between EGR1 expression and neuronal plasticity, the down-regulation of EGR1 in the PFC of depressed patients is particularly interesting and could represent one of the substrates for the anatomical and functional alterations observed in major depressive disorders in this brain area (Krishnan and Nestler, 2008; Koenigs and Grafman, 2009; Lefaucheur et al., 2017). In this context, a similar dysregulation of EGR1 expression is observed in other neuropsychiatric disorder characterized by functional alterations in PFC activity such as schizophrenia, where *Egr1* mRNA levels are also found down-regulated in the dorsolateral prefrontal cortex (Yamada et al., 2007; Kimoto et al., 2014). Interestingly, EGR1 levels in the PFC of schizophrenia patients are positively correlated with the mRNA levels for the glutamic acid decarboxylase 1 (GAD1), whose down-regulation is a robust molecular feature of schizophrenia subjects (Pérez-Santiago et al., 2012; Kimoto et al., 2014). Notably, although other IEGs such as c-Fos, c-jun, or EGR2 are also altered in schizophrenia, their expression levels are not correlated with GAD1 mRNA levels (Kimoto et al., 2014), which thus supports a specific role for EGR1 in GAD1 regulation and highlights its function as an important factor in the altered cortical GABA synthesis and cognitive functions observed in this neuropsychiatric disorder. Finally, in the search for schizophrenia biomarkers in peripheral tissues, EGR1 levels in whole blood samples was associated with schizophrenic symptoms such as high delusional states (Kurian et al., 2011). Similarly, EGR1 was among six genes identified as up-regulated in fibroblasts from schizophrenic patients, and the only one confirmed in peripheral blood cells as well (Cattane et al., 2015). Although this regulation is opposite to the down-regulation observed in the dorsolateral prefrontal cortex of schizophrenia patients (Kimoto et al., 2014), the up-regulation of EGR1 in blood cells was specific for schizophrenia when compared to major depressive disorder or bipolar disorder (Cattane et al., 2015), which confers EGR1 a particularly promising biomarker potential in a clinical environment. Altogether, these findings support a role for EGR1 in both the etiology and therapeutic interventions in schizophrenia.

In line with these clinical observations, *Egr1* mRNA levels are generally found down-regulated in specific brain areas in animal models inducing depressive- and anxiety-like states. For instance, the exposure of male mice to 14 days of chronic unpredictable stress leads to reduced levels of *Egr1* mRNA in the hippocampus associated with cognitive impairments in a water maze learning, novel object recognition and location tasks, CA1 basal dendrites atrophy, and altered ERK1/2 phosphorylation (Xu et al., 2015). Similarly, while an acute social defeat stress increases *Egr1* mRNA in the male mouse hippocampus (Rusconi et al., 2016), reduced *Egr1* mRNA levels in the mouse mPFC are found following repeated social defeat (Covington et al., 2010), a well-established animal model for depressive- and anxiety-like states (Hollis and Kabbaj, 2014). Notably, EGR1 expression is also reduced in the prefrontal cortex of human depressed subjects unmedicated or not responding to treatment, which thus suggests that EGR1 levels in the mPFC are directly associated with a depressive phenotype and could be seen as marker or a mediator of positive response to antidepressant treatment (Covington et al., 2010). Accordingly, reduced *Egr1* mRNA levels in the brain are commonly observed in another well-established animal model of depressive-like state, social isolation. Indeed, reduced EGR1 expression is observed in the PVN, mPFC, HPC, or extended amygdala of rats, mice and prairie voles following social isolation (Northcutt and Lonstein, 2009; Matsumoto et al., 2012; Hodges et al., 2014; Okada et al., 2014, 2015; Hodges and McCormick, 2015; Ieraci et al., 2016).

Despite the strong association of EGR1 expression levels with depression- and anxiety-like behaviors described above, the evidence for a functional link was obtained from the behavioral phenotype of EGR1-KO mice, which present with lower anxiety levels reflected by higher exploratory behavior in the open arms of an elevated plus maze (Ko et al., 2005). Since then, the role of EGR1 in regulating anxiety has been further described and targeted to the mPFC, although other structures such as the amygdala or ventral HPC are likely to contribute. In particular, we demonstrated that EGR1 expression levels in the rat mPFC control the social interaction behavior, an indicator of social anxiety, and was sufficient to explain sex differences in social interactions observed in Sprague-Dawley rats (Stack et al., 2010). Indeed, the lower levels of social interaction displayed by females when compared to males are paralleled by lower levels of EGR1 mRNA and proteins in the mPFC. Furthermore, antisense-mediated EGR1 knockdown in the mPFC of males reduced their social interaction levels to those of females (Stack et al., 2010). Conversely, viral-mediated EGR1 overexpression in the mPFC prevents deficits in social interactions induced castration in male rats (Dossat et al., 2017). Similarly, the intracerebroventricular injection of locked-nucleic acid-modified antisense nucleotide knocking down miR-124—which inhibits EGR1—reverses the social interactions impairments in EPAC-KO mice (Yang et al., 2012). Notably, it is particularly interesting to note that partial changes in EGR1 protein levels seen in the studies described above, especially in the mPFC, are sufficient to substantially alter complex behaviors such as social interactions. In addition to indicating that variations in EGR1 levels are critical in determining anxiety levels, this suggests that endogenous variations in EGR1 protein levels in the mPFC such as those occurring throughout the female estrous cycle (Duclot and Kabbaj, 2015) are likely to be associated with variations in anxiety-related behaviors. Accordingly, estrous cycle-dependent variations in anxiety-like behaviors are reported in female rodents (Donner and Lowry, 2013; Barth et al., 2015).

In addition to its association with the development of anxiety- and depression-like states, EGR1 is actively regulated by several classes of antidepressant treatments throughout the brain. While behavioral antidepressant effects are typically observed following chronic, but not acute, treatment, it is surprising to find an up-regulation of EGR1 in the rat hippocampus following a single dose of the tricyclic antidepressant desipramine (Dahmen et al., 1997), or in

the rat amygdala following an acute dose of fluoxetine, imipramine, mirtazapine, or lithium chloride (Slattery et al., 2005). Nevertheless, no effects were observed in other brain regions analyzed, suggesting that this effect was relatively constrained (Slattery et al., 2005). Following chronic treatment regimen, the effect is more robust as EGR1 is up-regulated following a wide variety of antidepressant treatments—ranging from the classical antidepressants imipramine and fluoxetine, to electroconvulsive seizures (ECS)—and in multiple key brain areas for antidepressant effects such as the mPFC and hippocampus (Morinobu et al., 1995, 1997; Bjartmar et al., 2000). Interestingly, this effect may not be restricted to neurons as imipramine application on cultured rat astrocytes up-regulates EGR1 expression in a MAPK-dependent manner, which then binds to the glial cell line-derived neurotrophic factor (*gdnf*) gene promoter and activates its expression (Kim et al., 2011). Moreover, while these effects were observed in unstressed systems, which could thus be considered at baseline, experimental interventions exerting an antidepressant-like effect, such as environmental enrichment, FGF2, or fluoxetine, have also been reported to reverse or protect from induction of anxiety- and depressive-like states in multiple models (Monsey et al., 2014; Novaes et al., 2017; Salmaso et al., 2016). Although causality still remains to be clearly established, the up-regulation of EGR1 following antidepressant treatment thus emerges as a key feature of antidepressant response. This is particularly interesting in light of the reduced EGR1 expression found in the frontal cortex of depressed patients who remained symptomatic despite being medicated, as this further suggests that EGR1 up-regulation could represent a reliable marker for positive therapeutic response to antidepressants (Covington et al., 2010).

Interestingly, despite its low expression levels early in the development, EGR1 has been identified as an important mediator of the effects of early-life experience through its transcriptional control of the *gr* gene. For instance, the levels of maternal care received by rat pups during the first week of life determines their neuroendocrine response to stress later in adulthood, through DNA methylation at the hippocampal *gr* promoter located on an EGR1 binding site (Weaver, 2007). As evidence suggests that maternal care triggers serotonin release in the hippocampus, it is particularly interesting that EGR1 knockdown by RNA interference prevents serotonin-induced increase in GR expression in cultured rat hippocampal neurons (Weaver et al., 2007), which thus suggests that the extent of maternal care received by the pup during the first week of life will influence EGR1 binding to the *gr* promoter, which will in turn determine GR expression in a long-lasting manner through epigenetic mechanisms (Weaver, 2007). Notably, children exposed to physical maltreatment—a known risk factor for the development of mood-related alterations in adulthood (Shackman et al., 2007; Shackman and Pollak, 2014)—present with greater DNA methylation of the *gr* promoter, including at the EGR1 binding site (Romens et al., 2015), indicating that such EGR1 control of GR expression by maternal care could also be observed in humans. Moreover, other early-life stressful experiences have similarly been reported to impact EGR1 expression. Maternal separation of C57Bl/6 mice from postnatal day 14–16, for instance, induces a rapid increase in EGR1 expression and its target ARC in the hippocampus through histone acetylation at their respective promoter (Xie et al., 2013). Although causality remains to be determined, these changes are associated with greater dendritic complexity and spine number in the hippocampal CA3 area (Xie et al., 2013), suggesting that early-life experiences can affect neuronal architecture and organization through EGR1. The timing of such manipulation is critical, however, as maternal separation in the same C56Bl/6 strain from postnatal day 2–15 leads to a marked reduction in EGR1 expression in the forebrain neocortex (Navailles et al., 2010).

In addition to shape response to stress later in adulthood, early-life experiences can also impact the development of neuropsychiatric disorders such as schizophrenia. Indeed, adult rats having received high levels of maternal care present with higher GAD1 mRNA hippocampal levels than individuals raised by dams providing low levels of maternal care (Zhang et al., 2010). Notably, this regulation is mediated by EGR1 binding, along with higher H3K9 acetylation and lower DNA methylation, at the *gad1* promoter (Zhang et al., 2010), and thus directly implicates EGR1 in the regulation of GAD1 expression in the brain, which is of particular interest in the context of neuropsychiatric illness in light of the positive correlation between GAD1 and EGR1 expression levels in schizophrenia patients (Kimoto et al., 2014). While the molecular underpinnings of EGR1 alterations in schizophrenia remain unknown, knockdown in cultured hippocampal GABA neurons of the histone deacetylase 1 (HDAC1) and its co-repressor DAXX, whose expressions are also altered in schizophrenia, results in increased GAD1 and *Egr1* mRNA levels, which opens the possibility for an HDAC1/DAXX-mediated repression of EGR1 expression leading to GAD1 inhibition (Subburaju et al., 2016). Furthermore, beyond its etiology, EGR1 is also associated with response to antipsychotic drugs (MacGibbon et al., 1994; Robbins et al., 2008; Bruins Slot et al., 2009; Wheeler et al., 2014; de Bartolomeis et al., 2015), or the psychomimetic phencyclidine in rats (Tamminga et al., 1995; Näkki et al., 1996).

Altogether, the above experimental evidence highlights the important role played by EGR1 in mediating or modulating the stress response and the development of various stress-related disorders. The upstream regulators involved, however, remain unclear and it thus becomes interesting to further consider the link between glucocorticoids released following chronic stress, and EGR1 expression in the central nervous system. Indeed, while EGR1 is a direct regulator of *gr* transcription, activation of GR leads to EGR1 up-regulation in the mouse and rat hippocampus through intracellular signaling pathways involving MAPK (Revest et al., 2005, 2010) or the serum and glucocorticoid regulated kinase 1 (SGK1; Tyan et al., 2008). Notably, the regulation of EGR1 expression by SGK1 involves well-defined mechanisms of *Egr1* transcriptional regulation via the activation by phosphorylation of SRF and CREB, and has been linked to spatial memory formation in rats (Tyan et al., 2008). As its expression in the rodent hippocampus and mPFC

rodents is strongly regulated by acute (Bohacek et al., 2015; Mifsud and Reul, 2016) or chronic stress (Anacker et al., 2013; Miyata et al., 2015; Skupio et al., 2015; Cattaneo and Riva, 2016; Wei et al., 2016), SGK1 emerges as a particularly interesting candidate in mediating EGR1 regulations in response to various stress paradigms. In this context, it is particularly interesting to note that SGK1 expression levels are down-regulated in the PFC of post-traumatic stress disorder patients—or increased in the peripheral blood of unmedicated depressed patients (Anacker et al., 2013)—and that SGK1 inhibition in the rat mPFC induces depressive-like behaviors in rodents associated with abnormal dendritic spine morphology and synaptic dysfunction (Licznerski et al., 2015). Altogether, these experimental observations delineate a hypothetical working model in which glucocorticoids release following chronic stress exposure alters SGK1 expression in key brain areas including the hippocampus and mPFC, which in turns regulates *Egr1* transcription through activation of SRF and CREB transcription factors. Although the requirement of EGR1 in SGK1's effects on neuronal plasticity remains to be determined, EGR1 could in turn orchestrate, through its wide array of targets, the neuronal and synaptic plasticity events underlying the long-term behavioral effects of stress that influence the development of stress-related disorders such as depression or PTSD.

Drug Reward, Withdrawal and Relapse

Exposure to substance of abuse is a powerful environmental stimulus that triggers a strong neuronal response throughout the brain, but mainly targeting the mesolimbic dopaminergic system, and bears the ability to reorganize existing neuronal connections in a long-lasting manner. IEGs such as EGR1 have thus been repeatedly associated with the neuronal response to large number of compounds with rewarding or addictive properties. EGR1's involvement in response to cocaine, for instance, are now relatively well-described and reviewed elsewhere (Veyrac et al., 2014). We will thus focus the following section on two distinct classes with rewarding properties.

Opiates, for instance, are known triggers for EGR1 expression in various brain areas. In particular, an acute heroin injection up-regulates *Egr1* mRNA levels in the core and shell of the nucleus accumbens, the dorsal striatum, and the cingulate cortex of C57Bl6 mice (El Rawas et al., 2009). Similarly, increased EGR1 expression levels are observed in the extended amygdala, dorsal striatum, nucleus accumbens shell, and cingulate cortex following an acute morphine injection (Hamlin et al., 2007; Ziółkowska et al., 2012, 2015). Notably, the latter is observed 4 h and 6 h following injection, which suggests that in this context, EGR1 up-regulation is part of a second wave of gene regulations, and is not associated with the rapid hyperlocomotor effects of morphine (Ziółkowska et al., 2015). They do suggest, however, that EGR1 can be involved in neuroadaptations underlying long-lasting effects of morphine exposure, such as withdrawal and relapse. Accordingly, naloxone-induced morphine withdrawal in rats induces an EGR1 up-regulation in the cerebral cortex, hippocampus, thalamus, cerebellum, and brainstem 60 min following the withdrawal (Beckmann et al., 1995). Similarly, EGR1 and its target ARC are up-regulated in the rat dentate gyrus upon morphine-withdrawal memory retrieval which, in light of its established role of in contextual memory reconsolidation in the hippocampus (Lee et al., 2004), suggests that EGR1 could be involved in the synaptic plasticity events underlying reconsolidation of the morphine withdrawal-context (García-Pérez et al., 2016). Under a similar perspective, EGR1 expression is increased in the rat basolateral but not central amygdala during reconsolidation of withdrawal memory, whereas its down-regulation by antisense oligodeoxynucleotides within the basolateral amygdala reduces the withdrawal memory-mediated suppression of heroin seeking (Hellemans et al., 2006), thereby indicating a functional role for EGR1 in encoding heroin seeking in the amygdala. In line with the tight interplay between the amygdala and the mPFC during cue-associated memory reactivation, extinction, or reconsolidation, *Egr1* mRNA levels are also found up-regulated in the rat mPFC following 14 or 30 days of heroin-seeking incubation (Kuntz et al., 2008; Kuntz-Melcavage et al., 2009; Fanous et al., 2013), which thus strengthen further the importance of EGR1 in regulating multiple aspects of opiates dependance.

Similar to opiates, alcohol consumption triggers a marked EGR1 response throughout the brain. In adult rats and mice, acute ethanol exposure leads to increased EGR1 expression in the mPFC, central amygdala, medial amygdala, supraoptic nucleus, PBN, lateral part of the caudate putamen, prelimbic and infralimbic cortices, orbitofrontal cortex, hippocampus and nucleus accumbens (Thiriet et al., 2000; Faria et al., 2008; Hansson et al., 2008; Lindholm et al., 2008; Liu and Crews, 2015). Repeated exposure for 15 days, however, or chronic intermittent exposure, reduces *Egr1* mRNA levels in the mPFC, hippocampus, and nucleus accumbens (Repunte-Canonigo et al., 2007; Faria et al., 2008), which thus indicates that EGR1's regulation by ethanol depends on the nature and duration of the exposure. Moreover, this dynamic regulation of EGR1 by ethanol is further illustrated upon withdrawal. Indeed, *Egr1* mRNA and protein levels are up-regulated in the cerebral cortex, olfactory bulb, inferior colliculus, brainstem, and hippocampus of ethanol-dependent rats at 12 h and 15–24 h, respectively, following withdrawal (Matsumoto et al., 1993), a process associated with increased EGR1 DNA binding activities in the cerebral cortex from 16 h to 72 h following withdrawal (Beckmann et al., 1997). Interestingly, while withdrawal-induced anxiety-like behaviors emerge within the same period, between 8 h and 17 h following withdrawal (Matsumoto et al., 1993), withdrawal-induced anxiety-like behaviors in mice, which rise on the 1st day of withdrawal and last up to 21 days later, are positively correlated with the increase in EGR1-positive cells in the central amygdala and bed nucleus of the stria terminalis (Lee et al., 2015), which thus link the increase in EGR1 expression in the amygdala to the development of anxiety-like symptoms upon ethanol withdrawal.

CONCLUSIONS

In this review article, we summarized and discussed the regulations and functions of the IEG EGR1 in the central nervous system relevant to neuropsychiatric disorders. Situated

downstream of general signaling pathways activated by neuronal activity, EGR1 has been found regulated by a wide variety of environmental events that position EGR1 as a critical integrator and mediator of environmental influences on neuronal activity. Furthermore, due to its very large range of potential transcriptional targets identified so far, the reach of EGR1's functions in neurons continues to expand. In particular, without considering eventual indirect effectors, EGR1 can alter the expression of genes related to every level of synaptic plasticity, from vesicular transport and release of neurotransmitters, to synaptic architecture, endocytosis, and protein degradation (**Figure 2**). Notably, in line with its sex- and estrous cycle-dependent expression in the rat mPFC, it is important to consider that this control of synaptic plasticity by EGR1 is likely to substantially vary between sexes in an estrous cycle-dependent manner. Despite such wide array of synaptic plasticity-related potential targets and its well-known association with neuronal activity. however, the current knowledge of the precise mechanisms by which EGR1 influences synaptic and neuronal plasticity, as well as the direct targets involved, remains paradoxically unclear and requires to be clearly described and validated *in vivo*. Nevertheless, EGR1 is tightly associated to neuronal activity throughout the brain and can thus be used as a reliable tool for mapping neuronal activity in response to a given environmental event. In this context, it is possible to consider that a substantial amount of EGR1's regulations described in this review simply reflect neuronal responses in a given structure to a given behavioral stimulation. It is important to note, however, that EGR1 governs specific neuronal processes, which can be reflected, for instance, by its specific involvement in the maintenance but not induction of LTP, or, under appropriate conditions, memory reconsolidation but not acquisition, for instance. In line with its crucial role in shaping neuronal response, EGR1 is associated with the etiology and treatment of most common neuropsychiatric disorders such as major depressive disorder, anxiety disorders, schizophrenia, or addiction. Therefore, despite its widespread mode of regulation, EGR1 functions in the central nervous system are complex and represent a valuable candidate for investigating gene × environment interactions.

AUTHOR CONTRIBUTIONS

FD and MK participated equally in the article design and outline; FD then wrote the first draft. After a few revisions and editing by both authors, the article was submitted.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Reward Network Immediate Early Gene Expression in Mood Disorders

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Over the past three decades, it has become clear that aberrant function of the network of interconnected brain regions responsible for reward processing and motivated behavior underlies a variety of mood disorders, including depression and anxiety. It is also clear that stress-induced changes in reward network activity underlying both normal and pathological behavior also cause changes in gene expression. Here, we attempt to define the reward circuitry and explore the known and potential contributions of activity-dependent changes in gene expression within this circuitry to stress-induced changes in behavior related to mood disorders, and contrast some of these effects with those induced by exposure to drugs of abuse. We focus on a series of immediate early genes regulated by stress within this circuitry and their connections, both well-explored and relatively novel, to circuit function and subsequent reward-related behaviors. We conclude that IEGs play a crucial role in stress-dependent remodeling of reward circuitry, and that they may serve as inroads to the molecular, cellular, and circuit-level mechanisms of mood disorder etiology and treatment.

Keywords: depression, reward system, immediate early gene (IEG), FosB/ΔFosB, CREB, accumbens, hippocampus, mood disorders

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INTRODUCTION

Neurocircuitry has evolved to reward behaviors that contribute to evolutionary fitness with feelings of pleasure, motivating individual organisms to value, and repeat actions that increase the likelihood of propagating their genetic material. These may include having sex, eating certain foods, caring for offspring, or engaging in social activity. However, the modern human environment, replete with abundant resources and access to pleasurable stimuli, may allow increased reward processing to induce maladaptive pursuits, such as overeating or addiction to drugs or sex (Berridge and Kringelbach, 2015). Conversely, deficiency in reward processing contributes to the anhedonic symptoms of mood disorders like depression (Nestler, 2015a; Luking et al., 2016), and current treatment and research in mood disorders focuses on the circuitry underlying reward and the mechanisms that may contribute to defective reward processing.

Rewarding behaviors become favored because they are reinforced. This process requires that they: (1) give rise to positive emotions (pleasure), (2) induce learning, and (3) produce additional consummatory behavior (i.e., eating, copulating, interacting, etc.). Thus, the reward circuitry must integrate information from brain structures that drive feelings of pleasure, formation and storage of memories, and decision-making and behavioral output. It has become increasingly clear over the last two decades that changes in gene transcription within this reward circuitry contribute to the development of mood disorders (Nestler, 2015a). These disease-related changes can involve mechanisms as diverse as histone and DNA modification, non-coding RNA expression, and

transcription factor induction and activity (Dalton et al., 2014; Geaghan and Cairns, 2015; Nestler, 2015a). The expression of many transcription factors involved in these processes is tightly regulated by neuronal activity, and such transcription factors belong to a class of molecules termed immediate early genes (IEGs). These IEGs represent a particularly attractive mechanism for diseases involving anhedonia, as reward circuit neuronal activity is altered in many models of depression (Russo and Nestler, 2013; Lammel et al., 2014), and thus the expression of many IEGs is dysregulated in the same models (Reul, 2014; Nestler, 2015a). Therefore, to fully unravel the etiology of human mood disorders, it is critical that we uncover the regulation of IEGs in the reward circuitry under both basal and disease conditions. This review will cover progress in identifying the regulation and downstream targets of IEGs within the brain regions comprising the reward circuitry, and the current evidence linking reward circuitry IEGs to stress responses and mood disorders.

THE CORTICO-BASAL GANGLIA REWARD NETWORK

The central feature of the reward circuitry is the release of dopamine (DA) from the ventral tegmental area (VTA) neurons into limbic brain regions that control prediction, perception, and processing of rewarding stimuli. VTA DA neurons have major projections to the prefrontal cortex (PFC; the mesocortical pathway) and to the nucleus accumbens (NAc; the mesolimbic pathway), but also project to hippocampus, amygdala, and several other forebrain regions. Mesocortical DA is thought to be involved in emotional responses and control of cognition (Nestler et al., 2015), while mesolimbic DA is traditionally linked to reward and motivated behaviors. Mesolimbic DA release activates dopamine receptors (DRs) on NAc medium spiny neurons (MSNs), GABAergic cells comprised of two largely separate populations that express predominantly either D1 or D2 DRs (Surmeier et al., 2007; Lobo, 2009). D1 MSNs comprise the "direct" pathway, which ultimately increases thalamocortical drive, while D2 MSNs make up the "indirect" pathway, which results in reduced thalamocortical drive. Because D1 DRs increase responsiveness to glutamatergic excitation while D2 DRs decrease this glutamate excitability, VTA DA release facilitates the direct pathway while putting a brake on the indirect pathway, with the combined effect of increased cortical drive.

NAc MSNs receive glutamatergic inputs from several cortical and limbic structures, including medial and lateral divisions of the PFC, ventral hippocampus (vHPC), basolateral amygdala (BLA), and medial thalamus (Sesack and Grace, 2010; Floresco, 2015). PFC inputs onto NAc regulate goal-directed behaviors, such as seeking and consuming substances/activities associated with reward, including food, sex, drugs, and social interactions (Kalivas et al., 2005; Gruber et al., 2009), providing the "executive control" required for planning and performing actions to obtain rewards. vHPC inputs onto NAc presumably provide information regarding affective valence of locations in space and previous experience generated from emotional learning. This applies to both positive and negative emotional states, i.e., reward- and aversion-based learning, including context-dependent fear conditioning, feeding behavior, and responses to drugs of abuse (Vezina et al., 1989; Fanselow, 2000; Kanoski and Grill, 2017). While general BLA activity and BLA projections to many other brain regions regulate fear-related learning and behavior, glutamatergic inputs from BLA onto NAc MSNs increases reward seeking and supports positive reinforcement (Ambroggi et al., 2008; Stuber et al., 2011; Janak and Tye, 2015).

Many of these NAc glutamatergic input regions also project to each other, and NAc MSNs send and receive GABAergic projections to and from the VTA as well. This results in a complex cortico-basal ganglia reward network (Sesack and Grace, 2010; Floresco, 2015), a simplified version of which is presented here (**Figure 1A**). The ultimate function of this network is to regulate and integrate cortical/limbic glutamatergic signals representing executive control, memory, and emotion with dopaminergic reward processing to control the thalamocortical outputs that drive behavior. Critically, many of the regions involved in this circuit undergo long-term changes in gene expression, and cell function, often as a result of stress exposure, that may drive mood-related disorders, and these changes result, in part, from aberrant expression and function of IEGs. This is particularly evident in stress-induced changes in the structure of reward network neurons.

Chronic social defeat stress, a rodent model of depression, causes an increase in dendritic spine density in NAc MSNs (**Figure 1B**). MSN dendritic spines are the structural correlate of glutamatergic inputs, and the number and shape of spines represent the number and strength of those individual inputs. The increased spine density observed in the NAc after chronic social defeat stress (CSDS) is due primarily to an increase in the number of stubby spines, which are immature, and there is no change in mature mushroom-shaped spines (Christoffel et al., 2011). Stubby spines are associated with smaller postsynaptic densities (PSDs) and weaker responses to glutamate, but the increase in their density after stress may represent an increase in glutamatergic signaling to the NAc, and it is indeed accompanied by an increase in the number (but not amplitude) of miniature excitatory postsynaptic potentials (mEPSPs; Christoffel et al., 2011). In addition to stress paradigms such as CSDS, the administration of psychostimulants such as cocaine also increases dendritic spine density, mainly due to an increase in the number of thin spines (Robinson and Kolb, 1999; Russo et al., 2010), a shape also considered immature. However, in contrast to stress, stimulant drug administration increases dendritic spine complexity in NAc MSNs, with many spines showing branching with multiple heads (Robinson and Kolb, 1999; **Figure 1B**). This increase in complexity may represent a reorganization and increase in synaptic signaling, indicating a change in circuit function after drug experience. Many gene products may be involved in the regulation of dendritic spines in the stressed and drug-exposed states, including several of the IEGs discussed below (e.g., ΔFosB, CREB; Maze et al., 2010; Russo et al., 2010). A better understanding of the links between IEG expression and structural and functional plasticity of the reward network is critical to

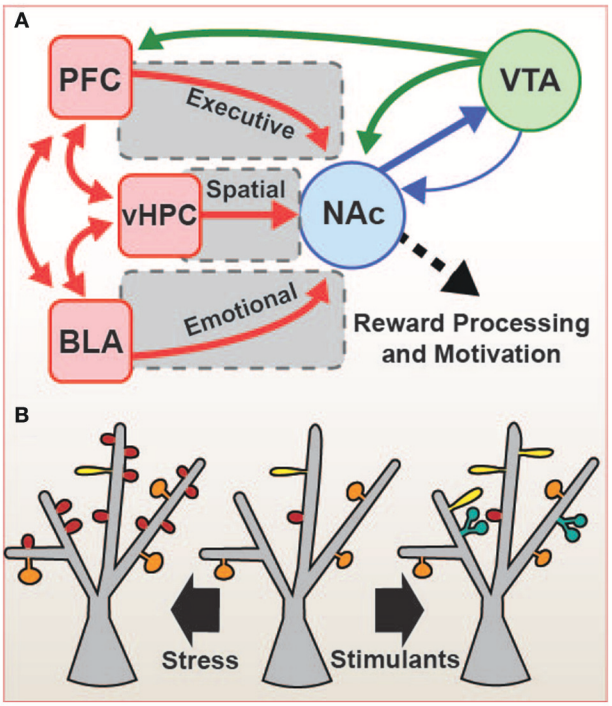

FIGURE 1 | Cortico-basal ganglia reward network. (A) The nucleus accumbens (NAc) integrates glutamatergic inputs (red) which regulate spatial (ventral hippocampus, vHPC), emotional (basolateral amygdala, BLA), and executive (prefrontal cortex, PFC) behaviors, and these inputs are modulated by dopamine (green) from the ventral tegmental area (VTA). BLA, vHPC, and PFC are also interconnected, allowing for further integration of the circuitry. The sum of these inputs results in reward-related learning and decision-making. Stress can cause alteration in the expression of IEGs throughout these brain regions, altering the function and structure these connections, and this may result in pathological changes in reward perception and motivation, including the anhedonia or despair common to many mood disorders. **(B)** Strength and number of glutamatergic connections correlate with changes in the shape and number of dendritic spines in NAc. In chronic social defeat stress (CSDS, left), an increase in the number of stubby (red) spines is observed, while in stimulant drug administration (e.g., cocaine and amphetamines, right), the numbers of thin (yellow), and branched (blue) spines are increased. These structural changes may be mediated by IEGs, and may represent a key factor in the circuit-level changes observed in the depressed and addicted disease states.

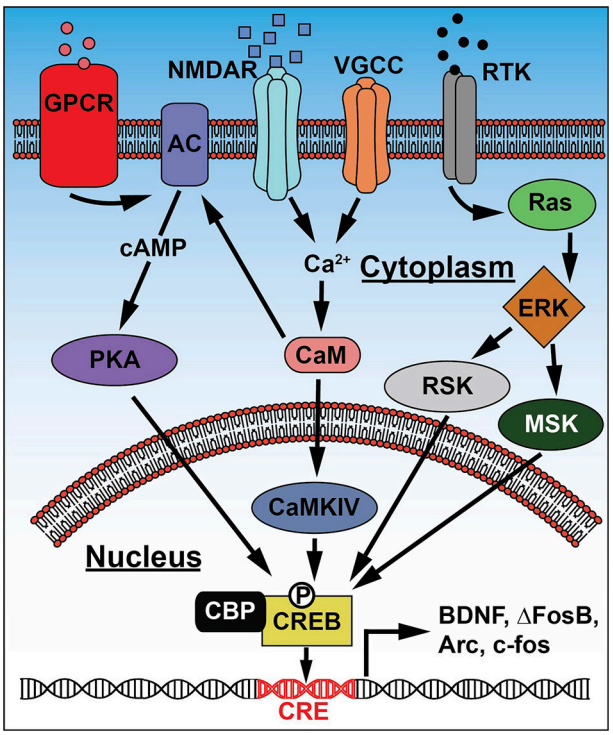

FIGURE 2 | Signaling pathways leading to CREB activation. Extracellular signals and changes in membrane potential activate receptors and channels including: G-protein coupled receptors (GPCR), NMDA-type glutamate receptors (NMDAR), voltage gated Ca^{2+} channels (VGCC), and receptor tyrosine kinases (RTK). These generate increases in second-messenger (cAMP and Ca^{2+}) or MAPK signaling that converge on kinase activation: protein kinase A (PKA), Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV), extracellular signal-regulated kinase (ERK), ribosomal S6 kinase (RSK), and mitogen and stress-activated kinase (MSK). Subsequent phosphorylation at Ser133 activates CREB and promotes interaction with CREB-binding protein (CBP), causing CREB to bind cAMP response elements (CRE) in the promoter regions of target genes and increase expression of proteins that regulate neuronal function, like BDNF and IEGs such as Arc, ΔFosB, and c-fos.

the development of our understanding of mood and addiction pathologies.

cAMP RESPONSE ELEMENT-BINDING PROTEIN (CREB)

CREB is a transcription factor that binds to the canonical cAMP response element (CRE) in DNA in response to activation of signaling pathways involving cAMP, Ca^{2+}/calmodulin, or various growth factors and/or cytokines. CREB activation of target gene transcription (**Figure 2**) is controlled by phosphorylation at serine 133 by protein kinase A (PKA, downstream of cAMP), Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV, downstream of Ca^{2+}), and/or MAP kinase signaling (downstream of growth factors and cytokines; Kida and Serita, 2014). Ser133 phosphorylation promotes interaction with CREB-binding protein (CBP), a critical step for transcriptional activation (Chrivia et al., 1993). The earliest and most extensive studies of CREB's role in neuronal function centered on its control of gene transcription underlying long-term potentiation of synapses and memory formation. CREB is critical for memory and synaptic plasticity in the invertebrate sea slug *Aplysia* (Dash et al., 1990; Kandel, 2012) and fruit fly (Yin et al., 1994), and long-term memory is impaired in CREB loss-of-function mice, but enhanced in CREB gain-of-function mice, primarily due to its role in the hippocampus (summarized in Kida and Serita, 2014).

CREB is stimulated in NAc by exposure to various stressors, and its activation in NAc has been linked to a variety of emotional responses, with the general consensus being that chronic activation of CREB in NAc leads to anhedonia while inhibition of CREB function in NAc promotes reward (Barrot et al., 2002; Carlezon et al., 2005). Moreover, reduced CREB

activity in NAc appears to have antidepressant-like effects in multiple stress models (Pliakas et al., 2001; Conti et al., 2002; Newton et al., 2002; Covington et al., 2011), suggesting that stress-induced CREB activation in NAc may contribute to the etiology of depression. However, the opposite appears true with regard to anxiety-like behaviors, as increased NAc CREB activity appears anxiolytic while inhibition of NAc CREB promotes anxiety (Barrot et al., 2002, 2005; Wallace et al., 2009), indicating that manipulation of NAc CREB activity may not be a simple therapeutic inroad to treatment of mood disorders.

In contrast to its function in NAc, CREB activation in hippocampus produces an antidepressant effect (Chen et al., 2001), and it is indeed induced in the hippocampus by a variety of antidepressant treatments (Nibuya et al., 1996; Thome et al., 2000). One of the many identified target genes of CREB is brain-derived neurotrophic factor (BDNF), and BDNF is also induced in hippocampus by antidepressants (Nibuya et al., 1995) and it is a key transducer of antidepressant effects (Björkholm and Monteggia, 2016). This CREB-BDNF pathway has been postulated to induce hippocampal neurogenesis as a crucial step in antidepressant action (Duman, 2004; Carlezon et al., 2005). It therefore follows that dysfunction of CREB in hippocampus may underlie both depression and some of the cognitive dysfunction linked to chronic stress that are often comorbid with mood disorders (Bortolato et al., 2014). It is also critical to note that CREB regulates the expression of many other IEGs linked to stress responses and depression, including FosB, c-fos, and Arc (see below), and so may act as a master regulator of the activity-dependent transcriptional response to stress throughout the reward circuitry.

AP-1 PROTEINS—c-fos, FosB/ΔFosB, JUN

Activator protein 1 (AP1) is a complex composed of heterodimers between Fos family proteins, Jun family proteins, Jun dimerization proteins, and/or activating transcription factor (ATF) proteins that, when assembled, act as potent and specific regulators of gene transcription. A typical AP1 complex consists of Fos-Jun heterodimers that utilize leucine zippers present in both proteins for dimerization and a basic region that interacts with DNA. The Fos family of transcription factors is comprised of c-fos, FosB (and its splice variants, ΔFosB and Δ2ΔFosB), Fra1, and Fra2, all of which are induced by neuronal activity. c-fos is transiently and robustly induced, with a half-life ranging from minutes up to a couple hours (Sheng and Greenberg, 1990; Kovács, 1998; Ferrara et al., 2003), and is hypothesized to target a wide variety of genes associated with cell differentiation, cell and synapse development, synaptic plasticity, and learning (Alberini, 2009; West and Greenberg, 2011). Its clear connection to cellular activity has led to its use as a marker of brain region activation in a range of behavioral and physiological conditions, however conclusive evidence for c-fos-specific gene targets has not yet been provided, and its direct role in neuronal function remains obscure. It is induced throughout the reward circuitry by virtually all emotionally salient stimuli (Kovács, 1998; Cruz et al., 2015; Nestler, 2015b), but its functional role in mood disorders and antidepressant responses is not well-understood.

FosB is encoded by the *FosB* gene and shares many characteristics with c-fos: FosB has low basal expression and is transiently and robustly induced by neuronal activity (Nestler et al., 1999), with a similar short half-life in cells to that of c-fos (Dobrazanski et al., 1991; Ferrara et al., 2003; Ulery et al., 2006). Splice variation of *FosB* gene transcripts produces a premature stop codon resulting in the truncated ΔFosB protein, which lacks two c-terminal degron domains lending it increased stability (Carle et al., 2007). Most other IEGs have a half-life of a few hours, while ΔFosB has an unusually long-half life, up to 7 days *in vivo* (Hope et al., 1994; Andersson et al., 2003; Ulery-Reynolds et al., 2009), making it a marker of *chronic* neuronal activity. ΔFosB is induced throughout the reward circuitry by chronic stress (Perrotti et al., 2004) and chronic antidepressant exposure (Vialou et al., 2015), and like CREB (which is essential for ΔFosB induction, Vialou et al., 2012), the behavioral effects of its expression differ by brain region. In the NAc, ΔFosB is induced by chronic social defeat stress, and its induction is greater in animals resilient to the behavioral effects of stress than in those susceptible to the depression-like phenotype (Vialou et al., 2010a). Moreover, ΔFosB induction in NAc promotes resilience to chronic stress and is necessary for the antidepressant effects of SSRIs like fluoxetine (Vialou et al., 2010a), apparently through modulation of AMPA receptor subunit expression and epigenetic regulation of CaMKIIα expression (Vialou et al., 2010a; Robison et al., 2014). Its induction by stress in resilient mice appears specific to D1-type MSNs in NAc, while a lower level of induction is seen in D2-type MSNs of susceptible mice (Lobo et al., 2013). Indeed, the specific overexpression of ΔFosB in D1 MSNs appears to have antidepressant effects (Vialou et al., 2010a; Muschamp et al., 2012; Donahue et al., 2014), and it alters the structure of glutamatergic synapses on these specific neurons. ΔFosB promotes the expression of immature thin and stubby dendritic spines, and a concomitant increase in silent synapses, in D1 but not D2 MSNs (Grueter et al., 2013), suggesting that it selectively alters glutamatergic inputs onto NAc direct pathway output neurons, directly modulating reward processing.

In the medial PFC, ΔFosB is selectively induced in mice susceptible to chronic social defeat stress (Vialou et al., 2014). Further, in direct opposition to its effects in NAc D1 MSNs, ΔFosB inhibition in mPFC neurons promotes resilience to chronic stress, while ΔFosB overexpression drives susceptibility, at least in part through induction of the cholecystokinin-B receptor (Vialou et al., 2014). The effect appears to be mediated by ΔFosB expression in mPFC neurons that project to NAc, emphasizing the critical nature of activity-dependent gene expression within the circuitry of reward. We recently reported that ΔFosB expression in hippocampus is critical for multiple forms of learning (Eagle et al., 2015), but the role of hippocampal ΔFosB in stress responses and mood disorders, both locally and in projections to NAc or PFC, remains unknown.

SERUM RESPONSE FACTOR (SRF)

SRF is a transcription factor that binds specifically to the serum response element found in the promoters of many other IEGs and a number of cardiac-specific genes (Knöll and Nordheim, 2009). In the adult brain, SRF is required for activity-induced gene

expression and synaptic plasticity but not for neuronal survival (Ramanan et al., 2005). Through its mediation of the expression and function of cytoskeletal-associated proteins, SRF seems to be instrumental in converting synaptic activity into plasticity-associated structural changes in neuronal circuits (Knöll and Nordheim, 2009), making it a potential player in the activity-dependent gene expression underlying stress-induced changes in reward circuitry. Indeed, SRF is induced in the NAc of resilient mice after chronic social defeat stress, and it binds to the *FosB* promoter and increases transcription of the gene (Vialou et al., 2010b). The subsequent SRF-dependent stress induction of ΔFosB is critical for the resilient phenotype, and, unlike cocaine-dependent induction of ΔFosB, appears independent of CREB actions at the *FosB* promoter (Vialou et al., 2010b, 2012).

EARLY GROWTH RESPONSE PROTEIN-1 (Egr-1)

Egr-1, also known as zinc finger protein 268, is an activity-dependent neuronal transcription factor that binds DNA via three distinct zinc finger domains. It appears to play a role in neuronal plasticity (Knapska and Kaczmarek, 2004), perhaps through its regulation of the expression of synaptobrevin II (Petersohn and Thiel, 1996). Egr-1 is induced in hippocampus by acute stress, like forced swim in rats, through activation of a complex epigenetic mechanism stemming from hippocampal glucocorticoid receptor (GR) activation (summarized in Reul, 2014). MAPK signaling downstream of GRs drives MSK1 and Elk-1 activity, a pathway also upstream of CREB and c-fos induction. This favors Ser10 phosphorylation and Lys14 acetylation of histone 3 at the Erg-1 gene promoter, leading to relaxed chromatin compaction, changes in DNA methylation, and Erg-1 expression (Gutièrrez-Mecinas et al., 2011; Saunderson et al., 2016). This effect lasts at least days in the brain, and may be responsible for subsequent altered responses to forced swim, perhaps underlying long-term stress-induced despair, a hallmark of mood disorders. Indeed, Egr-1 expression is reduced in both hippocampus and PFC by social isolation (Ieraci et al., 2016), indicating that it may contribute to long-term changes in mood due to prolonged stress. In the future, it will be critical to determine whether the effects of Egr-1 expression in hippocampus occur due to alteration of hippocampal projections to or from other reward circuitry components, such as NAc.

Egr-3, which colocalizes with Egr-1 and is also induced in an activity-dependent manner, has recently been implicated in multiple mood disorders. Egr-3's many targets include Arc (Li et al., 2005), discussed below, as well as NMDA and GABA receptor subunits (Roberts et al., 2005; Kim et al., 2012), suggesting that it may contribute to excitatory/inhibitory balance in reward circuitry. Initial studies using SNPs in the Egr-3 gene found a potential association with child bipolar disorder (Gallitano et al., 2012). A more recent study used large-scale microarray data and found that Erg-3 may play a critical role in dysregulation of PFC transcriptional networks in patients with bipolar depression (Pfaffenseller et al., 2016). Moreover, rodent studies suggest that Egr-3 may underlie some of the effects of clozapine in treating both psychosis and bipolar symptoms (Gallitano-Mendel et al., 2008; Williams et al., 2012), suggesting that further study of Egr-3 may yield critical insights into the etiology of mood disorders.

NPAS4

Neuronal PAS domain protein 4, or NPAS4, is an activity-dependent transcription factor expressed exclusively in neurons. It is necessary for normal development of inhibitory interneurons as well as neuronal plasticity in response to experience (Lin et al., 2008; Ploski et al., 2011; Ramamoorthi et al., 2011; Sim et al., 2013). Since NPAS4 is induced in both excitatory and inhibitory neurons and initiates distinct cascades in each cell type (Spiegel et al., 2014), it is thought to regulate excitatory and inhibitory balance within circuits (Bloodgood et al., 2013). Identified downstream targets of NPAS4 include brain-derived neurotrophic factor (BDNF) in excitatory neurons, and FERM and PDZ domain-containing protein 3 (Frmpd3) in inhibitory neurons (Spiegel et al., 2014).

In HPC, NPAS4 induction by both synaptic potentiation and depression protocols requires MAPK and PI3K pathways (Coba et al., 2008), suggesting a link to activation of other IEGs, like CREB. Stress directly mediates NPAS4 activation, as agonist bound glucocorticoid receptor binds to the NPAS4 promoter to downregulate its expression during acute stress (Furukawa-Hibi et al., 2012). After chronic stress, NPAS4 mRNA is significantly decreased in the hippocampus of juvenile mice, and these NPAS4-deficient juveniles developed cognitive deficits in adulthood (Ibi et al., 2008; Yun et al., 2010; Coutellier et al., 2015). These long-term changes may arise through epigenetic regulation, as the NPAS4 promoter has several CpG islands, and stress increases methylation at these sites (Furukawa-Hibi et al., 2015). Several animal strains, including SERT knockout rats and the Flinders Sensitive Line, have shown correlations between low NPAS4 expression, depressive-like behaviors, and antidepressant resistance (Guidotti et al., 2012; Bigio et al., 2016). Much of this work has been done in HPC, and further studies are needed to characterize the role of NPAS4 in NAc and other reward circuitry areas in the context of the same depression models. Moreover, NPAS4 is upregulated in NAc after exposure to drugs of abuse (Guo et al., 2012), but is role in drug responses or behaviors underlying addiction remains unknown.

ACTIVITY-REGULATED CYTOSKELETON-ASSOCIATED PROTEIN (Arc)

Arc is a flexible, modular, multidomain protein that interacts with many partners (Myrum et al., 2015; Zhang et al., 2015). Through these interactions, Arc serves to maintain the phosphorylation of the actin depolymerization factor cofilin, preserving its inactive form, and thus favors the polymerization of actin (Messaoudi et al., 2007). In this manner, Arc promotes the induction of thin, immature dendritic spines, a function shared with ΔFosB (see above). Importantly, Arc is also localized

to the postsynaptic density where it plays a critical role in internalization of AMPA receptors (Chowdhury et al., 2006) and promotes formation of immature dendritic spines (Peebles et al., 2010) and long-term depression (LTD; Bramham et al., 2010).

Recent evidence suggests that Arc expression and function may be tied to multiple aspects of depression. In a variety of rat and mouse paradigms, Arc is consistently induced throughout the cortex and hippocampus by acute stress, but may be up- or down-regulated by chronic stressors depending on the paradigm (Elizalde et al., 2010; Molteni et al., 2010; Boulle et al., 2014). In addition, the vast majority of studies suggest that chronic antidepressant treatment induces Arc expression throughout rodent cortex and hippocampus, and stress-induced Arc expression in specific brain regions appears to predict the subsequent effects of stress on cognitive function (summarized in Li et al., 2015). Thus, it seems possible that stress- or antidepressant-induced Arc may be critical for remodeling of reward circuitry synapses, perhaps in glutamatergic inputs to NAc or connections between other cortical and basal-ganglia regions, but further study will be required to determine the exact contribution of Arc expression to stress responses and mood disorders.

HOMER1A

Homer1 proteins act primarily as scaffolds mediating the interactions and locations of other neuronal proteins, including metabotropic glutamate receptors (e.g., mGluR1 and mGluR5), IP_3 receptors, Shank, and others. The short splice variant of Homer1, Homer1a, is induced by neuronal activity and acts as a dominant negative to block interactions of the long, constitutively active splice variants (Homer1b and Homer1c) with their normal ligands via competition for EVH1 binding sites. For example, Homer1a has been shown to uncouple mGluR receptors from downstream signaling (Tu et al., 1998) as well as cause a decrease in the size and density of dendritic spines (Sala et al., 2003) via inhibition of Shank targeting to synapses. The *Homer1* gene is implicated in the pathogenesis of major depression through genome-wide association as well as neuroimaging studies (Rietschel et al., 2010). In a repeated forced swim mouse model of depression, Homer1a is reduced in cortex, and this is reversed by antidepressant exposure (Sun et al., 2011). Interestingly, Homer1b and 1c are induced in HPC by social defeat stress (Wagner et al., 2015), and increasing their levels in proportion to Homer1a may act as a mechanism of resilience. This is because overexpression of Homer1a in mouse HPC promotes susceptibility to social defeat stress, with overexpressing animals showing increased behavioral despair and less active coping behavior (Wagner et al., 2015). In the accumbens, Homer1a is induced by antipsychotics that act at dopamine receptors (reviewed in Iasevoli et al., 2009), but any role of Homer1a in accumbens-mediated behavioral responses to stress and drugs of abuse remains to be uncovered.

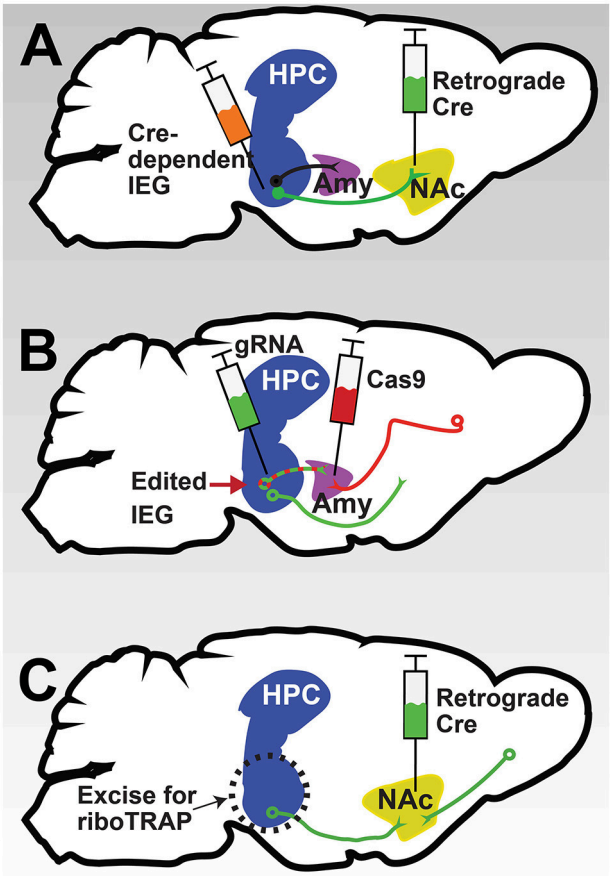

FIGURE 3 | Potential methods for circuit-specific interrogation of IEG function. (A) Schematic depicting the combination of a retrograde Cre virus (green) injected into a target region like NAc with a local virus expressing an IEG in a Cre-dependent manner injected into a projecting region like ventral HPC. Such a strategy would result in overexpression of the IEG only in HPC neurons projecting to NAc. **(B)** Schematic of a converse strategy: the combination of a retrograde virus expressing Cas9 (red) in a target region like amygdala (Amy) with a local virus expressing a guide RNA targeting an IEG (green) in a projecting region like ventral HPC could be used to silence an IEG in a specific circuit. **(C)** Schematic of a strategy for uncovering circuit-specific gene expression. Using a retrograde Cre virus in a target region to induce expression of GFP-tagged ribosomes in the projection region allows the use of TRAP to determine gene expression changes in the circuit. By combining this approach with mice floxed for a specific IEG, circuit-specific IEG transcriptional targets could be revealed.

OUTSTANDING QUESTIONS AND FUTURE DIRECTIONS

Despite accumulating evidence of IEG induction within reward circuitry in rodent models and patients with mood disorders, we still don't fully understand the contribution of IEGs to reward circuitry function and pathological behavior. A critical next step is to target IEGs in specific neural circuits. Such an approach has been difficult using classical techniques, but recent advances in cell labeling and cell- and circuit-specific manipulation provide exciting avenues to address some critical outstanding questions.

Are There Distinct Roles for IEGs in Specific Neuronal Subtypes?

Do IEGs perform the same functions in all neuronal cell types? Because some IEGs are induced more sparsely compared to others (e.g., NPAS4), the relevance of IEG expression to mood disorders may be tied to their induction in specific cell populations. Transgenic mouse lines allowing selective overexpression or knockout of IEGs in neurons that produce specific neurotransmitters (i.e., DAT-Cre or GAD-Cre) or express specific receptors (i.e., D1-Cre or D2-Cre) will be a critical tool in future studies. Moreover, coupling these lines with Cre-dependent viral vectors will address the role of IEGs in individual neuronal subtypes with both spatial and temporal specificity.

What Is the Role of IEGs in Specific Brain Circuits?

Although IEGs may be activated in many brain regions in response to stress or drugs, their relevance in circuits underlying addiction and depression behaviors is not fully understood. To assess the contribution of activated IEG suites within mesolimbic and cortical circuitry to cell function and animal behaviors, novel retrograde viral vector approaches will be critical. For instance, by combining a retrograde virus expressing Cre injected into a target region such as NAc with a locally expressing virus overexpressing an IEG of interest in a Cre-dependent manner injected into ventral HPC, one could measure the effects of the IEG on the function of HPC neurons specifically projecting to NAc, as well as subsequent behavior of the animal (**Figure 3A**). Alternatively, by combining retrograde expression of the Cas9 enzyme with a local expression of guide RNA, CRISPR-mediated editing of an IEG could be used to determine its circuit specific role (**Figure 3B**), an approach currently being piloted by our group and others. Of course, these techniques could be combined with transgenic Cre driver lines described above to allow cell-type *and* circuit-specific manipulation of IEGs, critical steps for our understanding of their role in the pathophysiology of psychiatric disease.

What Are the Gene Targets of IEGs in Specific Cell Types and Circuits?

Although it is critical to understand the roles of IEGs in specific cell types, neuronal ensembles, and specific circuits, many IEGs make unlikely pharmacological targets for treatment of psychiatric disease, as they often play critical roles in non-disease-related brain regions and other tissues. However, uncovering the gene targets of IEG transcription factors, like Fos family proteins or NPAS4, may reveal critical mediators of pathophysiology that are more amenable to pharmacological manipulation. New advances in gene expression profiling, like translating ribosomal affinity purification (TRAP; Heiman et al., 2014), are sufficiently flexible and robust to be applied to Cre-dependent cell- and circuit-specific approaches described above (Lobo, 2009; McCullough et al., 2016), and primed for use in Cre-dependent ensemble-specific approaches (Sakurai et al., 2016). Utilizing Cre-dependent reporter mouse lines expressing GFP-tagged ribosomes in combination with retrograde Cre viruses will allow circuit-specific TRAP profiling of gene expression (**Figure 3C**). Combining such an approach with mice floxed for a specific IEG will then allow assessment of the contribution of that IEG to circuit-specific gene expression in the context of stress or drugs. We predict that such techniques will uncover novel gene products underlying mood or substance use disorders that could be pharmacologically accessible targets for novel treatments.

CONCLUSIONS

It is clear that exposure to stressful events in life increases risk for mood disorders, and the many preclinical and fewer postmortem studies summarized here suggest that this may arise in part from stress-induced remodeling of reward circuitry driven by IEG expression. For some of these IEGs, like CREB, Homer1a, and ΔFosB, evidence abounds for their roles in stress responses, multiple aspects of mood disorders, drug addiction, and even antidepressant treatment, and the challenge now lies in integrating their functions across the brain regions and cell types involved and determining their downstream targets in order to uncover potential novel drug targets. For other IEGs, such as Egr-1, NPAS4, and Arc, their induction by stress makes them molecules of interest in mood disorder research, but causal connections to depression-related behaviors have not yet been uncovered, and continued study of their role in reward circuitry function is needed. In all cases, it has become clear that stress-dependent remodeling of the reward circuitry, and particularly of glutamatergic inputs to NAc, is a critical component in the development of depression- and addiction-related phenotypes, and that IEGs play a crucial role in this process and may provide a pathway to the molecular, cellular, and circuit-level mechanisms of mood disorder etiology and treatment.

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CM, EW, and AR researched, wrote, and edited the manuscript.

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MINI REVIEW
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Beyond Neuronal Activity Markers: Select Immediate Early Genes in Striatal Neuron Subtypes Functionally Mediate Psychostimulant Addiction

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Immediate early genes (IEGs) were traditionally used as markers of neuronal activity in striatum in response to stimuli including drugs of abuse such as psychostimulants. Early studies using these neuronal activity markers led to important insights in striatal neuron subtype responsiveness to psychostimulants. Such studies have helped identify striatum as a critical brain center for motivational, reinforcement and habitual behaviors in psychostimulant addiction. While the use of IEGs as neuronal activity markers in response to psychostimulants and other stimuli persists today, the functional role and implications of these IEGs has often been neglected. Nonetheless, there is a subset of research that investigates the functional role of IEGs in molecular, cellular and behavioral alterations by psychostimulants through striatal medium spiny neuron (MSN) subtypes, the two projection neuron subtypes in striatum. This review article will address and highlight the studies that provide a functional mechanism by which IEGs mediate psychostimulant molecular, cellular and behavioral plasticity through MSN subtypes. Insight into the functional role of IEGs in striatal MSN subtypes could provide improved understanding into addiction and neuropsychiatric diseases affecting striatum, such as affective disorders and compulsive disorders characterized by dysfunctional motivation and habitual behavior.

Keywords: striatum, IEGs, psychostimulants, ΔFosB, c-Fos, Egr3, MSNs, cocaine

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INTRODUCTION

Immediate early genes (IEGs) are activated transiently and rapidly throughout the brain by many cellular stimuli including psychostimulants. Traditionally, IEGs are used as markers of neuronal activity, in striatum and other brain regions, in response to psychostimulants. The striatum consists of the dorsal striatum, which regulates actions and habits vs. ventral striatum (a.k.a.- nucleus accumbens- NAc), which is involved in motivation and reinforcement (Voorn et al., 2004; Everitt and Robbins, 2013). Both striatal regions mediate psychostimulant-induced behavior as observed through motor, reward, motivational and habitual behaviors (Voorn et al., 2004). The main projection neurons in striatum are medium spiny neurons (MSNs), which are composed of two subtypes, those enriched in dopamine receptor 1 (D1) vs. dopamine receptor 2 (D2), as well as several other genes (Gerfen et al., 1990; Lobo et al., 2006; Heiman et al., 2008). The D1-MSNs vs. D2-MSNs are further distinguished by their projections through the brain (Gerfen, 1984, 1992; Smith et al., 2013). Early studies examining IEG gene and/or protein expression

identified striatal MSN subtypes that are activated by psychostimulants (Robertson et al., 1991; Young et al., 1991; Berretta et al., 1992; Cenci et al., 1992; Moratalla et al., 1996; Bertran-Gonzalez et al., 2008). However, in focusing on IEGs as activity markers in MSN subtypes, important information about the functional role of IEGs in psychostimulant-mediated behavioral and cellular plasticity is potentially missed. This review article will discuss the subset of research addressing this issue by summarizing the current insight into IEG function in D1-MSN and D2-MSN subtypes in psychostimulant action. These findings have implications for addiction, as well as neuropsychiatric diseases affecting MSN subtypes including affective disorders and compulsive disorders.

FosB IN D1-MSNs AS A MOLECULAR SWITCH FOR PSYCHOSTIMULANT ADDICTION

The most well studied IEG in MSN subtypes is FosB. FosB is induced in striatum by acute cocaine (Hope et al., 1992) but the long lasting ΔFosB, generated from the FosB primary transcript (Yen et al., 1991), persistently accumulates after chronic psychostimulant exposure (Hope et al., 1994). The persistent accumulation of ΔFosB is a consequence of a lack of the degron domain containing C-terminal and through CAMKIIα phosphorylation at the Ser37 stabilization site in ΔFosB, thus producing this stable version of FosB (Carle et al., 2007; Robison et al., 2013). This long lasting induction of ΔFosB by cocaine is dependent on D1 receptor signaling (Moratalla et al., 1996) implicating this induction occurs primarily in D1-MSN subtypes. Recent studies using D1-GFP reporter lines confirm ΔFosB induction occurs primarily in D1-MSNs after chronic cocaine (Lee et al., 2006; Lobo et al., 2013). Consistent with these findings, FosB mRNA was induced in D1-MSNs with acute and chronic cocaine using a ribosomal tagging approach (Heiman et al., 2008; Chandra et al., 2015).

Initial studies using a tetracycline responsive promoter (TetOp)-ΔFosB line crossed to a NSE-tetracycline transactivator (ttA) line resulted in expression of ΔFosB in striatal D1-MSNs (Kelz et al., 1999). This D1-MSN ΔFosB line displays enhanced locomotor and conditioned place preference (CPP) responses to cocaine (**Table 1**). Additionally, this line shows facilitated acquisition to cocaine self-administration at low threshold doses and enhanced effort to maintain self-administration of higher doses on a progressive ratio schedule of reinforcement (Colby et al., 2003; **Table 1**). These behaviors are occurring potentially through enhanced structural plasticity in D1-MSNs, since adenoassociated virus (AAV) mediated ΔFosB overexpression in NAc enhances MSN structural plasticity (Maze et al., 2010). Use of Cre-inducible herpes simplex virus (HSV) to overexpress ΔFosB in D1-MSNs in the NAc of D1-Cre mice confirmed the enhanced cocaine-mediated behavioral responses and showed that ΔFosB alone can enhance immature spine formation and reduce AMPAR/NMDAR ratios, in D1-MSNs (Grueter et al., 2013; **Table 1**). These structural and synaptic plasticity changes by ΔFosB are an indication of enhanced silent synapses, which are characteristic of cocaine effects on D1-MSNs (Graziane et al., 2016). Silent synapses are regarded as newly generated AMPAR-silent, NMDAR-only synapses, which are often present in the neonatal brain (Dong and Nestler, 2014). After withdrawal periods these synapses either retract or develop into fully functional synapses to induce new neural circuits (Dong and Nestler, 2014). A large body of evidence demonstrates that generation of these nascent synapses can promote behavioral responses to cocaine, such a locomotor sensitization, and that the maturation of these silent synapses can promote long-term behaviors associated with cocaine addiction, such as relapse (Russo et al., 2010; Dong and Nestler, 2014). Thus, ΔFosB may set the stage for long-term cocaine abuse by regulating the establishment of silent synapses in D1-MSNs during the initial stage of drug exposure. Whether ΔFosB in MSN subtypes continues to play a role in the long-term behaviors associated with drug addiction remains to be determined. Future studies performing these MSN subtype manipulations with prolonged abstinence and relapse models will help to answer this question. Finally, investigation of ΔFosB overexpression in D2-MSNs had no effect on cocaine-induced behaviors or spine formation but did enhance AMPAR/NMDAR ratios (Grueter et al., 2013; **Table 1**) suggesting that ΔFosB in these MSNs might play a role mature spine formation. This has implications for stress behavior since ΔFosB in increased in D2-MSNs in mice displaying stress susceptibility (Lobo et al., 2013).

A mechanistic role of ΔFosB in promoting behavioral and structural plasticity after cocaine has been examined. The TetOp-ΔFosB line displayed enhanced expression of GluR2 in NAc and GluR2 overexpression in NAc enhances cocaine CPP (Kelz et al., 1999). Robison et al. (2013) showed that ΔFosB increased CAMKIIα gene expression in NAc of the TetOp-ΔFosB line and the enhanced cocaine-mediated behavioral and structural plasticity effects of ΔFosB in NAc are CAMKIIα dependent (**Figure 1**). Along with regulating CAMKIIα, ΔFosB regulates a number of genes in NAc by chronic cocaine (McClung and Nestler, 2003; Renthal et al., 2009). Investigation of ΔFosB in other brain regions demonstrated unique targets, such as CCK and Cdk5 (Chen et al., 2000; Vialou et al., 2014) suggesting that ΔFosB may differentially regulate transcripts in different cell subtypes. Thus, ΔFosB and other IEG transcription factors could differentially regulate gene transcription in D1-MSNs vs. D2-MSNs. Future studies using neuronal subtype ChIP can provide improved understanding into the MSN subtype transcriptional role of ΔFosB in cocaine action.

c-Fos FUNCTION IN D1-MSNs IN COCAINE ACTION AND c-Fos AS AN ACTIVITY MARKER TO PROVIDE INSIGHT INTO FUNCTION

While FosB has been the most widely studied IEG in striatal circuits in psychostimulant action, a functional role for c-Fos in D1-MSN subtypes has been investigated. Previous rat studies demonstrate c-Fos induction in both MSN subtypes when a psychostimulant is given in a novel environment (Badiani

TABLE 1 | Medium spiny neuron (MSN) subtype manipulation of Immediate early genes (IEGs) in cocaine behaviors.

Molecule	Cell type	Brain region	Method	Effects mediated by cocaine	References
ΔFosB	D1-MSN	Striatum	NSE-tTA × TetOp-ΔFosB (Overexpression)	Increased CPP and locomotion	Kelz et al. (1999)
ΔFosB	D1-MSN	Striatum	NSE-tTA × TetOp-ΔFosB (Overexpression)	Enhanced cocaine acquisition and reinforcement (self-administration)	Colby et al. (2003)
ΔFosB	D1-MSN	NAc	HSV-LS1-ΔFosB + D1-Cre (Overexpression)	Increased locomotion and CPP	Grueter et al. (2013)
ΔFosB	D2-MSN	NAc	HSV-LS1-ΔFosB + D2-Cre (Overexpression)	No effect on locomotion and CPP	Grueter et al. (2013)
c-Fos	D1-MSN	Striatum	f/f-Fos-D1-Cre (Knockout)	Reduced locomotor sensitization, Reduced CPP extinction	Zhang et al. (2006)
Egr3	D1-MSN	NAc	AAV-Egr3-EYFP + D1-Cre (Overexpression)	Increased CPP and locomotion	Chandra et al. (2015)
Egr3	D2-MSN	NAc	AAV-Egr3-EYFP + D2-Cre (Overexpression)	Reduced CPP and locomotion	Chandra et al. (2015)
Egr3	D1-MSN	NAc	AAV-Egr3-microRNA + D1-Cre (Knockdown)	Reduced CPP and locomotion	Chandra et al. (2015)
Egr3	D2-MSN	NAc	AAV-Egr3-microRNA + D2-Cre (Knockdown)	Increased CPP and locomotion	Chandra et al. (2015)

et al., 1998; Ferguson and Robinson, 2004). Using D1-GFP and D2-GFP reporter mice, researchers demonstrate c-Fos induction by cocaine in a novel environment, occurs primarily in D1-GFP MSNs throughout striatum with a small induction in D2-GFP MSNs in dorsal striatum (Bertran-Gonzalez et al., 2008). c-Fos deletion, in D1-MSNs, blunted cocaine-induced locomotor sensitization and MSN dendritic spine formation (Zhang et al., 2006; **Table 1**). Interestingly, c-Fos deletion in D1-neurons did not alter cocaine CPP but it did prevent the extinction of CPP. These data, illustrate a dynamic role for c-Fos induction in D1-MSNs, however, one cannot rule out the differential behavioral effects as being mediated by other brain regions that express the D1 receptor.

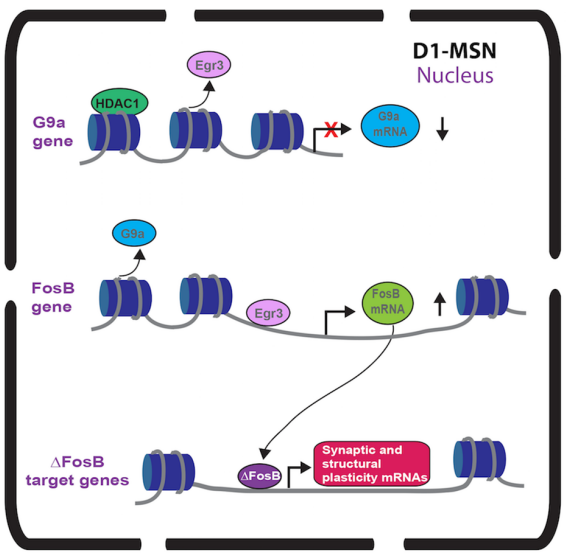

FIGURE 1 | Immediate early gene (IEG) transcriptional regulation in nucleus accumbens (NAc) dopamine receptor 1 (D1)-medium spiny neurons (MSNs) after repeated cocaine. Repeated cocaine causes reduced Egr3 binding to the G9a promoter (Chandra et al., 2015) and G9a transcription is repressed by mechanisms including HDAC1 (Kennedy et al., 2013). This causes reduced G9a in NAc D1-MSNs (Chandra et al., 2015). Repeated cocaine results in increased Egr3 binding to the FosB promoter causing increased FosB in NAc D1-MSNs (Heiman et al., 2008; Chandra et al., 2015). The truncated FosB isoform, ΔFosB, is increased in NAc D1-MSNs (Lee et al., 2006; Lobo et al., 2013) after repeated cocaine leading to increased binding of ΔFosB on synaptic plasticity and structural plasticity gene promoters (Maze et al., 2010; Robison et al., 2013).

While, a focus on c-Fos as a neuronal activity marker is broadly utilized across neuroscience, researchers use this role of c-Fos to gain functional insight into striatal neuronal ensembles in psychostimulant exposure. c-Fos-lacZ or c-Fos-GFP rodents demonstrate active striatal neuronal ensembles in context dependent cocaine locomotor sensitization. Ablation of these neuronal ensembles in NAc prevents this context-dependent sensitization (Koya et al., 2009). While these c-Fos neuronal ensembles express both D1-MSN and D2-MSN markers, they express higher levels of a D1-MSN enriched gene, prodynorphin (Pdyn) and lower levels of D2-MSN enriched genes, D2 and adenosine 2A (A2A) receptor (Guez-Barber et al., 2011) suggesting a greater number of D1-MSNs in this population. The c-Fos activated NAc ensembles display silent synapses after cocaine locomotor sensitization, which is dependent on a context-specific sensitization (Koya et al., 2012; Whitaker et al., 2016). Future, studies using these c-Fos neuronal ensemble approaches that target MSN subtypes could delineate a functional role for D1-MSN vs. D2-MSN active neuron populations in psychostimulant action.

A BIDIRECTIONAL ROLE OF Egr3 IN COCAINE ACTION THROUGH D1-MSN VS. D2-MSN SUBTYPES

While Egr1 (a.k.a. Zif-268) induction in striatum with acute psychostimulants is D1 receptor dependent (Daunais and McGinty, 1996; Steiner and Gerfen, 1996), there has been no investigation into a functional role of Egr1 in MSN subtypes. However, we recently examined the Egr family member, Egr3 in

MSNs in cocaine action (Chandra et al., 2015). Egr3 is induced in total striatum with acute cocaine through the activation of D1 receptors (Yamagata et al., 1994; Jouvert et al., 2002). Using a ribosomal-trapping method we observed an induction of Egr3 mRNA in NAc D1-MSNs while a reduction occurred in D2-MSNS after repeated cocaine (Chandra et al., 2015). Mimicking the effects of cocaine, by enhancing Egr3 in D1-MSNs and reducing Egr3 in D2-MSNs in NAc using Cre-inducible AAVs combined with D1-Cre and D2-Cre lines, potentiated cocaine CPP and cocaine-induced locomotion. In contrast, blunting the effects of cocaine, reducing Egr3 in D1-MSNs and enhancing Egr3 in D2-MSNs, reduced these behaviors (**Table 1**). Egr3 binding is enriched on promoters of CAMKIIα and FosB in NAc and mRNA of these genes is enriched in NAc D1-MSNs after repeated cocaine (Chandra et al., 2015; **Figure 1**) suggesting that Egr3 acts as a potential upstream regulator of the ΔFosB and CAMKIIα mediated effects in D1-MSNs (Kelz et al., 1999; Grueter et al., 2013; Robison et al., 2013). Additionally, Egr3 binding is reduced on the promoter of the repressive histone methylation enzyme, G9a and G9a is reduced in NAc D1-MSNs after repeated cocaine (Chandra et al., 2015; **Figure 1**). G9a binding and its histone mark, H3K9me2, is reduced on the FosB promoter after repeated cocaine and/or in the Tet-Op-ΔFosB D1-MSN line (Maze et al., 2010; **Figure 1**). Further, G9a overexpression prevents dendritic spine induction by repeated cocaine (Maze et al., 2010). Thus, Egr3 may act, in D1-MSNs, as an upstream regulator of ΔFosB induction and structural plasticity by direct transcriptional regulation at the FosB promoter and indirect regulation through reduced binding at the G9a promoter. This potentially leads to repression of G9A transcription through other factors, such as HDAC1 (Kennedy et al., 2013; **Figure 1**). Interestingly, a previous study showed reduced G9a in both D1-MSNs and D2-MSNs in the entire striatum after repeated cocaine and the effects of G9a were mediated through D2-MSNs (Maze et al., 2014). However, this could be a consequence of a G9A developmental knockout in MSN subtypes, which indeed led to D2-MSNs displaying a D1-MSN subtype identity. Finally, we recently demonstrated that Egr3 binding on the peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α promoter was increased after repeated cocaine exposure (Chandra et al., 2017). We observed an increase of PGC-1α in D1-MSNs and a reduction in D2-MSNs after repeated cocaine. Consistent with these findings, we observed bidirectional behavioral outcomes to cocaine when PGC-1α was overexpressed in NAc D1-MSNs vs. D2-MSNs. Although, the function of PGC-1α in MSN subtypes in cocaine action is unclear, previous research shows that PGC-1α can mediate dendritic spine plasticity in neurons (Cheng et al., 2012). Examination of Egr3's role in MSN structural and synaptic plasticity, as well as the role of G9a, ΔFosB and PGC-1α in mediating these effects through Egr3 will be important for understanding the cellular role of Egr3 in cocaine action.

CONCLUSION

While studies examining psychostimulant-mediated IEG function in D1-MSN vs. D2-MSN subtypes are sparse, they have provided some insight into mechanisms by which IEGs act in these neuron subtypes. This includes actions primarily through D1-MSNs in psychostimulant-mediated molecular, cellular and behavioral plasticity. Overall we focus on three select IEGs that have been examined in MSN subtypes in psychostimulant action. However, examination of other psychostimulant relevant IEGs, such as Arc and CREB (Carlezon et al., 1998; Salery et al., 2017), in MSN subtypes will provide a more comprehensive understanding of IEG function in striatal neuron subtypes in psychostimulant abuse. These MSN subtype specific studies have been restricted to non-contingent behaviors or the acquisition phase of self-administration, in the case of ΔFosB. Nonetheless, they provide potential mechanistic insight into the early stages of drug exposure before the shift to the addictive state. ΔFosB is involved in the generation of early plasticity processes, such as silent synapses, in D1-MSNs in psychostimulant exposure that set the stage for long-term neural circuit reorganization and the enduring behaviors occurring in addiction. These processes occur in c-Fos expressing neurons and Egr3 has been shown to transcriptionally regulate FosB, as well as molecules involved with structural plasticity. Thus c-Fos and Egr3, along with ΔFosB, likely play a role, in D1-MSNs, in mediating the nascent spine formation in the early stages of drug abuse that can ultimately give rise to stable spines and the long-term behaviors associated with addiction. Whether these IEGs are necessary to maintain the long-term circuit remodeling and relapse behaviors in addiction remains to be determined. Future studies examining these IEGs in MSN subtypes using more relevant models of addiction including self-administration with abstinence and relapse behavior will provide improved understanding of IEGs in striatal MSN subtypes in addiction. Finally, while studying these IEGs alone has provided important information, a more detailed probing of IEG transcriptional targets in MSN subtypes after drug exposure will provide improved information into the functional consequence of IEGs in addiction. Insight into how these IEG targets are regulating synaptic plasticity, structural plasticity, neural circuit remodeling and ultimately behavior could provide potential molecules that could be therapeutically targeted in addiction. These studies also have implications for neuropsychiatric diseases affecting striatal based behavior, including affective disorders and compulsive or stereotypy disorders.

AUTHOR CONTRIBUTIONS

MKL and RC both contributed to the writing of the manuscript, as well as the preparations of the figure and table.

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frontiers in Behavioral Neuroscience

REVIEW
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Schizophrenia: What's Arc Got to Do with It?

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Human studies of schizophrenia are now reporting a previously unidentified genetic convergence on postsynaptic signaling complexes such as the activity-regulated cytoskeletal-associated (Arc) gene. However, because this evidence is still very recent, the neurobiological implication of Arc in schizophrenia is still scattered and unrecognized. Here, we first review current and developing findings connecting Arc in schizophrenia. We then highlight recent and previous findings from preclinical mouse models that elucidate how Arc genetic modifications might recapitulate schizophrenia-relevant behavioral phenotypes following the novel Research Domain Criteria (RDoC) framework. Building on this, we finally compare and evaluate several lines of evidence demonstrating that Arc genetics can alter both glutamatergic and dopaminergic systems in a very selective way, again consistent with molecular alterations characteristic of schizophrenia. Despite being only initial, accumulating and compelling data are showing that Arc might be one of the primary biological players in schizophrenia. Synaptic plasticity alterations in the genetic architecture of psychiatric disorders might be a rule, not an exception. Thus, we anticipate that additional evidence will soon emerge to clarify the Arc-dependent mechanisms involved in the psychiatric-related dysfunctional behavior.

Keywords: behavior, RDoC, dopamine, glutamate, immediate early gene, Arg3.1, mice

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SCHIZOPHRENIA

Neuropsychiatric disorders are still defined by an ensemble of different behavioral abnormalities appearing with a certain intensity and duration that strongly disrupt the normal life of the affected individuals (American Psychiatric Association, 2013). However, these behavioral alterations present huge heterogeneity within and between subjects in terms of intensity, timing, penetrance depending on the sex and age of the subjects as well as assessment by different health care providers and responses to treatments. "Schizophrenia spectrum and other psychotic disorders" is a definition given when clinical features categorized as positive (e.g., delusions, hallucinations, disorganized thinking, grossly disorganized or abnormal motor behavior) and negative symptoms (e.g., diminished emotional expression and avolition) are evident in an individual (Lewis and Gonzalez-Burgos, 2006; American Psychiatric Association, 2013). In addition, cognitive deficits, mainly in executive functions, are long-lasting traits in patients diagnosed with schizophrenia and constitute key prognostic factors for the long-term outcomes of the disease such as the level of functional capability, social and occupational ability and quality of life (Green, 1996; Green and Nuechterlein, 1999; Mueser and McGurk, 2004). Thus, it is clear that such a complex disorder uniquely defined by several different behavioral abnormalities is subject to different degrees of heterogeneity.

Consistent evidence indicates that many psychiatric disorders such as schizophrenia have a strong genetic contributing factor with heritability estimated at up to 80% (Cardno and Gottesman, 2000; Sullivan et al., 2003). Because of this, over the last decade, an increasing effort has been

made to disentangle the possible impact of genetics in the development and heterogeneity of schizophrenia and of psychiatric disorders in general (Fromer et al., 2014; Purcell et al., 2014; O'Donovan and Owen, 2016). Despite other factors being implicated (e.g., environmental, epigenetics etc.), the current hope in advanced genetic assessments is to improve the causal understanding of psychiatric disorders, to provide a better definition of them, and to ultimately identify better and more effective treatments.

ARC GENETICS IN SCHIZOPHRENIA

Thanks to recent improvements in genomic sequencing techniques, Fromer et al. (2014) were able to run an exome sequencing study scanning genes for the *de novo* mutation at the single-base resolution on genomic DNA of 623 schizophrenia proband trios. Furthermore, Purcell et al. (2014), focusing on a subset of almost 2500 genes that have been previously implicated in schizophrenia, have sequenced the currently largest sample of patients with schizophrenia (2536) and healthy controls (2543). Both these works have unexpectedly revealed a consistent convergence of genetic variations on a set of synaptic proteins that interact with the activity-regulated cytoskeleton associated protein (Arc; Fromer et al., 2014; Purcell et al., 2014). For example, among the 28 genes listed in the "Arc complex", it has been proven that Arc protein directly binds Wave1, GKAP, IQSEC2, GluNA2 (Myrum et al., 2015a; Zhang et al., 2015). However, further biochemical experiments should be performed to substantiate and better validate the mechanisms of interaction within this Arc complex. Nevertheless, in the same year, another study reported reduced expression of Arc mRNA in the prefrontal cortex (PFC) of individuals with schizophrenia (Guillozet-Bongaarts et al., 2014). Moreover, an investigation on variants within the Arc gene reported a direct association between the rs35900184 single-nucleotide polymorphism (SNP) and schizophrenia (Huentelman et al., 2015). To further support the possible implication of Arc in the pathophysiology of schizophrenia, the copy number variance (CNV) and schizophrenia working groups of psychiatric genomics consortium analyzed the CNV of the entire genome comparing more than 40,000 individuals among patients and healthy controls. Again, deletions in a subset of genes belonging to the Arc complex were significantly enriched in patients with schizophrenia (Marshall et al., 2017). Finally, different mouse models relevant to schizophrenia show reduced Arc expression, which is instead not evident in mouse models relevant to other psychiatric disorders such as ADHD or bipolar disorders (Matsuo et al., 2009; Takao et al., 2013; Takagi et al., 2015; Managò et al., 2016; Chen et al., 2017; Mereu et al., 2017). This might suggest that the Arc pathway may serve as a hub that functionally links numerous schizophrenia risk-related factors. Together, these findings point to a previously undetected association of the immediate early gene (IEG) Arc to schizophrenia. However, Arc causal implication in the neuropathophysiology of schizophrenia is just starting to be elucidated (following paragraphs).

ARC GENETICS IN BEHAVIOR

Psychiatric disorders, including schizophrenia, are characterized by abnormal behaviors. Thus, here we will first address the implication of Arc genetics in behavioral functions. In particular, we will follow the new Research Domain Criteria (RDoC) framework recently developed by the USA National Institutes of Health (NIH/NIMH, 2017). The RDoC system currently includes five distinct domains: (1) Cognitive Systems. (2) Systems for Social Processes. (3) Positive Valence System. (4) Negative Valence Systems. (5) Arousal/Regulatory Systems). The RDoC framework aim to integrate many levels of information (from genomics to self-report) with specific dimensions of behavioral functioning, overcoming the boundaries of mental diagnosis. Indeed, within a disease as defined by DSM-V, alterations of different brain circuits or neurotransmitters could affect the same behavior. Alternatively, in different psychiatric illnesses, the same biological alteration could lead to a common behavioral alteration.

To our knowledge, there are still no selective Arc genetic variations in humans proven to be functional (i.e., altering Arc mRNA and/or protein expression). Only one study reported a case of a 7-year old female with a 540 kb microdeletion in the 8q24.3 region, which included Arc but also several other genes (Hu et al., 2015). This patient showed developmental abnormalities, Intellectual Disabilities (ID), autism and attention deficit hyperactive disorder (ADHD). Similarly, the mother, who carried the same microdeletion, presented a milder phenotype, including learning disabilities, depression, panic disorder and obsessive tendencies (Hu et al., 2015). However, this microdeletion syndrome does not account for the selective impact of Arc genetic disruption in behavioral abnormalities. Due to the lack of human data on functional common genetic alterations selective for Arc, our discussion will be centered on the available information derived from preclinical studies addressing the impact of selective Arc functional genetic variations in behavioral functioning.

COGNITIVE SYSTEMS

Arc genetic variations were initially implicated in the formation of long-term memories. Indeed, compared to wild-type mice, Arc knockouts performed slightly worse in the spatial Morris water maze, were impaired in contextual and cued fear conditioning, showed reduced conditioned taste aversion, and impaired long-term novel object recognition memory (Plath et al., 2006). All these abnormal cognitive functions suggest that reduced levels of Arc might be related to hippocampus-dependent memory deficits. Indeed, blocking the expression of Arc selectively in the hippocampus produced the same pattern of performance in the above mentioned tasks, including reduced spatial and fear memory formation in the Morris water maze and fear conditioning task (Guzowski et al., 2000; Chia and Otto, 2013; Nakayama et al., 2015). Altered long-term memories might be present in patients with schizophrenia (Goldberg et al., 1989; Aleman et al., 1999; Ranganath et al., 2008), even if this is not considered a signature feature. For example, patients with

schizophrenia might present episodic memory deficits due to an altered pattern of hippocampal-PFC activity, but they do not show an amnesic syndrome (Ranganath et al., 2008). More recently, we found that partial and complete deletion of the Arc gene in mice produced recency memory deficits in the temporal order object recognition task as well as spatial memory deficits in the spatial object recognition task (Managò et al., 2016). In contrast, cognitive abilities assessed by a recent-memory novel object recognition task reported to be dependent uniquely on the perirhinal cortex (PRH; Barker et al., 2007) were intact (Managò et al., 2016). These findings parallel similar evidence from patients with schizophrenia who show impairments in temporal context memory related to objects as well as in spatial navigation, while no alterations are evident in the ability to recall and recognize target items (Schwartz et al., 1991; Rizzo et al., 1996; Dreher et al., 2001; Folley et al., 2010). These recent mouse studies (Managò et al., 2016) begin to suggest that Arc-dependent cognitive abnormalities might rely on altered PFC and hippocampal dysfunction in the context of a normal functioning of the PRH. In agreement, convergent genetic, molecular, clinical, neurophysiological, neuropsychological and imaging work confirmed the presence of an altered pattern of PFC and hippocampal activation in schizophrenia (Meyer-Lindenberg and Weinberger, 2006; Papaleo et al., 2012; Millan et al., 2014). Initial work did not find an association between Arc common genetic variants and general cognitive abilities in healthy subjects (Myrum et al., 2015b). However, the Arc genetic variations investigated were not shown to have a functional impact on Arc protein or mRNA expression. Thus, future studies will be needed in order to disentangle the selective implication of Arc functional genetic variations in working memory performance and executive functions, the two cognitive domains at the basis of schizophrenia neuropathophysiology.

SYSTEMS FOR SOCIAL PROCESSES

In recent years, there has been growing consensus that abnormalities in social cognition form part of the core symptoms in schizophrenia (Billeke and Aboitiz, 2013; Millan et al., 2014). Individuals with schizophrenia have marked impairments in processing non-verbal social affective information while showing normal affect sharing and emotion experience (Green et al., 2015). Notably, social cognitive impairments in these individuals have a more deleterious impact on daily functioning than non-social cognitive deficits (Fett et al., 2011). Arc knockout mice show impaired social abilities as demonstrated by reduced sociability and reduced preference for social novelty (Managò et al., 2016). In particular, in the 3-chamber paradigm, Arc knockout mice preferred to be in the chamber with an empty cup rather than with a novel conspecific. Moreover, Arc knockout mice were not able to discriminate between a novel and a familiar conspecific. These social measures were obtained in a well-established test for mice used to assess social avoidance and preference for social novelty (Moy et al., 2004, 2008). Decreased interaction with conspecifics is an index of social withdrawal reminiscent of what is observed in patients with schizophrenia. Indeed, low social reciprocity with others and deficits in social cognition (Harvey et al., 2006) are core features of schizophrenia negative symptomatology. Moreover, these symptoms are also enduring and less responsive to medication, not to mention among the most disabling features of this psychiatric illness. Therefore, the reduced sociability and preference for social novelty shown by Arc knockout mice is consistent with the deficits seen in patients with schizophrenia and represent further evidence supporting the role of reduced Arc levels in schizophrenia neuropathology. However, we should highlight that despite their extensive use and importance, currently available tasks assessing social functions in rodents are still limited in their equivalence to tasks used in the human clinical setting. Indeed, social cognitive processes such as theory of mind, facial perception/recognition, and emotion regulation are the social processes mostly impaired in schizophrenia (Green et al., 2015). These social cognitive functions are not yet directly and specifically testable in rodents. This will require consistent efforts in the field with a clear aim to prove the predictive translational validity of novel and more refined social cognitive tasks in rodents.

POSITIVE VALENCE SYSTEM

This domain involves processes such as motivation, responsiveness to reward and habit formation. In schizophrenia, the hedonic responses to reward and willingness to work for a reward (motivational state) are impaired (Gard et al., 2009). Unfortunately, as far as we know, there is little evidence demonstrating that genetic variations in Arc play a role in these processes. However, recent work has begun to address this domain. One study reported that Arc knockout mice develop a cocaine-conditioned place preference (Salery et al., 2017), at doses that are ineffective in wild-type mice (Contarino et al., 2017). This suggest that Arc genetic disruption might increase rewarding effects of psychostimulant drugs, but further work will be needed in this novel and interesting area of research.

NEGATIVE VALENCE SYSTEMS

No alterations in anxiety-like states have been found in Arc knockout mice as measured by the O-maze and light-dark tests (Plath et al., 2006). Similarly, reactivity to acute threats such as mild foot shocks (Plath et al., 2006) or sudden acoustic sensory stimuli (Managò et al., 2016) was not altered in Arc knockout mice. However, overall Arc genetic disruption as well as knocking down Arc expression selectively in the lateral amygdala was enough to produce a deficit in fear conditioning memories (Ploski et al., 2008). Thus, these data suggest a marginal role of Arc genetic variations in the negative valence domain, with more direct involvement in the storage and expression of aversive memories.

AROUSAL/REGULATORY SYSTEMS

This domain includes processes responsible for generating activation of neural systems as appropriate for various contexts, and providing appropriate homeostatic regulation

(subcategories: arousal, circadian rhythms and sleep/wakefulness). Arousal represent the time of perception of internal/external stimuli related to the coding of relevant vs. non-relevant stimuli of the environment. Hippocampal CA1 recordings of local field potential during locomotion revealed a reduced power in the gamma and beta-2 range in Arc knockout mice compared to wild-type, indicating a disruption in the neuronal synchronization during active behavior (Malkki et al., 2016). In agreement, Arc knockout mice show altered activity when exposed to a newly-presented environment. In particular, both a slightly hyperactive (Managò et al., 2016) or hypoactive (Salery et al., 2017) phenotype have been reported. However, it is worth noting that the experimental setting of the latter study might have produced misleading and less sensitive data in locomotor activity as it was based on the breaking of only four beams placed at 90 degree points of a circular corridor. More consistent instead, Arc knockout mice showed increased locomotor sensitivity to dopaminergic psychostimulants including amphetamine (Managò et al., 2016) and cocaine (Salery et al., 2017). Moreover, repeated exposure to amphetamine produce, in the dorsal striatum and nucleus accumbens, a selective increase in a subset of mRNAs including Arc (Biever et al., 2017). Finally, the psychostimulant-induced increase in Arc expression seems to be evident mostly in D1-positive medium spiny neurons as well as in NMDA-positive neurons in striatal regions (Biever et al., 2017; Salery et al., 2017). Overall, these evidence point to Arc as an integrator of D1 and NMDA signaling and demonstrate that Arc genetic disruption causes a predisposition to higher sensitivity to psychostimulants.

Psychostimulant super-sensitivity is used as a rodent correlate of schizophrenia-like positive symptoms (Arguello and Gogos, 2006; van den Buuse, 2010) and is relevant to the arousal domain of the RDoC system. In particular, amphetamine exacerbates psychotic experiences in patients with schizophrenia and can be psychotogenic in normal subjects (Laruelle et al., 1999). Thus, Arc knockout mutants' locomotor activity phenotypes are consistent with an increased arousal state to external stimuli and might be seen as a proxy of schizophrenia-like positive symptoms. Possibly due to different arousal states, Arc knockout mice also show prepulse-inhibition (PPI) deficits (Managò et al., 2016). PPI is considered a measure of sensorimotor gating consistently conserved from rodents to humans (Braff and Geyer, 1990). There have been numerous reports of PPI deficits in patients with schizophrenia (Swerdlow et al., 2008), their unaffected first degree relatives (Cadenhead et al., 1993, 2000), and patients with schizotypal personality disorder (Cadenhead et al., 1993). Thus, the PPI deficits in Arc knockout mice are consistent with a schizophrenia-relevant behavioral endophenotype.

Related to sleep processes instead, initial studies reported that Arc knockout mice do not show any differences in the composition of sleep (Malkki et al., 2016). This suggest a marginal implication of Arc genetics in relationship to sleep and wakefulness, and a negligible implication of "off-line" processing (e.g., during post-behavioral sleep) in cognitive deficits.

ARC BIOLOGY

Arc is only present in Ca^{2+}/Calmodulin-dependent kinase II alpha (CaMKIIa) expressing neurons in the hippocampus, neocortex and striatum (Vazdarjanova et al., 2006; Miyashita et al., 2008). Its expression is tightly regulated. Indeed, after a novel experience, Arc mRNA moves to the dendrites in the active synapse where is translated (Link et al., 1995; Lyford et al., 1995; Jakkamsetti et al., 2013). Here, Arc protein plays a critical role in long-lasting forms of synaptic plasticity, including long-term potentiation (LTP), long-term depression (LTD) and homeostatic scaling (Plath et al., 2006; Rial Verde et al., 2006; Shepherd et al., 2006; Park et al., 2008; Waung et al., 2008; Jakkamsetti et al., 2013). Thus, Arc might be considered as an integrator of different inputs coming from the nervous system in order to lead to a proper synaptic connection. In particular, Arc might work as a downstream regulator, and functional Arc genetic variations might represent a direct genetic bridge between different schizophrenia-related signaling systems. In this context, we will discuss possible molecular mechanisms of Arc in the modulation of glutamatergic and dopaminergic pathways, two systems extensively implicated in the schizophrenia neuropathology.

ARC AND GLUTAMATE

Arc has been consistently linked to the glutamatergic system and reduced Arc protein expression alter glutamate-mediated processes such as learning and memory formation, cognition and neuronal plasticity (Guzowski et al., 2000; Park et al., 2008; Jakkamsetti et al., 2013; Wang et al., 2016). In particular, when Arc protein translation is disrupted, a high-frequency burst in the hippocampus is able to induce LTP; however, the second phase of consolidation of synaptic LTP is disrupted (Guzowski et al., 2000). In agreement, Arc has a fundamental role in the stabilization of actin filament at the synaptic site (Messaoudi et al., 2007). Moreover, Arc is implicated in the synaptic scaling of AMPA receptors for the induction of LTD, interacting with dynamin and endophilin (Chowdhury et al., 2006). In particular, Arc facilitates the endocytosis of AMPA receptor, a process that is implicated in the induction of LTD (Chowdhury et al., 2006; Shepherd et al., 2006). Notably, Arc can accumulate also at the inactive synapses binding to the inactive form of CamKIIbeta, consequently leading to the endocytosis of AMPA receptors (Okuno et al., 2012). Arc-dependent synaptic plasticity (LTP and LTD) is induced by the activation of mGluR1 or R5 (mGluR type I; Park et al., 2008; Kumar et al., 2012; Wang et al., 2016), and requires the involvement of eEF2 and FMRP that are implicated in the translation of Arc mRNA to protein (Park et al., 2008; Wang et al., 2016). However, despite the established involvement of Arc in mGluR-dependent plasticity (Park et al., 2008; Waung et al., 2008), its role in NMDA-dependent plasticity is still controversial. For instance, the localization of Arc mRNA at active synapses on the dendrites requires NMDA activation (Steward and Worley, 2001; Bloomer et al., 2008). Furthermore, consolidation of memories leads to an increased Arc protein level (Guzowski et al., 2000; McIntyre et al., 2005), and blocking

NMDA receptor reduced Arc expression induced by a learning process (Czerniawski et al., 2011). However, other evidence indicate that NMDA-induced LTP or LTD is Arc-independent (Park et al., 2008; Waung et al., 2008). More recently, Arc was involved in experience-induced cortical firing patterns correlated with Arc-dependent increase of NMDA activity (Ren et al., 2014). Overall, these findings highlight the importance of Arc in the consolidation of some types of NMDA-dependent memory formation. Thus, when Arc functioning is diminished, NMDA-dependent signaling is expected to be partially disrupted.

The glutamatergic system has been often implicated in the manifestation of schizophrenia-relevant clinical symptoms. Noncompetitive NMDA/glutamate receptor antagonists such as PCP, ketamine or MK801 have psychomimetic effects (Halberstadt, 1995; Andiné et al., 1999; Frohlich and Van Horn, 2014) reproducing many behavioral alterations reminiscent of positive, negative and cognitive symptoms of schizophrenia in healthy humans and exaggerating positive and negative symptoms in patients with schizophrenia (Coyle, 2006; Kantrowitz and Javitt, 2010). Moreover, from recent genome-wide association studies (GWAS), several genes belonging to the glutamatergic system were part of the 108 list of implicated loci (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). In particular, genes that encode subunits of NMDA and AMPA receptors were significantly coming out as being strongly implicated (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). In agreement, a number of pre-clinical studies in rodents reported that an alteration of NMDA or AMPA transmission might recapitulate different behavioral alterations in a number of RDoC domains that might possibly be related to schizophrenia-relevant endophenotypes (Wiedholz et al., 2008; Papaleo et al., 2012). Despite this, and the consequent remarkable effort of the academics and the industry, clinical results related to new treatments for schizophrenia targeting the glutamatergic system have been disappointing (Iwata et al., 2015; Bugarski-Kirola et al., 2016). In this context, Arc being a downstream effector of glutamatergic receptors, it might be a better target and a more consistent cause of the development of schizophrenia-relevant behavioral alterations.

ARC AND DOPAMINE

The long-standing pathophysiological hypothesis of schizophrenia involves a dysregulated dopaminergic system (Weinstein et al., 2017). In particular, the current hypothesis highlights that a hyperactive mesolimbic system through an aberrant activation of D2 receptors might be more related to the so-called "positive symptoms". Instead, a hypoactive mesocortical dopaminergic system with a lower stimulation of D1 receptor in the PFC can lead to schizophrenia negative and cognitive symptoms (Winterer and Weinberger, 2004; Simpson et al., 2010; Slifstein et al., 2015). Notably, the most common first-line treatments for acute and chronic therapy for schizophrenia are antipsychotic drugs, all of which interact with dopamine/D2 receptors (D2) brain pathways (Miyamoto et al., 2005; Hasan et al., 2013). Finally, D2 receptors have been confirmed as one of the major schizophrenia-association genetic hits in the most recent GWAS studies (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014).

Up until last year, there has been no evidence implicating Arc genetic variations as modulators of the dopaminergic system. Indeed, the only data available were those reporting changes in Arc expression induced by dopamine agonists or antagonists as just a marker for neuronal activity (Moro et al., 2007; Banerjee et al., 2009; Fumagalli et al., 2009). In contrast, we have now demonstrated that Arc genetic disruption result in selective alterations on different aspects of the dopaminergic system. In particular, Arc knockout mice have reduced amphetamine-induced dopamine release within the medial PFC (mPFC) and, in agreement, two-photon calcium imaging revealed a reduced mPFC activation following electrical stimulation of the ventral tegmental area (VTA; Managò et al., 2016). Treatment with a D1 agonist rescued the altered mPFC activity as well as recency memory deficits, demonstrating that the mPFC hypofunction was D1-dependent (Managò et al., 2016). Alternatively, infusing the D2 antagonist eticlopride directly into the nucleus accumbens of Arc knockout mice rescued their supersensitivity to amphetamine in terms of dopamine release and locomotor activity, unraveling a D2-dependent hyperactive dopaminergic mesolimbic system (Managò et al., 2016). These Arc-dependent effects were evident in the mPFC and in the nucleus accumbens, but not in the dorsal striatum. The source of dopamine in both the mPFC and nucleus accumbens is the VTA, while in the dorsal striatum it is the substantia nigra (Beckstead et al., 1979). Furthermore, amphetamine injection in Arc knockout mice produced opposing dopamine-release phenotypes in the mPFC compared to that in the nucleus accumbens. These contrasting effects in mesocortical and mesostriatal dopaminergic pathways might then suggest an Arc-dependent circuital dysfunction that will require further investigations. In conclusion, Arc function seems to be crucial for establishing a proper activity balance between mesocortical and mesostriatal dopaminergic circuits. Importantly, these alterations are reversible by selectively targeting D2 receptors in the ventral striatal regions and D1 receptors in the PFC.

Despite this previously unexpected evidence, the mechanisms underlying the peculiar effects of Arc genetics in the dopaminergic system are as yet unclear. Previous studies have reported that PFC dopaminergic inputs show protracted postnatal maturation through adolescence and are susceptible to activity-dependent modification during this period (Kalsbeek et al., 1988; Lewis and O'Donnell, 2000; Mastwal et al., 2014). Recurrent network activity in frontal-striatal loops can also affect striatal circuit maturation (Kozorovitskiy et al., 2012). As Arc protein is abundantly expressed in cortical excitatory and striatal GABAergic projection neurons (but not detected in midbrain dopamine neurons; Shepherd and Bear, 2011), it may regulate activity-dependent maturation of the VTA-PFC-striatal circuits during postnatal development. Considering the well-known role of Arc in modulating glutamate receptors (Shepherd and Bear, 2011; Jakkamsetti et al., 2013; Ren et al., 2014), and the balance between the glutamatergic and dopaminergic systems

which tightly regulate each other, our recent findings raise the possibility that Arc-dependent changes in glutamatergic signaling might be the effector of the changes in the dopamine system. However, further studies are needed to unravel these issues and how Arc alterations at the single-cell level might affect these circuits.

BEYOND SCHIZOPHRENIA

Findings from genetics studies might be applied to discrete behavioral domains (e.g., RDoC framework) overcoming the boundaries of psychiatric diagnosis. The current system for diagnosing psychiatric illnesses, based on DSM guidelines, relies on defining a constellation of signs and symptoms, each of which may be present in a number of different disorders, and none of which is, by itself, diagnostic. In support of this idea, recent findings indicate that different psychiatric disorders such as schizophrenia, autism, ADHD, intellectual disability and bipolar disorder, might share common genetic variations (McCarthy et al., 2014; Goes et al., 2016; Zhao and Nyholt, 2017). In this context, and because of its major modulatory impact in synaptic plasticity (Tzingounis and Nicoll, 2006; Bloomer et al., 2008; Park et al., 2008; Waung et al., 2008; Bramham et al., 2010; Gao et al., 2010; Ren et al., 2014; Wang et al., 2016), a role of Arc genetics in a number of different neurological and psychiatric disorders is not surprising (Greer et al., 2010; Cao et al., 2013; Ebert and Greenberg, 2013; Li et al., 2015). Indeed, Fromer et al. (2014) found that schizophrenia, autism spectrum disorder and ID share common genetic variations in the Arc complex. Despite this, to date, there have been no studies directly associating Arc genetic variations in other psychiatric disorders beyond schizophrenia. However, as already mentioned, one case with a rare microdeletion (8q24.3) encompassing the Arc gene demonstrated autistic traits, ID and ADHD (Hu et al., 2015). Moreover, genetic modifications associated with different syndromes such as the fragile X, Angelman and Autism Spectrum Disorder concern genes that encode for proteins involved in the regulation of Arc expression (Smith et al., 2011; Niere et al., 2012; Cao et al., 2013).

Patients with the Fragile X Syndrome (FXS) carry a triplet repeat expansion in the FMR1 gene that lead to reduced translation of the FMRP protein (Garber et al., 2008). The FMRP is a protein synthesis regulator and one of its targets is Arc (Park et al., 2008). In agreement, FMR1 knockout mice display higher production of Arc and consequent abnormal LTD (Niere et al., 2012; Ebert and Greenberg, 2013). The FXS is characterized by social impairments, cognitive disabilities, mood disorders and hyperactivity (Garber et al., 2008), which are all behavioral domains affected by Arc genetic variations (see above). Thus, it might be plausible that altered Arc expression is one of the causes of these behavioral abnormalities.

The Angelman Syndrome (AS) is caused by the deletion or inactivation of the maternal copy of the Ube3a gene (Williams et al., 2010). The *Ube3a* gene encodes for a brain-specific E3 ubiquitin ligase which has Arc as one of its substrates. In agreement, loss of Ube3A in mice cause an increase in Arc levels (Cao et al., 2013). The core symptoms of this pathology are delayed motor milestones, mental retardation, seizures, movement or balance disorders (Williams et al., 2010), once again asserting a potential implication of Arc-dependent mechanisms. Similarly, a genetic variation characterized by the appearance of Ube3A extra copies have also been associated with the autism spectrum disorder (Smith et al., 2011). Indeed, patients with this mutation present impaired social and communication deficits as well as repetitive behaviors (Smith et al., 2011; Bourgeron, 2015). Similarly, transgenic mice with three copies of the Ube3A manifest social deficits and increased self-grooming compared to the control group. Moreover, this mutation produced an impairment in the glutamatergic transmission and decreased Arc availability (Smith et al., 2011). Because these pathologies share common behavioral alterations in cognitive and social functions modulated by Arc genetics, we might hypothesize Arc as a converging downstream signaling output.

Finally, it seems that Arc could be involved also in Alzheimer's disease (AD). Indeed, Arc can directly bind presenilin1 to regulate γ-secretase activity in order to form more β-amyloid peptides, participating in the formation of neuritic plaques. Furthermore, the same study has reported increased Arc protein levels in patients with AD (Wu et al., 2011). Despite the potential direct role of Arc in the formation of β-amyloid peptides, both increased (Wu et al., 2011) and decreased (Bi et al., 2017) Arc expression have been reported in the cortex of patients with AD. Moreover, initial GWAS on European and American subjects did not reveal any association between Arc genetic variation and AD (Lambert et al., 2013). Nonetheless, a more recent study has described a possible association of a SNP (rs10097505) in the 3'UTR of the Arc gene with susceptibility to AD (Bi et al., 2017). Thus, further work will be needed to understand the possible involvement of Arc genetics in the AD pathology and especially in its cognitive manifestations.

CONCLUSIONS AND FUTURE DIRECTIONS

The evidence discussed here highlight the consistent implication of Arc genetic variations in the development and manifestation of a number of behavioral abnormalities relevant to schizophrenia and other psychiatric disorders. In particular, mouse studies indicate a preponderant role of Arc in behavioral domains including cognitive, social and arousal processes, which might depend on the alterations of the glutamatergic and dopaminergic systems (**Figure 1**).

Despite this, it is still unknown how disruption of Arc can recapitulate so divergent and selective alterations in the dopaminergic system. For example, it is not clear if the cause of the dopamine system dysfunction is driven by Arc disruption of the glutamatergic signaling or if Arc might exert a direct influence on dopaminergic pathways. Furthermore, it is still unclear if Arc might play a role in behavior directly altering it or through developmental processes or both. This will be particularly relevant as early detection and early intervention of cognitive and social deficits could be potentially more effective

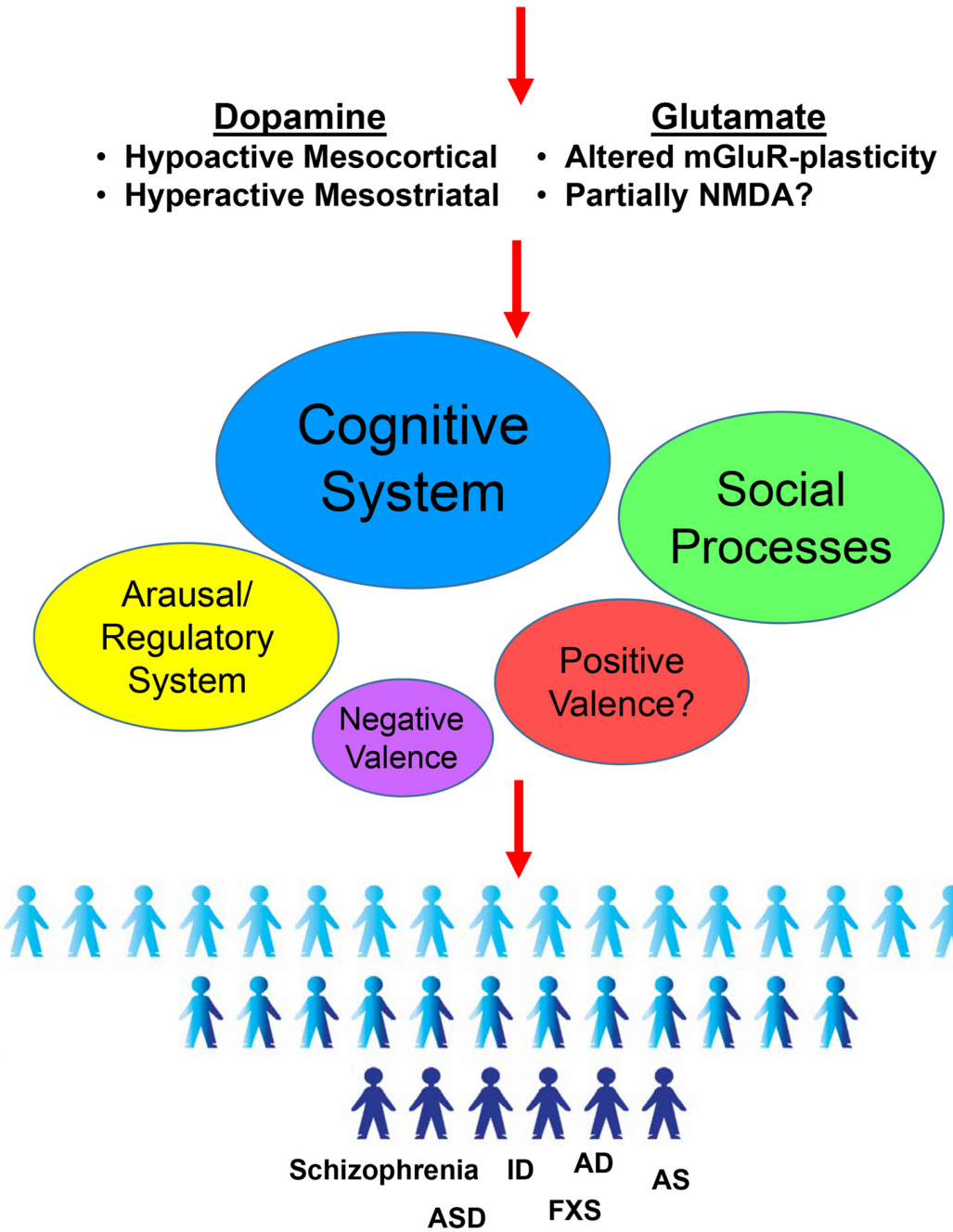

FIGURE 1 | Activity-regulated cytoskeletal-associated (Arc)-dependent effects in Research Domain Criteria (RDoC) behavioral domains and its putative role in psychiatric disorders. Arc genetic disruption have been reported to alter both the dopaminergic and glutamatergic systems in a very selective way. This Arc-dependent altered neurotransmission results in deleterious effects in different behaviors. In particular, following the RDoC framework (NIH/NIMH, 2017), altered levels of Arc induce consistent impairments mainly in cognitive systems, but also in social processes and in arousal/regulatory systems. More investigations are needed for the positive valence system. No major influence seems to be evident for the negative valence system. Ultimately, Arc-dependent alterations in these behavioral processes might converge in a pathological state. In agreement, genetic variations suggested to alter Arc expression have been implicated in different diseases such as Schizophrenia (Schizophrenia), Autism (ASD), Intellectual Disabilities (ID), Fragile X Syndrome (FXS), Angelman Syndrome (AS) and Alzheimer's Disease (AD).

in mitigating the pathological trajectories and ultimately the life quality of individuals with schizophrenia-vulnerability. In this context, mouse models will be useful tools in the development and testing of early diagnosis and early treatment strategies, at the same time strictly controlling for environmental and genetic factors. An aberrant maturation of the PFC has been reported in schizophrenia (Lewis and Levitt, 2002) and it is well known that the final maturation of dopaminergic terminals in the PFC is only reached after puberty (Manitt et al., 2011). Arc mRNA expression starts to increase after postnatal day 7 in the cortex, and its activation depends on the correct dopaminergic input coming from the VTA (Ye et al., 2016). Thus, we might hypothesize that this dopamine-induced Arc expression during postnatal development could be important for the correct establishment of synaptic connectivity within the mesocortical circuit. However, we cannot exclude an involvement of Arc in the prenatal developmental process as the presence of Arc in the brain has been detected since embryonic stages (Alberi et al., 2011). Identifying the developmental functions of Arc would also be relevant to other neurodevelopmental disorders such as autism, FXS and AS as discussed above. Therefore, studying the role of Arc in brain development will be important.

In conclusion, a concerted effort between clinical and preclinical genetic and mechanistic studies focused on Arc modulation of behavioral outputs looks to be a promising area of investigation. Indeed, this could greatly advance our knowledge on the causes of schizophrenia, especially in the areas of cognitive and social alterations. Notably, a better understanding of genetic variations that affect Arc, or its binding partners, might help to pave the way to more efficient treatments and prevention strategies in keeping with the promises of precision medicine. In particular, individual variability in Arc genetics could provide valuable tools to better address abnormalities in cognitive and social processes.

AUTHOR CONTRIBUTIONS

FP and FM found the materials and wrote the article.

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Cocaine and Caffeine Effects on the Conditioned Place Preference Test: Concomitant Changes on Early Genes within the Mouse Prefrontal Cortex and Nucleus Accumbens

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Caffeine is the world's most popular psychostimulant and is frequently used as an active adulterant in many illicit drugs including cocaine. Previous studies have shown that caffeine can potentiate the stimulant effects of cocaine and cocaine-induced drug seeking behavior. However, little is known about the effects of this drug combination on reward-related learning, a key process in the maintenance of addiction and vulnerability to relapse. The goal of the present study was thus to determine caffeine and cocaine combined effects on the Conditioned Place Preference (CPP) test and to determine potential differential mRNA expression in the Nucleus Accumbens (NAc) and medial prefrontal cortex (mPFC) of immediate-early genes (IEGs) as well as dopamine and adenosine receptor subunits. Mice were treated with caffeine (5 mg/kg, CAF), cocaine (10 mg/kg, COC), or their combination (caffeine 5 mg/kg + cocaine 10 mg/kg, CAF-COC) and trained in the CPP test or treated with repeated injections inside the home cage. NAc and mPFC tissues were dissected immediately after the CPP test, after a single conditioning session or following psychostimulant injection in the home cage for mRNA expression analysis. CAF-COC induced a marked change of preference to the drug conditioned side of the CPP and a significant increase in locomotion compared to COC. Gene expression analysis after CPP test revealed specific up-regulation in the CAF-COC group of *Drd1a*, *cFos,* and *FosB* in the NAc, and *cFos*, *Egr1,* and *Npas4* in the mPFC. Importantly, none of these changes were observed when animals received same treatments in their home cage. With a single conditioning session, we found similar effects in both CAF and CAF-COC groups: increased *Drd1a* and decreased *cFos* in the NAc, and increased expression of *Drd1a* and *Drd2,* in the mPFC. Interestingly, we found that *cFos* and *Npas4* gene expression were increased only in the mPFC of the CAF-COC. Our study provides evidence that caffeine acting as an adulterant could potentiate reward-associated memories elicited by cocaine. This is associated

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with specific changes in IEGs expression that were observed almost exclusively in mice that received the combination of both psychostimulants in the context of CPP memory encoding and retrieval. Our results highlight the potential relevance of caffeine in the maintenance of cocaine addiction which might be mediated by modifying neural plasticity mechanisms that strengthen learning of the association between drug and environment.

Keywords: caffeine, cocaine, immediate-early genes, nucleus accumbens, prefrontal cortex, learning

INTRODUCTION

Addictions are brain disorders that affect neural pathways involved in reward, motivation, and memory (Volkow et al., 2012). Recent neurobehavioral studies have shown that drug users show a number of cognitive deficits that may be secondary to drug-induced changes in brain structure and function (Cadet and Bisagno, 2016). Learned associations between the rewarding effects of a drug, and contextual stimuli present at the time of drug use, are a central component of acquiring and maintaining drug seeking behavior, and are capable of inducing relapse (Volkow et al., 2012). Understanding the mechanisms that underlie these unwanted memories should provide insight into effective therapies aimed at reducing context-induced relapse.

Cocaine is a highly addictive psychostimulant and is rarely obtained on the illegal market in its pure form (Cole et al., 2011). Forensic analyses of seized illicit cocaine samples usually report variable quantities of caffeine (López-Hill et al., 2011; Sena et al., 2017). Caffeine deserves a special consideration on its own. Energy drinks marketed as dietary supplements or beverages usually contain caffeine as their active ingredient (Sena et al., 2017). Such supplements are taken to enhance cognitive performance but might also influence mood and sleep (Childs, 2014). In clinical settings, caffeine intake is positively correlated with substance-use disorders (Kendler et al., 2006) and has been shown to increase illicit drug use (Miller, 2008).

In preclinical studies, we have previously demonstrated that caffeine can potentiate cocaine-mediated motor (Prieto et al., 2015; Muñiz et al., 2016) and motivational effects (Prieto et al., 2016). Also, caffeine potentiates striatal reactive astrocytosis induced by cocaine (Muñiz et al., 2016) or MDMA (Khairnar et al., 2010) and cocaine-mediated testicular toxicity (González et al., 2015).

Expression of immediate-early genes (IEGs) has been shown to be induced by activity-dependent synaptic plasticity or behavioral training and is thought to play an important role in memory storage (Cruz et al., 2015). IEGs encode regulatory transcription factors and a variety of proteins that act on diverse cellular processes (Cruz et al., 2013). Several IEGs have been of particular interest due to their association with synaptic plasticity and learning. These include *Egr1* (early growth response gene 1) (Veyrac et al., 2014) or *Npas4* (neuronal Per-Arnt-Sim domain protein 4) (Spiegel et al., 2014).

Findings reviewed above suggest that distinct neuroadaptations can emerge in the central nervous system (CNS) with the combination of caffeine and cocaine. At the basis of such neuroadaptations lie changes in the expression of IEGs. However, information is scarce regarding potential additive effects of these two psychostimulants on transcriptional regulators controlling genomic responses of activated neurons within the mesolimbic circuit. Therefore, in the present study we investigated changes in expression of IEGs (*cFos, FosB, cJun, Egr1, Npas4*) as well as Dopamine and Adenosine receptor subunits mRNA changes in the Nucleus Accumbens (NAc) and medial Prefrontal Cortex (mPFC), as a consequence of combined administration of caffeine and cocaine. We compared molecular changes induced by associative learning with both psychostimulants, using the conditioned place preference (CPP) test, with those obtained after repeated psychostimulant injections given in the home cage.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (10–12 weeks old) from the School of Exact and Natural Sciences of the University of Buenos Aires (UBA) were housed in a light- and temperature-controlled room. Mice had free access to food and water. We followed "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council (US) Committee on Guidelines for the Use of Animals in Neuroscience and Behavioral Research, 2003) and approved by IACUC Committee of the Faculty of Pharmacy and Biochemistry, Universidad de Buenos Aires (Protocol Number: EXP_UBA N° 40944/20151). Mice were euthanized by cervical dislocation performed by an individual proficient in this technique.

Pharmacological and Physiological Procedures

Cocaine hydrochloride and caffeine were purchased from Sigma-Aldrich, St. Louis, MO. Animals were assigned to four different groups: COC (Cocaine-only, 10 mg/kg), CAF (Caffeine-only, 5 mg/kg), CAF-COC (Combined, 10 mg/kg + Caffeine 5 mg/kg), both dissolved in sterile saline and co-administered in a single injection) or Control (sterile saline), see **Figure 1**.

Brain tissue, mPFC and NAc, were obtained immediately after CPP test ended (Experiment #1), or just after first conditioning session of the CPP test (Experiment #2). For Experiment #3, animals were injected with the same number of injections (saline and/or drug) used for CPP experiments but injections were made inside their home cage, and animals were sacrificed 48 h after last injection (following the same time schedule used for CPP, see **Figure 1**).

Behavioral Analysis

Conditioned Place Preference

A detailed plan of the training schedule is shown in **Figure 1**. CPP test was done in a custom-made plastic apparatus consisting

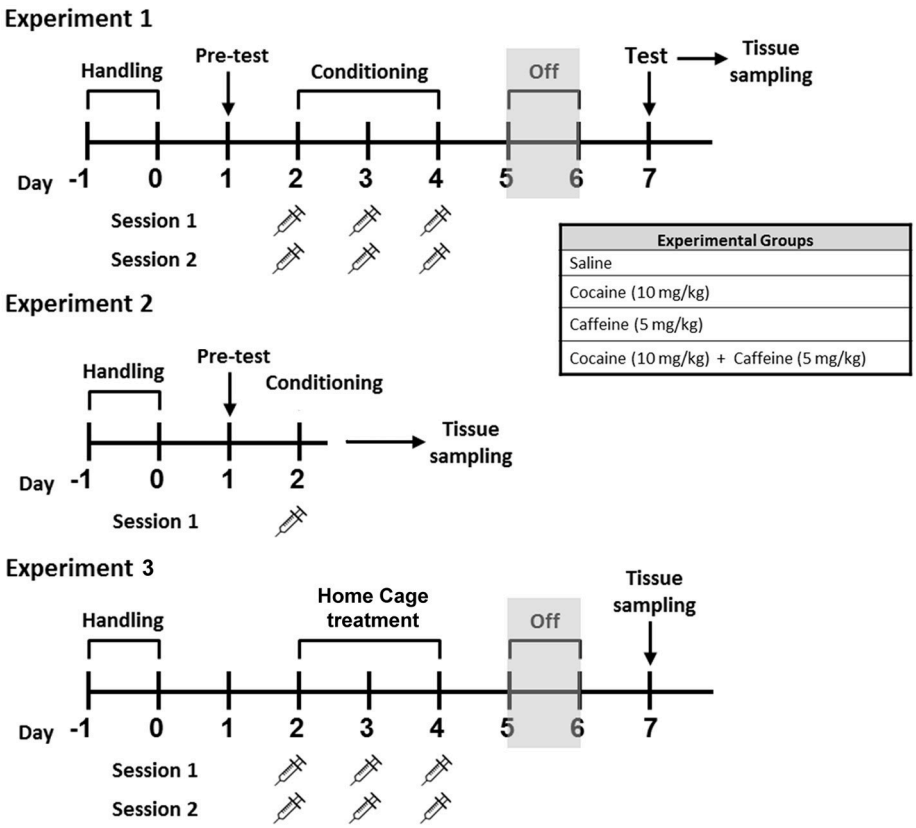

FIGURE 1 | Schematic representation of the experimental treatments for Experiments #1, #2, and #3 Male C57BL/6 mice were subjected to CPP learning with caffeine 5 mg/kg (CAF), cocaine 10 mg/kg (COC), and their combination (5 mg/kg caffeine + 10 mg/kg cocaine, CAF-COC). Tissue samples for RT-PCR were taken immediately after the first conditioning CPP session, (Experiment #2), or immediately after CPP test session, (Experiment #1). For Experiment #3, brain tissue was obtained 48 h after last injection.

of two equal size chambers (25 × 20 × 20 cm) connected by a smaller center chamber (25 × 7.5 × 18 cm) (see **Figure 2B**). Between the chambers were guillotine doors that were closed on the conditioning days and opened on the pretest and testing days. Both conditioning compartments had different visual, tactile and olfactory cues following a procedure previously described by Aguilar-Valles et al. (2014). First, animals were handled twice a day for 2 days before pretest trial. On the pretest session, animals were placed in the center chamber and allowed to explore freely for 15 min in order to establish the baseline preferences. Preference bias for any of the two conditioning compartments was declared based on the time spent in each compartment during the pretest session. Drug injections were paired with the initially non-preferred side (CS+ chamber), and saline was given in the preferred side (CS− chamber). On the following day, CPP conditioning started consisting in three consecutive training days with two daily training sessions (30 min). Injections were given just prior to placing the animal in the CS+ chamber and saline prior to the CS− chamber (chambers and time of day were counterbalanced between and within groups). Control animals received saline in both CS+ and CS− compartments. Forty-eight hours after the final training session, testing (15 min) consisted of giving mice free access to the apparatus in a drug-free state. CPP score was expressed as the time spent in the CS+ minus CS− compartments.

Experiment #2 was aimed to investigate changes during the initial encoding of the drug-context association. In this experiment, animals were exposed to the pretest session and then 24 h later received one CS+ pairing session in the morning. Brain tissue was collected immediately after the end of the first exposure to CS+.

Time spent in each chamber was recorded and analyzed using EthoVision XT 7.0 (Noldus) and confirmed by experimenter observation. Locomotor activity (total distance in cm) was recorded for all sessions while animals were inside CPP compartments.

Real Time PCR

Brain tissues were extracted after CPP test (Experiment #1), after CPP first conditioning (Experiment #2), or 48 h after last injection in the home cage (Experiment #3). Mouse brains were rapidly removed. mPFC and NAc were dissected, placed on dry ice, and then stored at −70°C in RNAlater (Qiagen) until further assays. Total RNA was isolated using

TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. Five hundred nanograms of RNA were treated with DNAseI (Invitrogen) and reverse-transcribed in a 20 μL reaction using M-MLV reverse transcriptase (Promega) and random hexamer (Biodynamics). For quantitative real-time PCR primers sets were designed for the specific amplification of murine *Drd1a*, *Drd2*, *Adora1*, *Adora2a*, *cFos, FosB, cJun, Egr1, Npas4,* and *Actb* as a housekeeping control gene (sequences listed in **Table 1**). Each sample was assayed in duplicate using 4 pmol of each primer, 1X SYBR Green Master Mix (Applied Biosystems), and 2–20 ng of cDNA in a total volume of 13 μL. Amplification was carried out in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems).

Statistical Analysis

Several statistical analyses were performed with ANOVAs in order to detect and better characterize possible differences between the experimental conditions. Before performing ANOVAs, data sets of each of the different experimental variables were inspected for homogeneity of variances and normal distribution among the four experimental groups. Transformation of data was applied to bring data to normal distribution when required. Kruskal Wallis ANOVA on Ranks was performed when data did not comply with the assumptions of parametric tests. When one-way ANOVA was performed, Tukey was used as the post-hoc test (http://www.infostat.com.ar/). Infostat software by default applies Tukey-Kramer test for unbalanced designs when needed. Data are expressed as

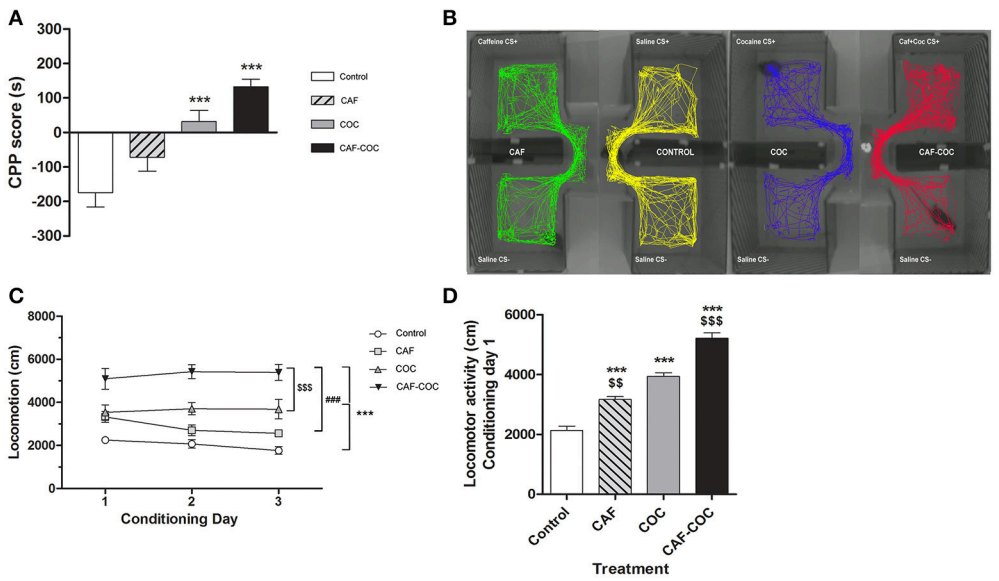

FIGURE 2 | Behavioral changes induced by caffeine, cocaine or the combination of both psychostimulants. **(A)** CPP score calculated as follows: CPP score (s) = time spent in the CS + chamber − time spent in the CS − chamber. Values indicate mean ± SEM. One-way ANOVA–Tukey: $^{***}p < 0.001$ different from Control. **(B)** Representative track plots (screen-captured from Ethovision file) of mice during CPP test. **(C)** Psychostimulant treatments induced locomotor activity during the conditioning phase in the CS+ chamber. Two-way ANOVA with repeated measures followed by Bonferroni test, CAF-COC $^{***}p < 0.001$ different from Control, $^{###}p < 0.001$ different from CAF and $^{\$\$\$}p < 0.001$ different from COC. **(D)** Locomotor activity measured on the first conditioning day session in the CS+ chamber. One-way ANOVA–Tukey. $^{***}p < 0.001$ different from Control; $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ different from COC.

TABLE 1 | Primer sequences.

Gene	Ac. Number	Primer forward	Primer reverse
Drd1a	NM_010076	TTCTTCCTGGTATGGCTTGG	GCTTAGCCCTCACGTTCTTG
Drd2	NM_010077	TATGCCCTGGGTCGTCTATC	AGGACAGGACCCAGACAATG
Adora1	NM_001039510	TAGACAGTTCAGGTGGCCAG	AGTACATTTCCGGGCACAGA
Adora2a	NM_009630	CGCAGAGTTCCATCTTCAGC	ACGTCCTCAAACAGACAGGT
cFos	NM_010234	TCCCCAAACTTCGACCATGA	AGTTGGCACTAGAGACGGAC
FosB	NM_008036	ACAGATCGACTTCAG GCGGA	GTTTGTGGGCCACCAGGAC
cJun	NM_010591	CATAGCCAGAACACGCTTCC	TTGAAGTTGCTGAGGTTGGC
Egr1	NM_007913	GATGGTGGAGACGAGTTAT	GATTGGTCATGCTCACG
Npas4	NM_153553	CATCTGGGCCACTCTATGGT	AGGGGTCTCTTCTCTCCAGT
Actb	NM_007393	TGTTACCAACTGGGACGACA	GGGGTGTTGAAGGTCTCAAA

the mean ± SEM. Differences were considered significant if $p < 0.05$.

For statistical analysis of locomotor activity of Experiment #1, two-way ANOVA with repeated measures (treatment and day effects) followed by Bonferroni test was applied using the software IBM SPSS Statistics V20.0.0. Bonferroni correction allows controlling α error by adjusting the level of significance of each individual contrast according to the number of comparisons.

RESULTS

Experiment #1: Cocaine and Caffeine-Induced Behavioral and Molecular Effects following the CPP Test

1a. CPP

All animals established a preference for one of the two conditioning compartments of the CPP box at the pretest day, i.e., before place conditioning was initiated. Animals assigned to the drug group were conditioned with drugs to the non-preferred compartment (CS+ chamber). As shown in **Figure 2A**, mice in the COC group developed a positive CPP for the cocaine associated compartment at the CPP test day [$F_{(3, 38)} = 14.48$ $p < 0.001$, $N = 9$–11, Tukey: $p < 0.001$ different from Control]. CAF-COC also induced a positive CPP demonstrated by a marked preference for the initially non-preferred side following association with drug administration (Tukey: $p < 0.001$ different from Control). For caffeine treated mice, no statistical differences were found compared to the Control group (Tukey: $p > 0.05$). **Figure 2B** shows representative track plots (screen-captured from EthoVision file) of mice during CPP test. Previous work has indicated that when combined, low-doses of cocaine and caffeine enhance approach behavior to cues paired with their administration in rats, suggesting that reinforcement induced by these drugs may be additive (Bedingfield et al., 1998). Our results also show a tendency of caffeine to increase CPP score values (compared with cocaine values) but the doses tested in this study did not show a clear additive effect (**Figure 2A**).

In addition, locomotor activity was measured during the conditioning phase in the CS+ chamber (**Figure 2C**). Two-way ANOVA with repeated measures showed significant main treatment effect [$F_{(3, 40)} = 29.18$, $p < 0.001$, Bonferroni test for multiple comparisons: CAF-COC $p < 0.001$ different from Control; $p < 0.001$ different from CAF; $p < 0.001$ different from COC; COC $p < 0.001$ different from Control]. However, no significant effect of day [$F_{(2, 80)} = 0.86$, $p > 0.05$, $N = 10$–12], or day by treatment interaction [$F_{(6, 80)} = 1.62$, $p > 0.05$] were observed. As depicted in **Figure 2D**, mice receiving the combination of CAF-COC on day one of conditioning were significantly more active than animals administered with any other treatment.

1.b. Gene Expression Changes Induced by Caffeine and Cocaine-Associated Memory Retrieval in the CPP Test

Tissue samples from the mPFC and NAc were obtained for gene expression analysis immediately following CPP testing, as shown in **Figure 1**. We measured mRNA expression of Dopamine and Adenosine receptor subunits and IEGs that are sensitive to drug exposure and associated with neuronal activation, plasticity and memory.

mPFC

Figure 3 shows gene expression effects following CPP with caffeine, cocaine, and their combination on Dopamine and Adenosine receptor subunit and IEGs mRNA expression in the mPFC. One-way ANOVA-Tukey indicated that cocaine given alone decreased Dopamine receptor subunits, *Drd1a* [$F_{(3, 19)} = 7.88$, $p < 0.01$, $N = 5$, Tukey: $p < 0.01$ different from Control] and *Drd2* [$F_{(3, 21)} = 5.73$, $p < 0.01$, $N = 5$–6, Tukey: $p < 0.05$ different from Control], in the mPFC. For the Adenosine receptor subunits, both groups that were trained in the CPP with caffeine had increased expression of *Adora1* [$F_{(3, 21)} = 6.97$, $p < 0.01$, $N = 5$–6, Tukey: CAF $p < 0.05$, CAF-COC $p < 0.01$ different from Control; CAF $p < 0.05$, CAF-COC $p < 0.01$ different from COC], whereas there were no changes in *Adora2a* gene expression [$F_{(3, 22)} = 0.82$, $p > 0.05$ $N = 5$–6]. Interestingly, three out of five IEGs including *cFos*, *Egr1,* and *Npas4* showed significant increases solely in the group that was trained with the combination of both psychostimulants {*cFos* [$F_{(3\ 22)} = 18.41$, $p < 0.001$, $N = 5$–6, Tukey: $p < 0.001$ different from Control; $p < 0.001$ different from COC]; *Egr1* [$F_{(3, 23)} = 5.27$, $p < 0.01$, $N = 6$, Tukey: $p < 0.01$ different from Control; $p < 0.05$ different from COC]; *Npas4* [$F_{(3, 23)} = 10.57$, $p < 0.001$ $N = 6$, Tukey: $p < 0.001$ different from Control; $p < 0.001$ different from COC]}. No significant differences were found for *cJun* [$F_{(3, 22)} = 0.42$, $p > 0.05$, $N = 5$–6] and *FosB* [$F_{(3, 22)} = 0.64$, $p > 0.05$, $N = 5$–6] expression in the mPFC.

NAc

Figure 4 shows gene expression effects following CPP with caffeine, cocaine, and their combination on Dopamine and Adenosine receptor subunit and IEGs mRNA expression in the NAc. Caffeine or cocaine given alone did not modify Dopamine receptor subunits *Drd1a* but the combination of both drugs (CAF-COC) induced and increase in *Drd1a* expression [$F_{(3, 19)} = 3.31$, $p < 0.05$, $N = 4$–5, Tukey: $p < 0.05$ different from Control]. For *Drd2*, both COC and CAF-COC groups induced an increase in *Drd2* [Kruskal-Wallis H = 11.68, $p < 0.01$, $N = 4$–5, paired comparisons: $p < 0.05$ different from Control] suggesting a cocaine-mediated effect. We did not find any significant effects for Adenosine receptor subunits *Adora1* [$F_{(3, 20)} = 0.87$, $p > 0.05$, $N = 5$–6] and *Adora2a* [$F_{(3, 19)} = 0.22$, $p > 0.05$, $N = 4$–6] for any experimental group in the NAc.

FosB and *cFos* only increased in the group that received the combination of both psychostimulants [*FosB* $F_{(3, 20)} = 5.272$, $p < 0.05$, $N = 5$–6, Tukey: $p < 0.05$ different from Control; $p < 0.05$, different from COC. *cFos* $F_{(3, 20)} = 18.41$, $p < 0.01$, $N = 5$–6, Tukey: $p < 0.05$ different from Control]. Also, both COC and CAF-COC groups showed increased *Egr1* [$F_{(3, 19)} = 5.27$, $p < 0.01$, $N = 4$–6, Tukey: $p < 0.01$ different from Control]. CAF-COC treatment did not produce significant changes in *Npas4* and *cJun* expression [*Npas4* $F_{(3, 20)} = 1.17$, $p > 0.05$, $N = 5$–6; *cJun* $F_{(3, 21)} = 0.45$, $p > 0.05$, $N = 5$–6].

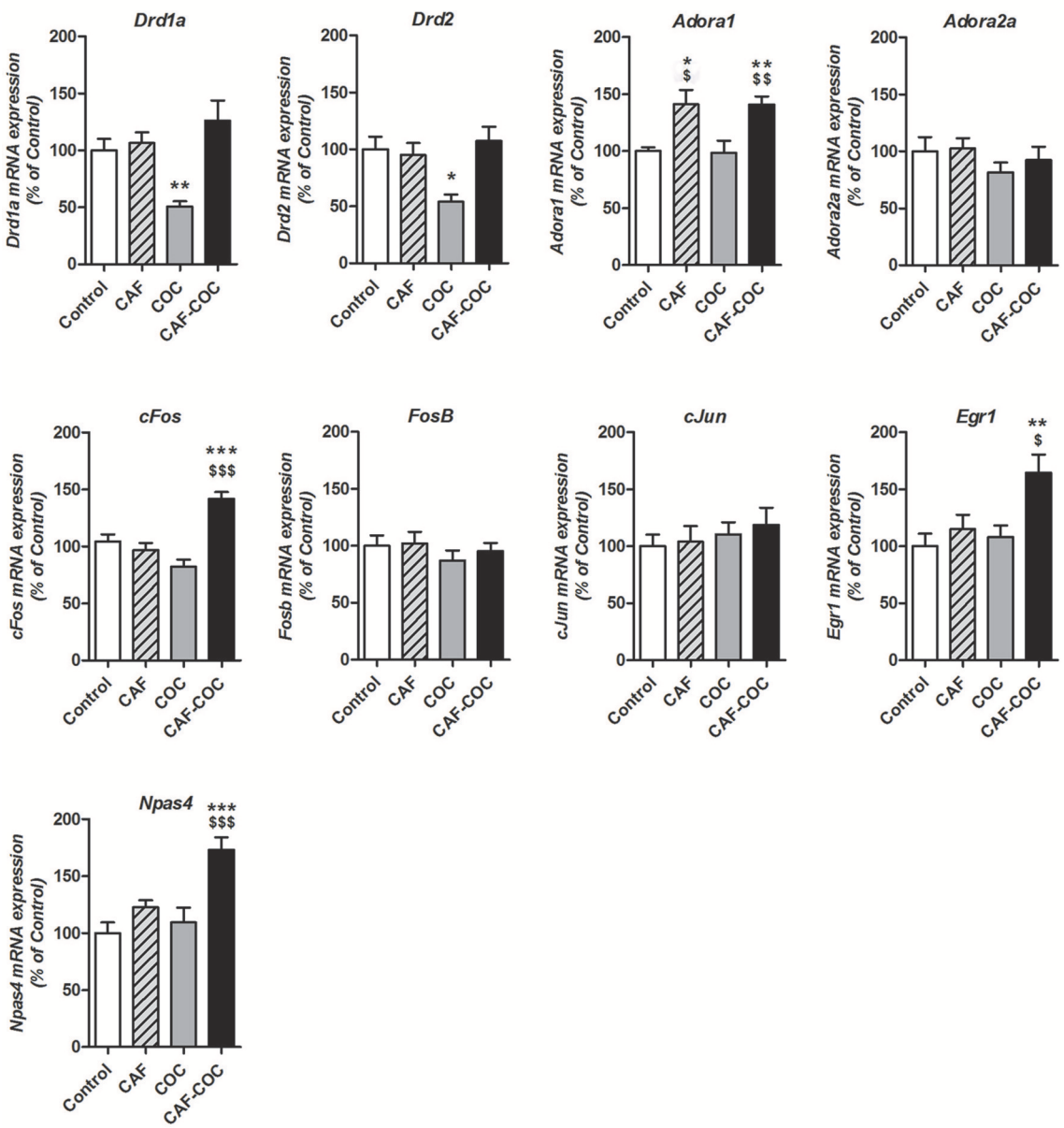

FIGURE 3 | Gene expression changes induced in the mPFC by caffeine and cocaine-associated memory retrieval in the CPP. Dopamine receptor subunits *Drd1a* and *Drd2*; adenosine receptor subunits *Adora1* and *Adora2a*; IEGs *FosB*, *cFos*, *cJun*, *Egr1*, *Npas4*. The values indicate mean ± SEM. One-way ANOVA–Tukey: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ different from Control; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ different from COC.

Experiment #2: Cocaine and Caffeine-Induced Behavioral and Molecular Effects following a Single Conditioning Session of the CPP Test

2a. Single Conditioning Session of the CPP Test

Mice were handled for habituation, exposed to CPP pre-test and then exposed to CPP first conditioning session 24 h later, see **Figure 1**. We measured mRNA levels of the same genes previously described in the mPFC and NAc but in this experiment brain tissues were obtained immediately after first CPP conditioning session. The main goal of this new experiment was to compare gene expression profile from a single exposure to the context with the drug or saline to the one observed following a full CPP test.

Treatments with both psychostimulants induced locomotor activity on the first conditioning session in the CS+ chamber, see

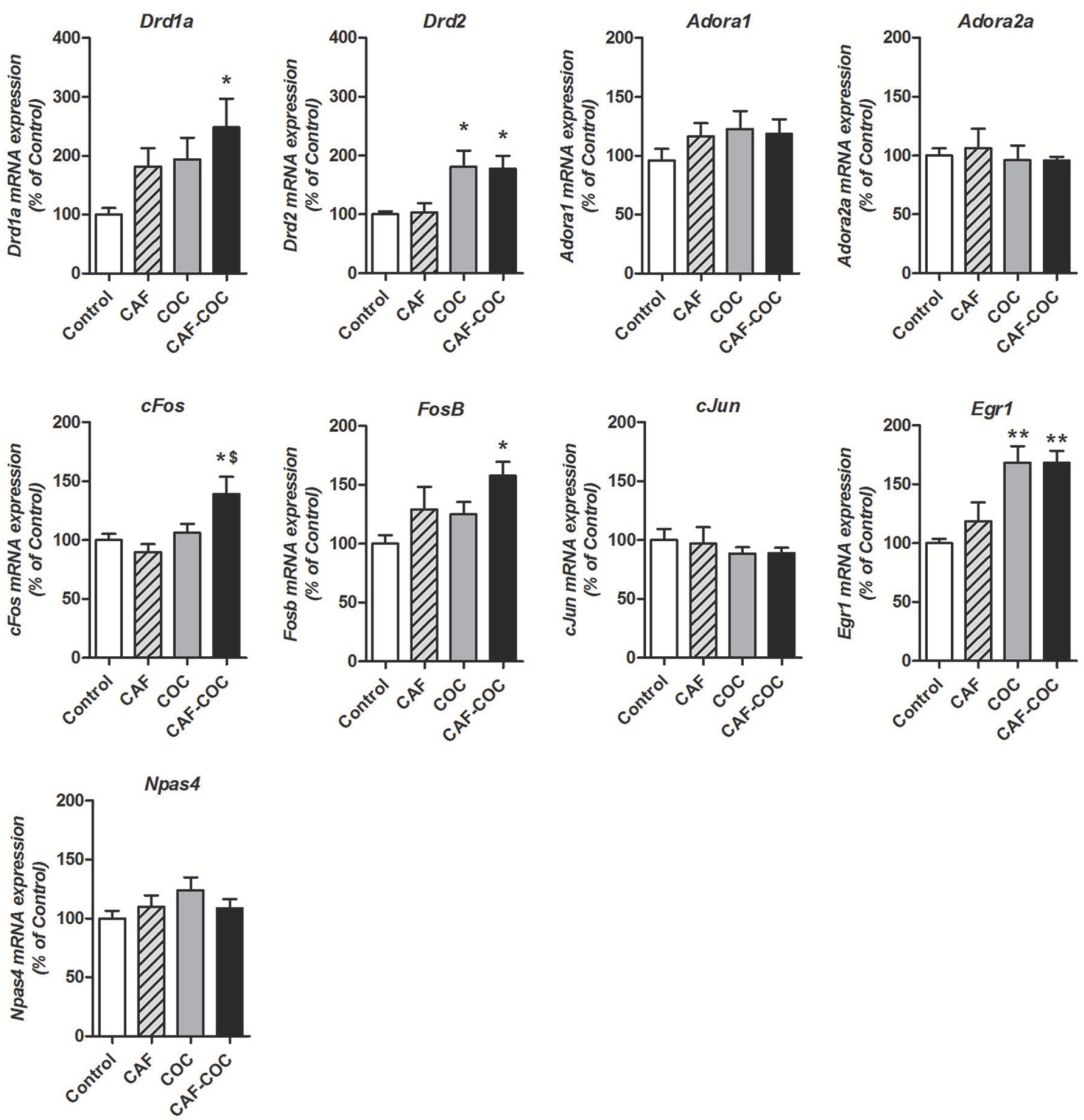

FIGURE 4 | Gene expression changes induced in the NAc by caffeine and cocaine-associated memory retrieval in the CPP. Dopamine receptor subunits *Drd1a* and *Drd2*; adenosine receptor subunits *Adora1* and *Adora2a*; IEGs *FosB*, *cFos*, *cJun*, *Egr1*, *Npas4*. Values indicate mean ± SEM. One-way ANOVA–Tukey. Kruskal–Wallis One–way ANOVA on ranks was used for *Drd2* data. $^{*}p < 0.05$, $^{**}p < 0.01$ different from Control. $^{\$}p < 0.05$, different from COC.

Figure 2D. One-way ANOVA-Tukey [$F_{(3, 26)} = 87.20$, $p < 0.001$, $N = 6$–7] indicated that all groups showed increased locomotion compared to Control ($p < 0.001$ different from Control). Also, CAF and CAF-COC groups were different from COC group ($p < 0.01$ and $p < 0.001$ respectively).

2b. Gene Expression Changes following a Single Conditioning Session of the CPP Test

mPFC

Figure 5 shows gene expression changes following a single CPP encoding session with caffeine, cocaine, and their combination on Dopamine and Adenosine receptor subunits and IEGs mRNA expression in the mPFC. One-way ANOVA-Tukey indicated that groups that received caffeine, given alone or in combination with cocaine, showed significant increase in Dopamine receptor subunits *Drd1a* and *Drd2* in mPFC [*Drd1a* $F_{(3, 21)} = 8.43$, $p < 0.01$, $N = 5$–6, Tukey: CAF $p < 0.05$, CAF-COC $p < 0.01$ different from Control; CAF $p < 0.01$ different from COC; *Drd2* $F_{(3, 21)} = 23.09$, $p < 0.001$, $N = 5$–6, Tukey: CAF and CAF-COC $p < 0.001$ different from Control; $p < 0.001$ different from COC]. For *Adora1* we found increased expression in the CAF group [$F_{(3, 21)} = 6.75$, $p < 0.01$, $N = 5$–6 Tukey:

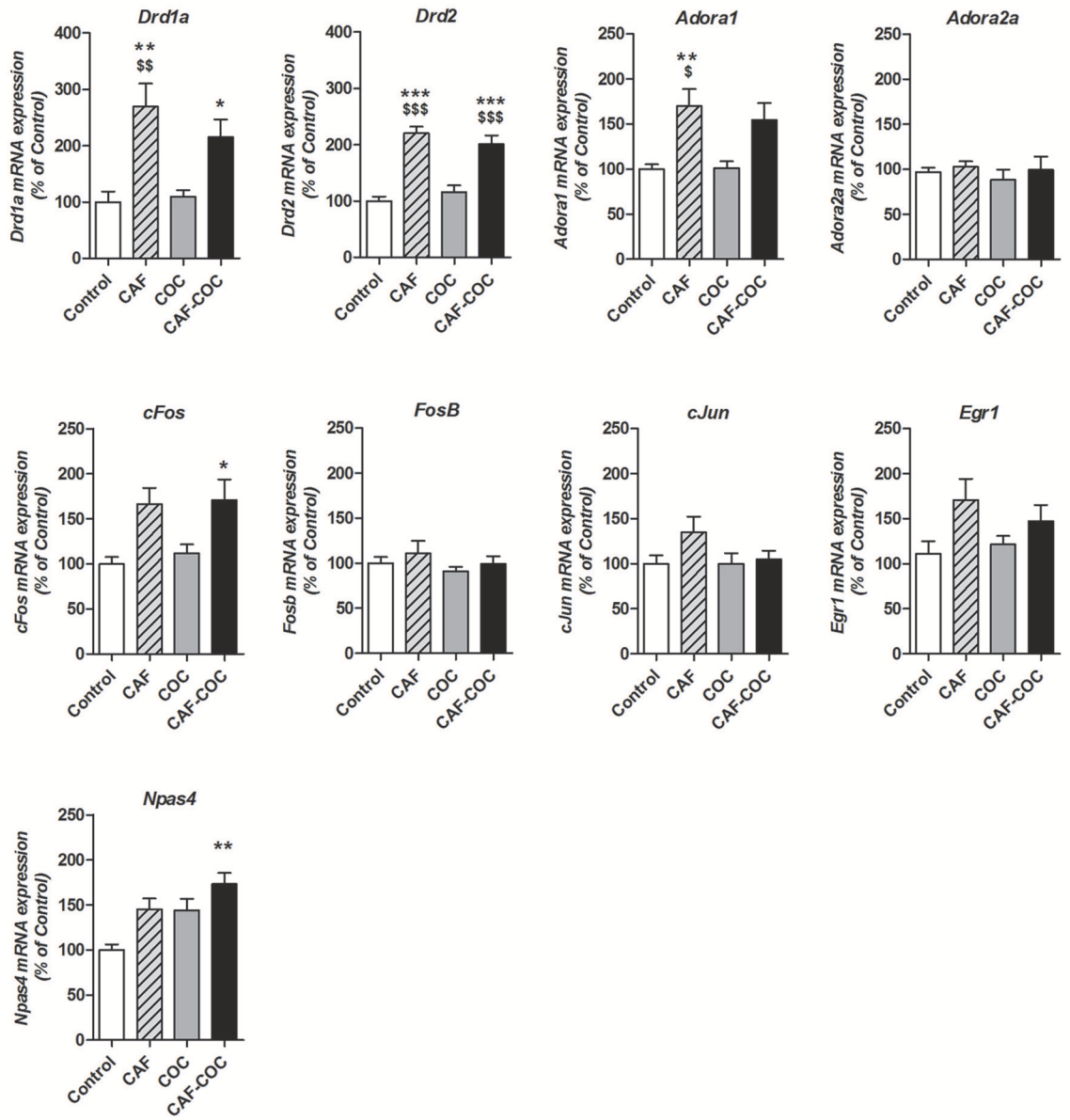

FIGURE 5 | Gene expression changes in the mPFC following a single conditioning session of the CPP. Dopamine receptor subunits *Drd1a* and *Drd2*; adenosine receptor subunits *Adora1* and *Adora2a*; IEGs *FosB*, *cFos*, *cJun*, *Egr1*, *Npas4*. Values indicate mean ± SEM. One-way ANOVA–Tukey: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ different from Control; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ different from COC.

$p < 0.01$ different from Control; $p < 0.05$ different from COC]. No changes were observed in *Adora2a* gene expression in any groups [$F_{(3, 22)} = 0.35$, $p > 0.05$, $N = 5$–6]. Concerning IEGs expression, for *cFos* and *Npas4* we observed the same trend that the one observed following full CPP, meaning that only groups that received the combination of CAF-COC showed increased expression [*cFos* $F_{(3, 20)} = 4.73$, $p < 0.05$, $N = 5$–6, Tukey: $p < 0.05$ different from Control; *Npas4* $F_{(3, 22)} = 6.46$, $p < 0.01$, $N = 5$–6, Tukey: $p < 0.01$ different from Control]. No significant differences were found for *cJun*, *Egr1* or *FosB* expression in the mPFC [*cJun* $F_{(3, 23)} = 1.82$, $p > 0.05$, $N = 6$; *Egr1* $F_{(3, 23)} = 2.55$, $p > 0.05$, $N = 6$; *FosB* $F_{(3, 23)} = 0.77$, $p > 0.05$, $N = 6$].

NAc

Figure 6 shows gene expression changes following a single CPP encoding session with caffeine, cocaine, and their combination

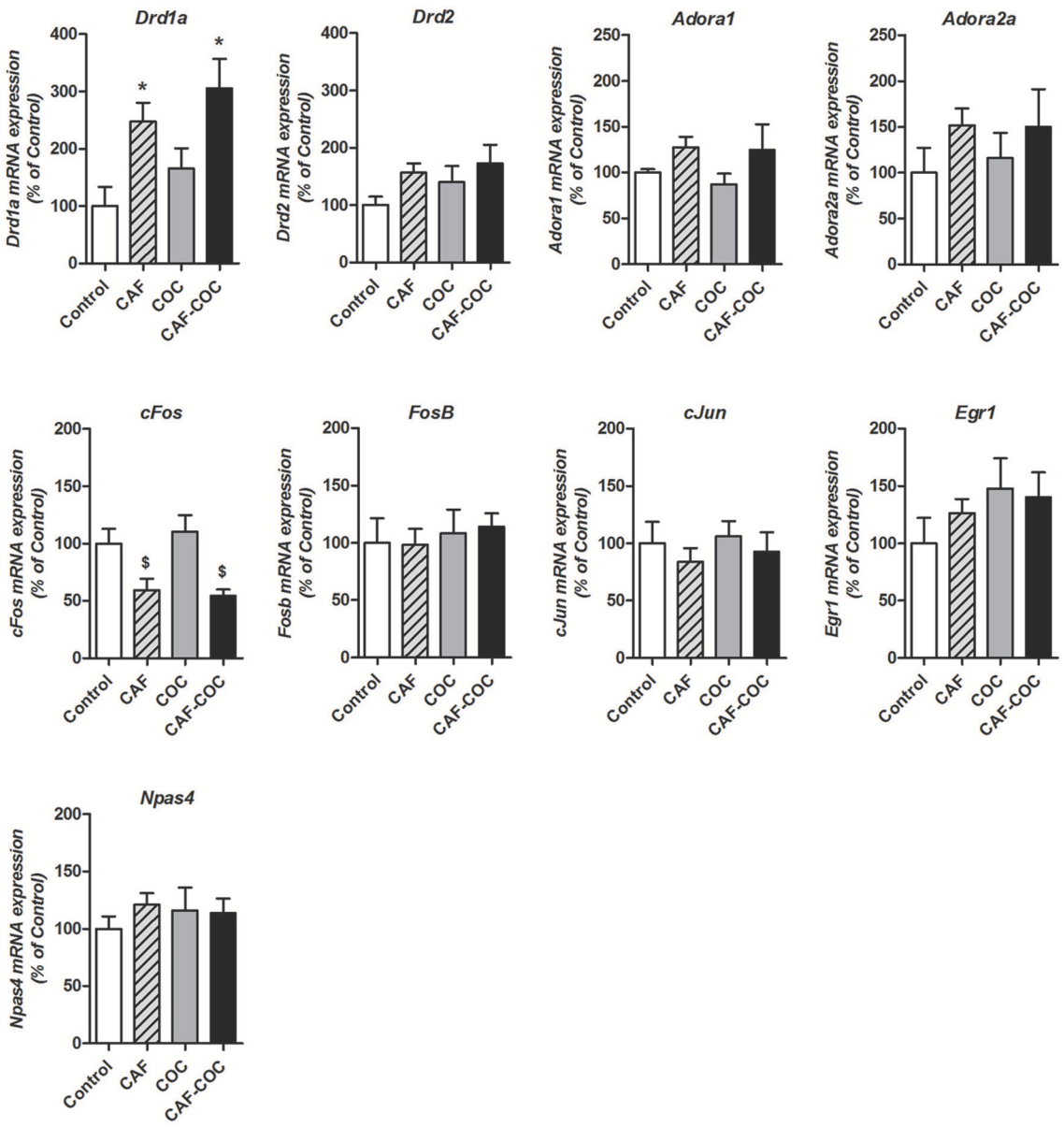

FIGURE 6 | Gene expression changes in the NAc following a single conditioning session of the CPP. Dopamine receptor subunits *Drd1a* and *Drd2*; adenosine receptor subunits *Adora1* and *Adora2a*; IEGs *FosB*, *cFos*, *cJun*, *Egr1*, *Npas4*. Values indicate mean ± SEM. One-way ANOVA–Tukey: $^{*}p < 0.05$ different from Control, $^{\$}p < 0.05$ different from COC.

on Dopamine and Adenosine receptor subunits and IEGs mRNA expression in the NAc. Overall, we found fewer gene expression changes (compared to the profile obtained after CPP test). The only genes that showed changes were *Drd1a* and *cFos*. For *Drd1a*, we found that caffeine induced increases in *Drd1a*, given alone or in combination [$F_{(3, 20)} = 5.68$, $p < 0.01$, $N = 4$–6, Tukey: CAF and CAF-COC $p < 0.05$ different from Control]. For *cFos*, we also observed a caffeine-mediated effect but in this case, caffeine-treated groups showed a decrease in its expression compared to COC group [$F_{(3, 19)} = 5.87$, $p < 0.01$, $N = 4$–6, Tukey: $p < 0.05$ different from COC]. We did not observe any other significant change on other genes: *Adora1* [$F_{(3, 19)} = 1.43$, $p > 0.05$, $N = 4$–5]; *Adora2a* [$F_{(3, 20)} = 0.65$, $p > 0.05$, $N = 4$–6]; *Drd2* [$F_{(3, 18)} = 1.11$, $p > 0.05$, $N = 4$–5]; *FosB* [$F_{(3, 21)} = 0.17$, $p > 0.05$, $N = 5$–6]; *cJun* [$F_{(3, 20)} = 0.46$, $p > 0.05$, $N = 4$–6]; *Egr1* [$F_{(3, 21)} =$

0.93, $p > 0.05$, $N = 5$–6; *Npas4* [$F_{(3, 21)} = 0.37$, $p > 0.05$, $N = 5$–6].

Experiment #3: Gene Expression Changes Induced by Caffeine and Cocaine Repeated Treatment in the Home Cage

In Experiment #3, mice received the same handling and the same doses and number of injections (one injection with saline plus one injection with drug or saline per day, for 3 consecutive days) as the groups that were trained and tested in the CPP (Experiment #1) but injections were given in the home cage, see **Figure 1**. Following the CPP protocol, animals were left undisturbed in their home cage for 2 days. Animals were sacrificed 24 h later (in a drug free state) and brain tissue collected for gene expression analysis. The objective of the Experiment #3 was to investigate whether changes observed following the CPP test (i.e., in Experiment #1) were dependent on context or linked to drug administration (i.e., a consequence of repeated drug injection).

mPFC

Figure 7 shows changes in gene expression following a 3-day repeated treatment given in the home cage with caffeine, cocaine, and their combination in the mPFC. We measured Dopamine and Adenosine receptor subunits and IEGs mRNA expression from brain tissues obtained 48 h after the last injection, in a drug-free state. We have found decreased *Drd2* expression for all treatments [$F_{(3, 21)} = 6.64$, $p < 0.01$, $N = 5$–6, Tukey: $p < 0.05$ and $p < 0.01$ different from Control] and decreased *Adora2a* expression in cocaine treated groups [$F_{(3, 22)} = 4.62$, $p < 0.05$, $N = 5$–6, Tukey: $p < 0.05$ different from Control]. Also, the group injected with the combination of CAF-COC showed a significant decrease in *cJun* mRNA in the mPFC [$F_{(3, 23)} = 3.28$, $p < 0.05$, $N = 6$, Tukey: $p < 0.05$ different from Control]. No other significant changes were observed for *Adora1* [$F_{(3, 22)} = 1.17$, $p > 0.05$, $N = 5$–6], *Drd1a* [$F_{(3, 22)} = 0.60$, $p > 0.05$, $N = 5$–6], *FosB* [$F_{(3, 21)} = 2.10$, $p > 0.05$, $N = 5$–6], *cFos* [$F_{(3, 21)} = 0.22$, $p > 0.05$, $N = 5$–6], *Egr1* [$F_{(3, 20)} = 0.29$, $p > 0.05$, $N = 5$–6] and *Npas4* [$F_{(3, 21)} = 0.48$, $p > 0.05$, $N = 5$–6].

NAc

Figure 8 shows gene expression changes following repeated treatment given in the home cage with caffeine, cocaine, and their combination, in the NAc. We measured Dopamine and Adenosine receptor subunits and IEGs mRNA expression from brain tissues obtained 48 h after the last injection, in a drug-free state. Overall, in Experiment #3 few changes were found on gene expression. No changes were found in many of the gene expression tested except for *FosB*. One-way ANOVA–Tukey indicated that the only gene demonstrating significant changes was *FosB,* a significant decrease was found [$F_{(3, 23)} = 4.42$, $p < 0.05$, $N = 6$ Tukey: $p < 0.05$ different from Control]. No other significant changes were observed for *Adora1* [$F_{(3, 23)} = 0.21$, $p > 0.05$, $N = 6$], *Adora2a* [$F_{(3, 20)} = 0.78$, $p > 0.05$, $N = 5$–6], *Drd1a* [$F_{(3, 19)} = 0.55$, $p > 0.05$, $N = 5$]; *Drd2* [$F_{(3, 20)} = 0.79$, $p > 0.05$, $N = 5$–6], *cFos* [$F_{(3, 22)} = 1.01$, $p > 0.05$, $N = 5$–6], *cJun* [$F_{(3, 22)} = 2.23$, $p > 0.05$, $N = 5$–6], *Egr1* [$F_{(3, 23)} = 0.06$, $p > 0.05$, $N = 6$] and *Npas4* [$F_{(3, 21)} = 0.45$, $p > 0.05$, $N = 5$–6].

Gene expression data for all experimental conditions are summarized in **Table 2**.

DISCUSSION

We report three main findings of the present study. (1) Combined administration of caffeine and cocaine induced a marked change of preference to the drug conditioned side of the CPP and a significant increase in locomotion. (2) Molecular changes observed at the time of CPP memory retrieval revealed specific effects on IEGs mRNA that were present almost exclusively in mice that received the combination of both psychostimulants. (3) Neuroadaptations mediated by both psychostimulants implicated different changes in mPFC or NAc, and were dependent on context (CPP or home-cage administration). These findings are of interest as neuroimaging studies involving human subjects have associated functional changes in the mPFC (Volkow et al., 2005) and NAc (Risinger et al., 2005) with cocaine craving.

Psychostimulants are drugs that can act as cognitive enhancers and potentiate learning and memory (Bisagno et al., 2016). Positive effects of caffeine are well known and include enhanced wakefulness, increased concentration, and stimulated activity (Daly and Fredholm, 1998). It is also known that the drug administration environment is critical in determining which neuronal ensembles are activated during cocaine treatment (Mattson et al., 2008). Moreover, formation and storage of memory representations rely on activity-driven changes in synaptic plasticity of neural networks mediated by IEGs (Veyrac et al., 2014). We found distinct changes in a subset of IEGs (*cFos, FosB, cJun, Egr1*, and *Npas4*) induced by caffeine and cocaine administration in the mouse brain that may underlie neuroplasticity in the NAc and mPFC circuits. The potential implications for these differential changes in gene expression are discussed below.

NAc

The NAc modulates the brain's natural reward system through changes in accumbal Dopamine released in response to rewarding stimuli that include motivation and incentivized learning (Cadet and Bisagno, 2016). CPP test trial responses depend on whether the animal remembers and/or retrieves the respective associations established during the drug-pairing sessions. In the present study we report that, while both groups that received cocaine showed positive CPP scores, only the group that received the combination of both psychostimulants during CPP training showed increased *cFo*s and *FosB* mRNA in the NAc. These changes appear to be context-induced since they did not occur in the groups treated with the same drug doses and schedule but in their home cage. In fact, we only found reduced *FosB* mRNA levels in the NAc of animals that received home cage treatment.

Drug-induced Dopamine can synergistically enhance *cFos* promoter activation in striatal neurons and those Fos-expressing neurons may act as a unit to form neuronal ensembles that

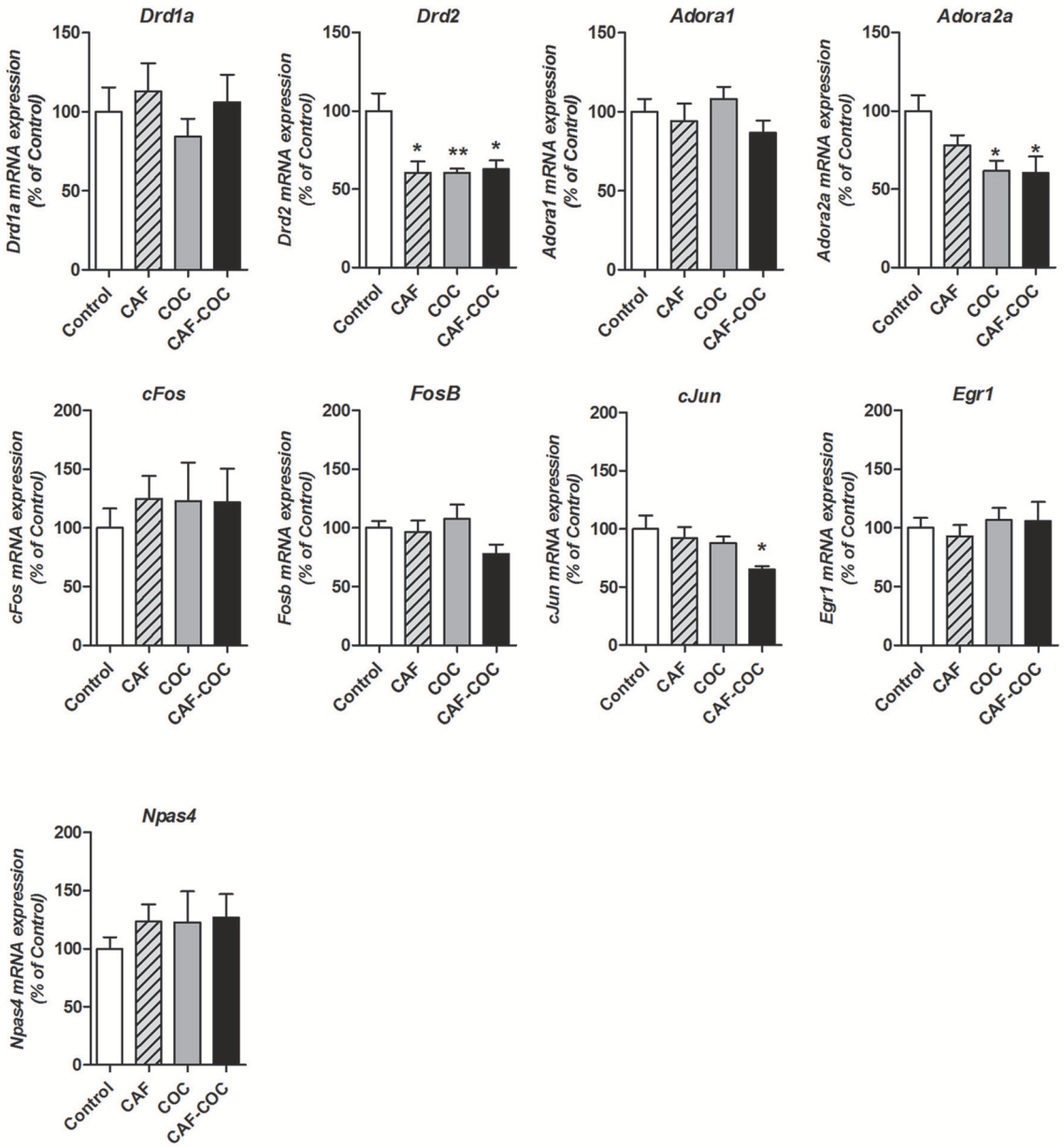

FIGURE 7 | Gene expression changes in the mPFC induced by caffeine and cocaine repeated treatment in the home cage. Dopamine receptor subunits *Drd1a* and *Drd2*; adenosine receptor subunits *Adora1* and *Adora2a*; IEGs *FosB*, *cFos*, c*Jun*, *Egr1*, *Npas4*. Values indicate mean ± SEM. One-way ANOVA–Tukey: $^{*}p < 0.05$, $^{**}p < 0.01$ different from Control.

encode conditioned drug behaviors (Cruz et al., 2015). ΔFosB, encoded by the FosB gene, is a member of the Fos family of IEGs induced in the NAc by the repeated administration of cocaine (Alibhai et al., 2007). It is important to note that the ability of ΔFosB to accumulate after a single exposure to cocaine is dependent on both the strength and duration of the stimulus (Kelz et al., 1999). This suggests that neuronal activity must reach a threshold for ΔFosB to accumulate in the NAc. This "all or nothing" theory was suggested for *cFos* since it is likely that only a small proportion of neurons experience enough strong activation so that calcium concentration surpasses the threshold for induction of *cFos* and other IEGs (Cruz et al., 2015). Our results suggest that the association of context with reward was not robust enough in mice receiving only

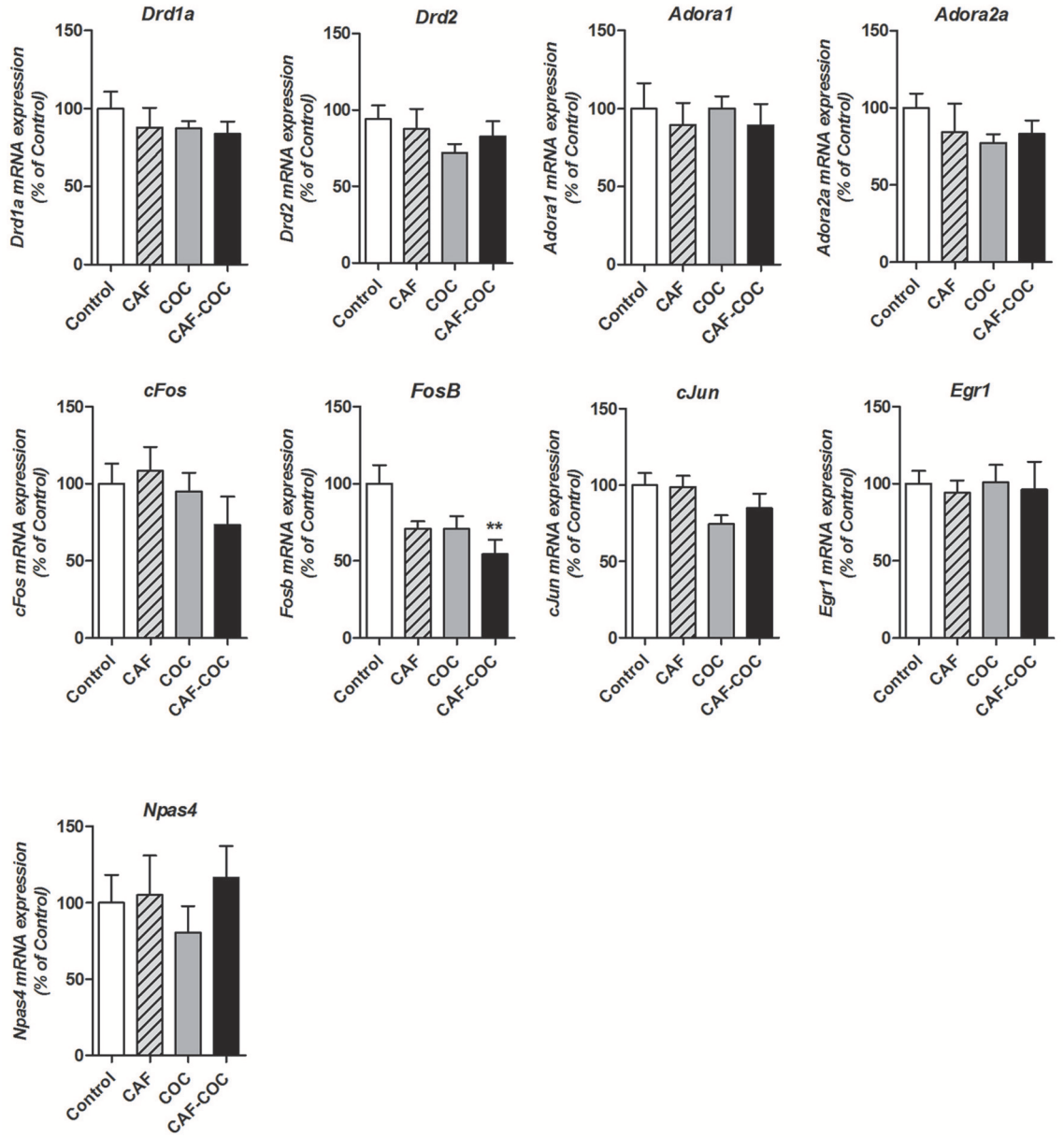

FIGURE 8 | Gene expression changes in the NAc induced by caffeine and cocaine repeated treatment in the home cage. Dopamine receptor subunits *Drd1a* and *Drd2*; adenosine receptor subunits *Adora1* and *Adora2a*; IEGs *FosB*, *cFos*, *cJun*, *Egr1*, *Npas4*. Values indicate mean ± SEM. One-way ANOVA–Tukey: $^{**}p < 0.01$ different from Control.

one psychostimulant (at the doses used in the present study) to increase *cFos* or *FosB*. Accordingly, CPP training with the combination of caffeine plus cocaine is likely to produce a stronger stimulus that is sufficient to induce expression of these IEGs.

Expression of *Egr1* in the NAc was shown to be responsive to CPP training with cocaine (Fritz et al., 2011). Our results are also in agreement with a recent report by Li et al. (2016) who showed that *Egr1* and *FosB* in the NAc mediate memory of cocaine-place association. Moreover, these authors suggest that both D1 and NMDA receptors regulate these two IEGs during drug memory consolidation (Li et al., 2016). Interestingly, we also found that following CPP, only caffeine plus cocaine treated animals showed increased D1 receptor mRNA in the NAc.

TABLE 2 | Gene expression change for all experimental conditions: FC (first conditioning CPP session), CPP (CPP test session) or HC (injections in home cage).

mPFC		FC			CPP			HC		
		Caf	Coc	Caf-Coc	Caf	Coc	Caf-Coc	Caf	Coc	Caf-Coc
	Drd1a	↑	–	↑	–	↓	–	–	–	–
	Drd2	↑	–	↑	–	↓	–	↓	↓	↓
	Adora1	↑	–	–	↑	↑	–	–	–	–
	Adora2a	–	–	–	–	–	–	–	↓	↓
	cFos	–	–	↑	–	–	↑	–	–	–
	FosB	–	–	–	–	–	–	–	–	–
	cJun	–	–	–	–	–	–	–	–	↓
	Egr1	–	–	–	–	–	↑	–	–	–
	Npas4	–	–	↑	–	–	↑	–	–	–
NAc	*Drd1a*	↑	–	↑	–	–	↑	–	–	–
	Drd2	–	–	–	–	↑	↑	–	–	–
	Adora1	–	–	–	–	–	–	–	–	–
	Adora2a	–	–	–	–	–	–	–	–	–
	cFos	↓	–	↓	–	–	↓	–	–	–
	FosB	–	–	–	–	–	↑	–	–	↓
	cJun	–	–	–	–	–	–	–	–	–
	Egr1	–	–	–	–	↑	↑	–	–	–
	Npas4	–	–	–	–	–	–	–	–	–

Symbol order: first symbol (↑, increase; ↓, decrease or –, no change) relates to Caffeine (green), second symbol to Cocaine (blue) and third symbol to Caffeine-Cocaine (red) groups.

Regarding caffeine-mediated effects, we found a decrease in *cFos* gene expression in the NAc of animals exposed to the first conditioning session that might be related to a decrease in NAc activation mediated by caffeine. It is important to note that the caffeine dose used in our study (5 mg/kg) is considered to be in the low range and other studies have reported that only high caffeine doses (i.e., 75 mg/kg) increase IEGs expression in the NAc (Bennett and Semba, 1998). In addition, significant increases in ΔFosB expression were observed in the NAc from animals exposed to caffeine mixed with alcohol, but not mice exposed to alcohol or caffeine (15 mg/kg) alone (Robins et al., 2016). These authors suggested that the combination of both drugs, i.e., caffeine and alcohol, increased Dopamine in the NAc to a higher degree than the stimulation produced by caffeine alone thereby increasing ΔFosB expression (Robins et al., 2016). We have also observed changes in the mRNAs for Adenosine and Dopamine receptors that appear to be sensitive to psychostimulants in striatal tissue (Ferré et al., 2010; Volkow et al., 2015; Muñiz et al., 2016).

mPFC

Ample evidence indicates that the mPFC has a significant role in the development and persistence of addictive behavior (Volkow et al., 2012). By integrating input from cortical and subcortical areas and conveying excitatory output to the NAc, the mPFC is thought to exert control over the motor circuitry to regulate drug responses and drug-associated stimuli (Cadet and Bisagno, 2013). In preclinical models, mPFC is associated with reward, including cocaine-CPP, (Tzschentke and Schmidt, 1999) and salience- and novelty-detection (Dalley et al., 2004).

In the present study, we found that a combination of psychostimulants influenced the outcome of CPP. Molecular results from this study suggest that caffeine might strengthen environment-drug association within the mPFC-NAc network. Indeed, we found a pattern of IEGs mRNA expression similar to the one obtained from NAc tissue, i.e., increased mRNA of *cFos* and *Egr1* in the mPFC in the group that received the combination of both psychostimulants during CPP training. *cFos* levels are reported to be increased in mPFC neurons following exposure to a cocaine-associated environment (Slaker et al., 2015). In addition, cocaine-conditioned stimuli were associated with increased *Egr1* expression in the PFC (El Rawas et al., 2012).

In addition, we observed a cocaine-mediated decrease in *Drd2* mRNA following CPP and after chronic treatment in the home cage (in Experiment #3, all psychostimulants reduced *Drd2* expression in the mPFC). This finding is probably linked to chronic cocaine effects, in agreement with previous studies demonstrating a decrease in D2 receptor function in the mPFC is related to cocaine-induced sensitization (Beyer and Steketee, 2002). However, following acute psychostimulant administration, on the first

conditioning day, we only found caffeine-mediated effects on *Drd1a* and *Drd2*. Both groups receiving caffeine, but not the group receiving cocaine alone, showed increased mRNA of these receptors in the mPFC. The differences in gene expression patterns seen in this study following acute and chronic drug administration suggest that specific neuroadaptations occurred in between those time points.

Among the differentially expressed IEGs seen in the mPFC compared to the NAc is *Npas4*. This IEG is expressed throughout the brain at a low level, though it is enriched in the frontal, parietal, and entorhinal cortices (Maya-Vetencourt et al., 2012; Spiegel et al., 2014). In our study, *Npas4* was only increased in the group that was injected with both psychostimulants during CPP training and following a single pairing CPP session, suggesting increases in mPFC activation.

Specific roles for *Npas4* in cocaine-mediated adaptations were reported by Ye et al. (2016). These authors demonstrated that there are brain-wide projection patterns separating mPFC cells responding to positive and negative experiences and that there was enrichment of *Npas4* expression in mPFC cells responding to cocaine. These authors also demonstrated that cocaine-induced increase of *Npas4* expression in mPFC is very rapid, within 30 min (Ye et al., 2016). It was proposed that *Npas4*, by controlling distinct networks of genes, differentially regulated synaptic input to excitatory and inhibitory neurons in order to facilitate circuit responses to sensory experience (Spiegel et al., 2014). Cognitive impairments are associated with decreased *Npas4* mRNA expression in the mouse frontal cortex (Qiu et al., 2016). Our results showing an association of CPP learning and increases in *Npas4* mRNA levels complement the decreases observed in cognitive decline.

We provide evidence here that caffeine may affect neuroplasticity through IEGs expression in circuits that are activated by drug use and associative learning. Our results also show that IEGs changes are specific to environmental context and that they are brain-region dependent. It is important for us to refocus from using these genes as activity markers, as has been done in the past, to exploring the role of these gene in establishing memory in mPFC cortical and cortical-subcortical circuits. Whilst this study describes specific changes in some early genes previously linked to memory and reward, it is difficult to establish the specific contribution of each of these genes to learning and memory since they may reflect general plasticity changes within the mPFC/NAc circuit.

Finally, it has been suggested that prevention of relapse might be achieved by using treatments that diminish the impact of drug-associated stimuli on drug seeking. Addiction is a brain disease and a learning disorder suggesting that drug-induced alterations can be undone or counteracted by a different learning strategy (Hyman et al., 2006). Interestingly, it has been reported that social interaction can prevent cocaine CPP (Prast et al., 2014).

This study also highlights the importance of considering caffeine effects as a potent adulterant in cocaine seized samples. Prieto et al. (2016) previously suggested that caffeine can act as a priming element for cocaine-related reward circuitry and as an effective discriminative stimulus for maintaining drug-seeking behavior in animals. In addition, caffeine intake is positively correlated with substance-use disorders (Kendler et al., 2006) and has been shown to increase illicit drug use (Miller, 2008). Thus, it seems reasonable to examine caffeine daily intake in psychostimulant-addicted patients undergoing treatment.

AUTHOR CONTRIBUTIONS

VB, FU, CS, and BG were responsible for the study design. JM, JP, MS, and BG performed the experiments and analyzed data. VB, FU, BG, JLC, and CS provided critical revision of the manuscript for important intellectual content. All authors have critically reviewed content and approved final version submitted for publication.

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Behavioral and Neurochemical Phenotyping of Mice Incapable of Homer1a Induction

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Immediate early and constitutively expressed products of the *Homer1* gene regulate the functional assembly of post-synaptic density proteins at glutamatergic synapses to influence excitatory neurotransmission and synaptic plasticity. Earlier studies of *Homer1* gene knock-out (KO) mice indicated active, but distinct, roles for IEG and constitutively expressed *Homer1* gene products in regulating cognitive, emotional, motivational and sensorimotor processing, as well as behavioral and neurochemical sensitivity to cocaine. More recent characterization of transgenic mice engineered to prevent generation of the IEG form (a.k.a *Homer1a* KO) pose a critical role for Homer1a in cocaine-induced behavioral and neurochemical sensitization of relevance to drug addiction and related neuropsychiatric disorders. Here, we extend our characterization of the *Homer1a* KO mouse and report a modest pro-depressant phenotype, but no deleterious effects of the KO upon spatial learning/memory, prepulse inhibition, or cocaine-induced place-conditioning. As we reported previously, *Homer1a* KO mice did not develop cocaine-induced behavioral or neurochemical sensitization within the nucleus accumbens; however, virus-mediated Homer1a over-expression within the nucleus accumbens reversed the sensitization phenotype of KO mice. We also report several neurochemical abnormalities within the nucleus accumbens of *Homer1a* KO mice that include: elevated basal dopamine and reduced basal glutamate content, Group1 mGluR agonist-induced glutamate release and high K^+-stimulated release of dopamine and glutamate within this region. Many of the neurochemical anomalies exhibited by *Homer1a* KO mice are recapitulated upon deletion of the entire *Homer1* gene; however, *Homer1* deletion did not affect NAC dopamine or alter K^+-stimulated neurotransmitter release within this region. These data show that the selective deletion of Homer1a produces a behavioral and neurochemical phenotype that is distinguishable from that produced by deletion of the entire *Homer1* gene. Moreover, the data indicate a specific role for Homer1a in regulating cocaine-induced behavioral and neurochemical sensitization of potential relevance to the psychotogenic properties of this drug.

Keywords: Homer1a, cocaine, glutamate, dopamine, nucleus accumbens, anxiety, sensitization

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1 INTRODUCTION

The mammalian gene *Homer1* possesses 10 exons, the regulated transcription of which results in both constitutively expressed and immediate early gene (IEG) products (Brakeman et al., 1997; Kato et al., 1998; Bottai et al., 2002). Exons 1–5 encode an Enabled/vasodilator-stimulated phosphoprotein (Vasp) homology 1 (EVH1) domain (Beneken et al., 2000). This EVH1 domain exhibits a RxxxxxGLGF sequence that enables Homer interactions with a proline-rich sequence (PPSPF) displayed by proteins regulating neuronal morphology, synaptic architecture, and glutamate receptor signaling/intracellular calcium dynamics (Shiraishi-Yamaguchi and Furuichi, 2007; Worley et al., 2007; Szumlinski et al., 2008a; Fagni, 2012; cf. Constantin, 2016). Exons 6–10 encode the carboxyl-tail of Homer proteins that consists of a coiled-coil (CC) domain, two leucine zipper motifs, and the 3′ UTR (Kato et al., 1998; Xiao et al., 1998; Soloviev et al., 2000). This CC-domain enables Homer proteins to tetramerize (Hayashi et al., 2006) – a property necessary for maintaining the functional architecture of glutamate synapses (for reviews, Shiraishi-Yamaguchi and Furuichi, 2007; Worley et al., 2007; Szumlinski et al., 2008a; Fagni, 2012; Constantin, 2016).

Alternative transcript splicing in regions downstream from exon 5 and premature termination of gene transcription has been reported for all three *Homer* genes (*Homer1*, *Homer2*, *Homer3*) (Soloviev et al., 2000). However, this phenomenon has been best-described for the *Homer1* gene (Brakeman et al., 1997; Bottai et al., 2002). The premature termination of *Homer1* transcription results in truncated or "short" Homer1 isoforms, so named because they lack the CC- and leucine zipper motifs necessary to multimerize, of which Homer1a and ania-3 have been identified in brain (Brakeman et al., 1997; Berke et al., 1998; Soloviev et al., 2000). The induction of these IEG Homer1 isoforms (i.e., Homer1a and ania-3) upon synaptic activity serves a dominant negative function by displacing CC-Homer proteins from their EVH1-bound partners. While this dynamic expression is predicted to cause a temporary reduction in the efficacy of post-synaptic glutamate transmission, by virtue of their inability to tetramerize, IEG Homer induction enables the trafficking to, and lateral movement within, the plasma membrane of glutamate receptors and associated signaling molecules. Thus, the induction of IEG Homer isoforms is critical for synaptic rearrangement and neuroplasticity (for reviews, Shiraishi-Yamaguchi and Furuichi, 2007; Fagni, 2012; Marton et al., 2015).

Converging evidence from clinical and behavioral/neural genetic studies in animals support active roles for members of the Homer family of post-synaptic scaffolding proteins in regulating various aspects of behavior, as well as drug-induced neuroplasticity relevant to addiction (for reviews, Szumlinski et al., 2008a; Marton et al., 2015). IEG Homer isoforms are induced in mesocorticolimbic structures by acute treatment with various drugs of abuse (see Szumlinski et al., 2008a; Marton et al., 2015). However, our understanding of their functional significance for addiction-related behavioral and neuropathology is rather limited. Earlier virus-mediated gene transfer studies failed to detect significant effects of Homer1a over-expression upon cocaine-induced locomotor hyperactivity/sensitization in rodent models (Lominac et al., 2005; Szumlinski et al., 2006). In contrast, behavioral phenotyping of transgenic mice over-expressing Homer1a selectively within striatal striosomes supported an active role for Homer1a in acute amphetamine-elicited locomotion, as well as in the expression of anxiety (Tappe and Kuner, 2006). The behavioral phenotype of striatal Homer1a over-expressing mice is consistent with those reported previously for *Homer1* whole-gene deletion (Szumlinski et al., 2004, 2005a; Lominac et al., 2005), as well as for rats infused intra-accumbens with oligonucleotides against *Homer1* (Ghasemzadeh et al., 2003), providing support for a competitive interaction between IEG and CC-Homer1 isoforms in regulating addiction-related emotional and motivational processing (Marton et al., 2015).

Herein, we provide a more complete behavioral and neurochemical phenotyping of a transgenic mouse engineered to prevent expression of Homer1a (*Homer1a*$^{-/-}$) while retaining expression of Homer1c (Hu et al., 2010). *Homer1a*$^{-/-}$ mice do not exhibit cocaine-induced locomotor or neurochemical sensitization within the NAC (Park et al., 2013). Here, we relate this resiliency to the dysregulation of NAC extracellular dopamine and glutamate and, demonstrate, using an adeno-associated virus (AAV) strategy, an active role for Homer1a induction within the NAC in regulating cocaine-induced neuroplasticity of relevance to addiction and related mental disorders.

2 MATERIALS AND METHODS

2.1 Subjects

Homer1a$^{+/+}$, *Homer1a*$^{+/-}$, and *Homer1a*$^{-/-}$ mice (on a mixed C57BL/6J × 129Xi/SvJ background; see Hu et al., 2010 for details), as well as *Homer1*$^{+/+}$ and *Homer1*$^{-/-}$ mice (on a mixed BALB/cJ × C57BL/6J × 129Xi/SvJ background; see Yuan et al., 2003 for details) were bred in-house at UCSB from mating of heterozygous breeder pairs and both male and female littermates were employed in all studies. The primers for genotyping *Homer1a*$^{-/-}$ mice are: H1F: CACCCGATGTGACACAGAAC, H1cR: CCAGTAATGCCACGGTACG, H1aR298: CACTGCTT CACATTGGCAGT. Wild-type band (H1F/H1cR) is at 602 bp and KO band (H1F/H1aR298) is at 211 bp. The primers for genotyping *Homer1*$^{-/-}$ mice are: 84: CAATGCATG CAATTCCTGAG, 85: CGAGAAACTTACATATATCCGCAAA, 86: GAACTTCGCGCTATAACTTCG. Wild-type band (H1F/H1cR) is at 602 bp and KO band (H1F/H1aR298) is at 211 bp. To reduce litter confounds, mice were selected from a minimum of four different litters within each replicate and testing began when mice were 7–8 weeks of age. Animals were group-housed in polyethylene cages in a temperature (25°) and humidity (71%) controlled vivarium under a 12 h dark/light cycle (lights off: 7:00 A.M.) with food and water available *ad libitum*. Experimental protocols, as well as housing and animal care, were consistent with the guidelines provided by the National Institute of Health (NIH) Guide for Care and Use of Laboratory Animals. All experiments were approved by the Institutional Animal Care and Use Committee of University of California, Santa Barbara.

2.2 Behavioral Test Battery

Male and female *Homer1a*$^{+/+}$, *Homer1a*$^{+/-}$, *Homer1a*$^{-/-}$ littermates were subjected to a behavioral test battery consisting of various conventional paradigms to assay anxiety-like behavior (elevated plus maze, reactivity to a novel object), depressive-like behavior (Porsolt swim test, saccharin preference), spatial learning and memory (Morris water maze), and sensorimotor processing/gating (acoustic startle and pre-pulse inhibition of acoustic startle/PPI). With the exception of the saccharin preference test, the procedures for each of these paradigms are detailed in our published work (see Szumlinski et al., 2004, 2005a; Lominac et al., 2005; Ary et al., 2013; Lee et al., 2015; Guo et al., 2016).

In brief, for the elevated plus maze, mice were placed on the center intersection of the maze with two white open arms and two black-walled arms 24 cm high. Each arm measured 123 cm long by 5 cm wide. Behavior in the apparatus was monitored for a 5 min trial (Lee et al., 2015; Guo et al., 2016). For the reactivity to a novel object test, mice were placed into a black Plexiglas (40 cm × 40 cm × 30 cm) open-field arena, containing an inedible object in the center. Mice were allowed to explore the arena for 2 min and the amount of time spent investigating the object, as well as the number of object contacts were recorded (Ary et al., 2013; Lee et al., 2015). For the Porsolt swim test, mice were placed into Plexiglas buckets (23 cm × 24 cm × 22 cm) and the behavior of the mice was scored in 30-s intervals using a checklist for swimming or floating over a 15-min period (Lominac et al., 2005; Szumlinski et al., 2005a; Ary et al., 2013; Guo et al., 2016). For the saccharin preference test, mice were presented with two 50 ml sipper tubes, one containing tap water and the other containing a 0.16% w/v saccharin solution for 24 h in the home cage. Bottle weights before and after the 24-h drinking period gauged the volume consumed and were used to calculate the preference for the saccharin solution. For the Morris water maze, we employed a stainless steel circular tank (200 cm in diameter, 60 cm in height; painted white on the inside and filled with room temperature water to a depth of 40 cm), with salient extra maze cues located on all four walls of the room in which the maze was located. A clear platform was placed in the tank and its location remained fixed throughout the course of the experiment. For 4 days, mice were trained four times a day (once at each compass point) to locate the hidden platform. During each trial, mice were randomly placed in the pool at one of the four compass points and swimming was recorded digitally by a video camera mounted on the ceiling directly above the pool (ANY-Maze, Stoelting). Training sessions were 120 s in duration; if the mice were unable to locate the platform during the allotted time, they were guided to the platform where they remained for 30 s. At 24 h after the last training trial, a 120-s memory probe test was performed in which the platform was removed and the amount of time taken by the mouse to swim toward the platform location and the time spent swimming in the platform quadrant was recorded (Lominac et al., 2005; Ary et al., 2013). For the acoustic startle/PPI experiment, we presented six different trial types: startle pulse (st110, 110 dB/40 ms), low prepulse stimulus given alone (st74, 74 dB/20 ms), high prepulse stimulus given alone (st90, 90 dB/20 ms), st74 or st90 given 100 ms before the onset of the startle pulse (pp74 and pp90, respectively), and no acoustic stimulus (i.e., only background noise was presented; st0). St100, st0, pp74, and pp90 trials were applied 10 times, st74 and st90 trials were applied five times, with trials randomly administered [average intertrial interval was 15 s (10–20 s)]. The background noise of each chamber was 70 dB. The data for startle amplitude were averaged across stimulus trials and the percent inhibition of the 110 dB startle by the 74 and 90 dB prepulse intensities was calculated for each animal to index PPI (Szumlinski et al., 2005a; Guo et al., 2016).

For studies of *Homer1a*$^{+/+,+/-,-/-}$ mice, behavioral testing occurred across days, with several tests conducted per day, spaced 4–6 h apart. The order of testing was the same for each cohort of animals: Day 1: PPI, reactivity to novel objects, Day 2: elevated plus-maze, and Porsolt swim test. Mice were then allowed 2–3 days recovery from the Porsolt swim test and then tested for their ability to habituate to repeated placement within a novel activity chamber (30 cm × 30 cm × 45 cm; 30 min sessions over 5 days). As the Porsolt swim test was predicted to reduce performance in a Morris water maze, a separate cohort of *Homer1a*$^{+/+,+/-,-/-}$ mice were trained (4, 2-min trials/day for 4 days) and then tested in the Morris water maze (2-min test) (see Lominac et al., 2005). Acoustic acuity was also assayed in a distinct cohort of male *Homer1a*$^{+/+,+/-,-/-}$ mice by randomly presenting acoustic stimuli ranging in intensity from 0 to 125 dB within the acoustic startle chambers during a 20-min session. Following this session, this cohort of animals were assayed for saccharin preference by presenting mice with two 50 ml sipper tubes in the home cage (water vs. 0.125% w/v saccharin) under continuous-access procedures over the course of 8 days. With the exception of this last cohort of mice, all behavioral experiments included approximately equal numbers of male and female subjects (minimum $n = 6$/sex/genotype) and sex was included as a between-subjects variable during data analysis by analyses of variance (ANOVA). However, with the exception of the novel object test, no sex differences in behavior were observed and thus, the data are presented collapsed across sex for the majority of the experiments.

For behavioral paradigms in which genotypic differences were observed, the experiment was replicated in *Homer1a*$^{+/+}$ and *Homer1a*$^{-/-}$ mice infused intra-NAC with an AAV carrying *Homer1a* cDNA (AAV-Homer1a) or a scrambled control (AAV-control; see section 2.4 below) to confirm an active role for this IEG isoform in regulating behavior. For these follow-up studies, behavioral testing commenced a minimum of 3 weeks following AAV infusion and mice were assayed for acoustic startle/PPI, followed by the Porsolt swim test. As behavior in the Morris Water Maze test relies heavily upon hippocampal, rather than accumbens, function, AAV-infused mice were not tested in this assay.

2.3 Cocaine-Induced Place-Conditioning

A separate cohort of male and female *Homer1a*$^{+/+,+/-,-/-}$ mice were used to index for genotypic differences in cocaine-conditioned reward using cocaine-induced place-conditioning.

The procedures to elicit place-conditioning were identical to those described previously by our group (e.g., Ary et al., 2013), with separate groups of mice injected with intraperitoneal (i.p.) injections of 3, 10, or 30 mg/kg cocaine (National Institute on Drug Abuse, Bethesda, MD, United States). Animals were habituated to both compartments of the two-compartment apparatus for 15 min during a pre-conditioning testing (PreTest) and then were subjected to daily, 15-min, conditioning sessions, alternating between cocaine and saline, over the course of 8 days. The day following the last conditioning session, animals were allowed free-access to both compartments in a post-conditioning test. The behavior of the mice was recorded throughout the experiment using a digital video-tracking system and ANYMaze software (Stoelting, Co., Wood Dale, IL, United States). The difference in the time spent in the cocaine- versus saline-paired compartment on a post-conditioning test served to index cocaine reward/aversion (Conditioned Place-Preference Score or CPP Score). The data were analyzed using a Genotype (+/+, +/−, −/−) × Sex × Dose (3, 10, and 30 mg/kg cocaine) × Side (paired vs. unpaired) ANOVA, with repeated measures on the Side factor. The data for locomotor activity during the PreTest and cocaine-induced locomotion were published previously in Park et al. (2013). A follow-up study employed identical place-conditioning procedures to assay the effects of intra-NAC AAV-Homer1a infusion upon genotypic differences in novelty-induced locomotion during the PreTest, habituation of locomotor activity (changes in saline-induced locomotion observed during the four saline-conditioning sessions), sensitization of cocaine-induced locomotor hyperactivity (changes in locomotor activity observed during the four cocaine-conditioning sessions; 10 mg/kg) and cocaine-conditioned reward (CPP Score following conditioning).

2.4 Construction of AAV-Homer1a

The rat Homer1a coding sequence was amplified using whole-brain cDNA, and the PCR was product expressed as an N-terminal fusion protein with the hemagglutinin (HA)-tag in a rAAV backbone containing the 1.1 kb CMV enhancer/chicken-actin (CBA) promoter, 800 bp human interferon scaffold attachment region inserted 5_ of the promoter, the woodchuck post-transcriptional regulatory element (WPRE), and the bovine growth hormone polyA flanked by inverted terminal repeats (AAV-Homer1a, AAV-Homer1c). The same AAV-CBA-WPRE-bGH backbone encoding the enhanced green fluorescent protein (EGFP) was used as control (AAV-GFP). As described previously (Hauck et al., 2003), AAV pseudotyped vectors virions containing a 1:1 ratio of AAV1 and AAV2 capsid proteins with AAV2 intertrigeminal regions were generated. For this, human embryonic kidney 293 cells were transfected with the AAV *cis*plasmid, the AAV1 (pH21) and AAV2 (pRV1) helper plasmids (pF6), and the adenovirus helper plasmid by standard calcium phosphate transfection methods. Cells were harvested at 48 h after transfection, and the vector purified using heparin affinity columns as described previously (During et al., 2003). As also described previously (Clark et al., 1999), genomic titers were determined using the Prism 7700 sequence detector system (Applied Biosystems, Foster City, CA, United States) with primers designed to WPRE.

2.5 Surgical Procedures and AAV Infusion

The NAC is a limbic structure implicated in both the psychomotor-activing and rewarding/reinforcing properties of drugs of abuse (e.g., Morein-Zamir and Robbins, 2015; Scofield et al., 2016). Homer1a is induced within the NAC by various drugs of abuse (Szumlinski et al., 2008a; Marton et al., 2015) and *Homer1a*$^{-/-}$ mice exhibit impaired cocaine-induced neurochemical sensitization within this region (Park et al., 2013). Thus, under isoflurane anesthesia, we conducted craniotomies to implant bilateral guide cannulae (20-gauge; 10-mm long), 2 mm above the NAC of mice (see Lominac et al., 2005; Cozzoli et al., 2009; Ary et al., 2013), for the purposes of conducting *in vivo* microdialysis measurement of glutamate and dopamine (see section 2.6 below) and/or infusing AAVs carrying either cDNA for Homer1a (AAV-Homer1a) or a scrambled DNA sequence as a control (AAV-control) to determine an active role for NAC Homer1a expression in regulating neurochemistry and behavior (see Lominac et al., 2005; Cozzoli et al., 2009 for AAV details). A Kopf stereotaxic device held the animal's head level and holes were drilled based on coordinates from bregma: (AP: +1.3 mm; ML: ±1.0 mm; DV: −2.3 mm) (Paxinos and Franklin, 2013). Guide cannulae were fixed to the skull with dental resin, surgical incisions were closed with a tissue adhesive.

For AAV-naïve mice undergoing microdialysis procedures, dummy cannulae (24 gauge; length equivalent to guide cannulae) was then placed inside the guide cannulae to prevent contamination or blockade and animals were allowed a minimum of 5 days recovery prior to neurochemical testing. Our AAVs are considered BSL1 and thus, standard protective personal equipment were employed during AAV infusion. For animals receiving AAV infusions, microinjectors (33-gauge, 12 mm long) where then lowered down the guide cannulae and the AAVs infused at a rate of 0.05 μl/min for 5 min (total volume/side = 0.25 μl). Microinjectors were left in place for an additional 5 min to allow for diffusion away from the microinjector tip prior to removal and occlusion of the guide cannulae. AAV-infused mice were allowed a minimum of 3 weeks recovery to achieve maximal neuronal transduction (Klugmann and Szumlinski, 2008). AAV transduction within the NAC was verified using standard immunohistochemical staining procedures for the hemagglutanin (HA) tag using a mouse anti-HA primary antibody (Covance, Princeton, NJ, United States), and straining visualized using a M.O.M. Detection Kit (Vector Laboratories, Burlingame, CA, United States), as conducted previously (Szumlinski et al., 2004, 2005b, 2017; Lominac et al., 2005; Cozzoli et al., 2009; Ary et al., 2013).

2.6 *In Vivo* Microdialysis

Homer1a$^{-/-}$ mice do not exhibit cocaine-induced dopamine or glutamate sensitization in the NAC (Park et al., 2013). Thus, a series of *in vivo* microdialysis experiments were conducted to relate this sensitization-resistant phenotype to biochemical indices of neuronal function within this region. For all in

vivo microdialysis experiments, a microdialysis probe (24 gauge; 12 mm in length with ∼1.0 mm active membrane) was lowered unilaterally into one of the guide cannulae and perfused with artificial CSF (146 nM NaCl, 1.2 mM $CaCl_2$, 2.7 mM KCl, 1.0 mM $MgCl_2$, pH = 7.4) at a rate of 2 μl/min. Dialysate collection began after 3 h of probe equilibration and occurred in 20 min intervals into vials containing 10 μl of preservative [10% methanol (v/v), 15% acetonitrile (v/v), 150 mM $NaPO_4$, 4.76 mM citric acid, 3 mM SDS, 50 mM EDTA, pH = 5.6]. Dialysate was stored at −80°C until analysis by HPLC (see section 2.6 below). Microdialysis probe localization within the NAC was verified using standard cresyl violet staining procedures (for AAV-naïve mice) or upon immunohistochemical localization of AAV transduction (see above), followed by examination of tissue under light microscope. In nearly all subjects, microdialysis probes were localized primarily to the shell subregion or to the interface between the shell and core subregions (see section 3).

2.6.1 DHPG-Stimulated Glutamate Release

Homer proteins are critical regulators of mGlu1/5 function *in vitro* and *in vivo* (cf. Ango et al., 2000, 2001; Shiraishi-Yamaguchi and Furuichi, 2007; Worley et al., 2007; Szumlinski et al., 2008a; Fagni, 2012; Constantin, 2016). Thus, we compared the effects of *Homer1a* versus pan-*Homer1* deletion upon an *in vivo* index of mGlu1/5 function – agonist-stimulated glutamate release (Swanson et al., 2001; Szumlinski et al., 2004, 2017). For this, *Homer1a*$^{+/+,-/-}$ and pan-*Homer1*$^{+/+,-/-}$ mice were perfused within increasing concentrations of the mGlu1/5 agonist DHPG [(S)-3,5-dihydroxyphenylglycine; 0, 3, 30, and 300 μM; Tocris Cookson, Ballwin, MO, United States], via the microdialysis probe in 1-h intervals. For each mutant line, the data were analyzed using Genotype (+/+ vs. −/−) × DHPG ANOVA, with repeated measures on the DHPG factor (four levels).

2.6.2 Depolarization-Induced Dopamine and Glutamate Release

To relate the distinct neurochemical phenotypes of *Homer1a* (Park et al., 2013) versus *Homer1* (Szumlinski et al., 2004, 2005a) mutants to the excitability of dopamine and glutamate terminals within the NAC, we compared depolarization-induced neurotransmitter release by locally infusing high K^+ solutions (50 and 100 mM; Sigma-Aldrich, St. Louis, MO, United States) through the microdialysis probe in 1-h intervals (e.g., He and Shippenberg, 2000). To conserve animal numbers, this microdialysis experiment was conducted 3–4 days following the DHPG study described in Section 2.6.1 above, using the other side of the head. The data were analyzed separately for each mutant line using a Genotype × K^+ ANOVA, with repeated measures on the K^+ factor (three levels).

2.6.3 Glutamate No Net-Flux Procedures

Pan-*Homer1* deletion reduces basal extracellular glutamate content within the NAC of mice (Szumlinski et al., 2005a) and prior conventional *in vivo* microdialysis studies suggested lower basal extracellular glutamate content within the NAC also of *Homer1a*$^{-/-}$ animals (Park et al., 2013). However, estimates of basal neurotransmitter levels assessed under conventional microdialysis procedures are subject to differences in probe recovery (Parsons and Justice, 1992). Thus, we quantified the effects of *Homer1a* deletion upon the basal extracellular glutamate content within the NAC using no net-flux procedures (Szumlinski et al., 2004, 2005a, 2017; Lominac et al., 2005; Ary et al., 2013). For this, increasing concentrations of glutamate (2.5, 5, and 10 μM) were infused through the probe in ascending order for 1 h each. Linear regression analyses were then conducted on the plot of the average net-flux of glutamate at each glutamate concentration versus the concentration of glutamate infused through the probe and the point of no net-flux (y = 0; estimate of basal extracellular levels of glutamate), as well as the slope of regression lines (estimate of glutamate clearance), were determined and analyzed using independent samples *t*-tests. A follow-up experiment determined how an intra-NAC AAV-Homer1a infusion altered the genotypic differences in our dependent measures in this assay. This follow-up study employed identical no net-flux procedures as those employed in the original genotypic comparison, with the exceptions that the *Homer1a*$^{+/+,-/-}$ mice were infused intra-NAC with either AAV-control or -Homer1a, a minimum of 3 weeks prior to probe insertion and the data were analyzed using an AAV (control vs. Homer1a) × Genotype (+/+ vs. −/−) ANOVA.

2.6.4 Cocaine-Induced Sensitization of Dopamine and Glutamate within the NAC

To determine whether or not Homer1a within the NAC actively regulates the development of cocaine-induced dopamine and glutamate sensitization in this region, *Homer1a*$^{+/+}$ and *Homer1a*$^{-/-}$ mice were infused with AAV-control or -Homer1a and then conventional *in vivo* microdialysis procedures were conducted a minimum of 3 weeks later. Low fecundity in our Homer1a colony at the time of study forced us to conserve animal numbers and thus, microdialysis procedures were conducted in the same AAV-infused mice as those tested for cocaine-induced locomotor sensitization/place-preference (see section 2.3). In order to accommodate both paradigms, mice first underwent a microdialysis session to assay the effects of AAV infusion upon the neurochemical response to an acute injection of 30 mg/kg cocaine (i.p.). Following a 3-h equilibration period, baseline sampling was conducted over an hour and then mice were injected with cocaine and samples collected for 3 h thereafter. At the end of this first microdialysis session, the probe was removed and mice were allowed to recover for 2 days prior to the 10-day place-conditioning procedure (consisting of four alternating injections of saline and 30 mg/kg cocaine). One to 2 days following the post-conditioning test, a second microdialysis session was conducted using the opposite side of the head to assay for cocaine-induced neurochemical sensitization.

In contrast to our prior study (Park et al., 2013), the *Homer1a*$^{+/+,-/-}$ mice in this study were treated with a total of six injections of 30 mg/kg cocaine. Due to technical

issues associated with subject attrition and sample loss due to technical difficulties with the HPLC, not all mice were tested for cocaine-induced neurochemical changes during both microdialysis sessions. Thus, in order to include all of the remaining the mice tested, Injection Number was treated as a between-subjects variable. As group differences were not observed for baseline glutamate levels on either injection, the data were expressed as a percent of baseline values for each group and to facilitate visualization of group differences in the magnitude of the cocaine-induced rise in dopamine and glutamate, the area under the curve (AUC) was employed in the statistical analyses of the results (Lominac et al., 2012; Shin et al., 2016). Thus, for both baseline neurotransmitter levels and the AUC for cocaine-induced changes in neurotransmitter levels, the data were analyzed using a Genotype × AAV × Injection Number between-subjects ANOVA.

2.7 High Pressure Liquid Chromatography

The high pressure liquid chromatography (HPLC) system and procedures for the electrochemical detection of glutamate and dopamine in the dialysate of mice, as well as the chromatographic analysis of the data, were identical to those described previously by our group (Lominac et al., 2012, 2014; Ary et al., 2013; Szumlinski et al., 2017). Each HPLC system consisted of a Coularray detector, a Model 542 autosampler and two Model 582 solvent delivery systems (ESA/Thermo-Fisher), with a detection limit of 0.01 fg/sample (20 μl/sample onto column). For analysis of dopamine, the MD-TM mobile phase was employed (ESA/Thermo-Fisher), and neurotransmitters in 30 μl from each 50 μl sample were separated using a MD-150 mm × 3.2 mm column (ESA/Thermo-Fisher). An ESA/Thermo-Fisher 5014B analytical cell was used for the detection of monoamines (oxidation and reduction electrode potentials of +220 and −150 mV, respectively). For glutamate, the mobile phase consisted of 3.5% acetonitrile (v/v), 22% methanol (v/v), 100 mM $NaPO_4$, pH = 6.75. A reversed phase column (50 mm × 3.0 mm Capcell PAK; Shiseido, Tokyo, Japan) was used to separate the amino acids, and precolumn derivatization with *o*-phthaladehyde (2.7 mg/ml) of the 20 μl from each 50 μl sample was performed using an ESA Model 540 autosampler (ESA/Thermo-Fisher). Glutamate was detected using an electrochemical analytical cell with an oxidizing potential of +550 mV. Glutamate and dopamine content in each sample were analyzed by peak height and compared with an external standard curve for quantification (glutamate standards: 2.5, 5.0, 10 mM; dopamine standards: 1.25, 2.5, 5 nM).

2.8 General Statistical Approaches

As detailed above, the majority of data were analyzed using ANOVAs, with repeated measures as appropriate for the experimental design. Significant interactions were deconstructed along the relevant factors, and significant main effects were followed by least significant differences (LSDs) *post hoc* tests or *t*-tests, as appropriate for the number of comparisons. $\alpha = 0.05$ for all analyses.

3 RESULTS

3.1 Negative Affect

Exposure to a novel environment induces Homer1a expression within forebrain (Vazdarjanova et al., 2002) and pan-*Homer1* KO mice exhibit robust signs of hyper-emotionality across a variety of behavioral paradigms (Szumlinski et al., 2004, 2005a; Lominac et al., 2005). Thus, we first assayed *Homer1a*$^{+/+}$ and *Homer1a*$^{-/-}$ mice for behavioral signs of negative affect using a comparable behavioral test battery as that employed in our previous work. As detailed below, the results do not support a consistent role for Homer1a induction in the manifestation of anxiety- or depressive-like behaviors in mice.

3.1.1 Elevated Plus Maze

When tested in the elevated plus maze test, no main effects or interactions with sex were detected ($p > 0.05$ for all variables); thus, the data for male and female mice were collapsed for data presentation. *Homer1a*$^{+/-}$ mice exhibited a significant increase in open arm entries, relative to both *Homer1a*$^{+/+}$ and *Homer1a*$^{-/-}$ mice (**Figure 1A**) [Genotype effect: $F(1,79) = 4.50$, $p = 0.01$; LSD *post hoc* tests]. A similar pattern of genotypic differences was observed for the time spent in the open arms, but the result was shy of statistical significance (**Figure 1B**; Genotype effect: $p = 0.1$).

3.1.2 Novel Object Test

In the novel object test assay, females exhibited lower behavioral indices of anxiety than males, as indicated by a significant increase in both the time in object contact [Sex effect: $F(1,59) = 18.91$, $p < 0.0001$], and number of object contacts [Sex effect: $F(1,59) = 36.50$, $p < 0.0001$]. No sex difference was observed for the latency to first make contact with the novel object ($p = 0.31$) and, as illustrated in **Figure 1C** (females) and **Figure 1D** (males), genotypic differences in exploratory behavior were not observed for either sex ($p > 0.20$ for all variables).

3.1.3 Porsolt Swim Test

No sex differences were observed for any of the variables measured during Porsolt Swim testing (Sex effects and interactions, *p*'s > 0.05). Thus, the data were collapsed across sex for presentation. *Homer1a* deletion reduced the latency to first float during both the 15-min exposure session, as well as during the 5-min re-exposure session conducted 24 h later (**Figure 1E**), with both *Homer1a*$^{+/-}$ and *Homer1a*$^{-/-}$ mice exhibiting a shorter latency to float, compared to controls [Test effect: $p = 0.08$; Genotype effect: $F(2,51) = 6.5$, $p = 0.003$; Genotype × Test: $p = 0.93$]. However, no genotypic differences were observed regarding the total floating during either session (**Figure 1F**) [Test effect: $F(1,51) = 3.93$, $p = 0.05$; Genotype effect and Genotype × Test: $p > 0.31$].

3.1.4 Saccharin Preference

Although the results of the Porsolt swim test suggested greater depressive-like behavior in *Homer1a*$^{-/-}$ mice, we failed to detect genotypic differences in the initial or average preference for a palatable saccharin solution over water (for both variables,

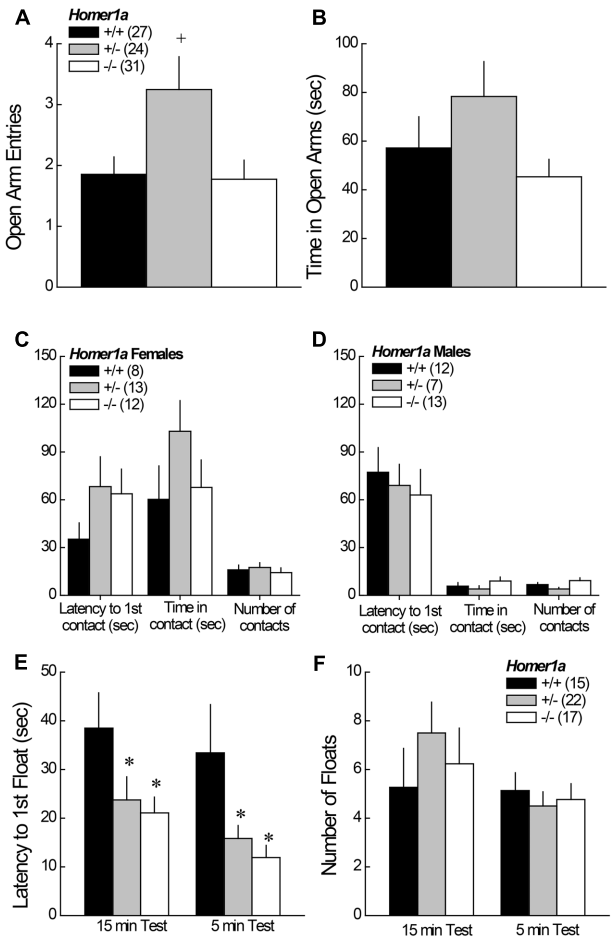

FIGURE 1 | Anxiety-like behavior in *Homer1a*$^{+/+}$, *Homer1a*$^{+/-}$, and *Homer1a*$^{-/-}$ mice. When compared in an elevated plus maze test, *Homer1a*$^{+/-}$ mice exhibited relatively lower anxiety as indicated by greater **(A)** number of open arm entries and **(B)** time spent in the open arms. While no sex differences in anxiety-like behavior were observed on the elevated plus maze, females **(C)** exhibited greater anxiety than males **(D)**, in the novel object test, but no genotypic differences were observed. No sex differences were apparent in the Porsolt Swim test, however, Homer1a deletion increased **(E)** the latency to first float, without influencing the total amount of floating **(F)**. The data represent the mean ± SEM of the number of mice indicated in parentheses. *$p < 0.05$ vs. +/+; +$p < 0.05$ vs. +/+ and –/– (LSD *post hoc* tests).

Genotype effects, $p > 0.25$), with the average preference exhibited by *Homer1a*$^{+/+}$, *Homer1a*$^{+/-}$, and *Homer1a*$^{-/-}$ mice being 43.1 ± 3.80, 50.90 ± 2.49, and 45.11 ± 4.48%, respectively.

3.1.5 Habituation to a Novel Environment

As the above data argue an inconsistent role for *Homer1a* in mediating the behavioral response to an acute mild stressor, we next determined whether *Homer1a* deletion might impair the ability to habituate to the repeated presentation of a mild stressor using a simple locomotor habituation paradigm. No significant main effects of, or interactions with, the Sex factor were observed (p's > 0.15). While there were trends for reduced overall locomotor activity in *Homer1a*$^{+/-}$ and *Homer1a*$^{-/-}$ mice, this genotypic difference was not significant nor were there genotypic differences in the extent to which animals habituated with repeated testing (data not shown) [Session effect: $F(4,208) = 23.41$, $p < 0.0001$; Genotype effect and Genotype × Session, $p > 0.05$]. Further, no genotypic differences were observed for the change in the latency to enter the center zone (data not shown) [Session effect: $p = 0.09$; Genotype × Session, $p > 0.05$] or in the number of entries into the center zone of the activity monitors with repeated testing (data not shown) [Session effect: $F(4,52) = 5.33$, $p < 0.0001$; Genotype × Session: $p > 0.95$].

3.2 Sensorimotor Processing

Pan-*Homer1* knock-out mice exhibit marked deficits in sensorimotor-gating (Szumlinski et al., 2004, 2005a; Lominac et al., 2005). Thus, we determined the effects of *Homer1a* deletion upon acoustic startle and pre-pulse inhibition of acoustic startle (PPI). Again, we failed to detect a sex difference in behavior (for all variables, no Sex effect or interactions, $p > 0.30$), thus, the data were collapsed across sex for presentation.

3.2.1 Acoustic Startle during PPI Testing

Relative to *Homer1a*$^{+/+}$ littermates, both *Homer1a*$^{+/-}$ and *Homer1a*$^{-/-}$ mice exhibited reduced startle responsiveness to the various acoustic stimuli (**Figure 2A**) [Genotype effect: $F(2,79) = 21.95$, $p < 0.0001$; Tone effect: $F(6,474) = 84.60$, $p < 0.0001$; Genotype × Tone: $F(12,474) = 14.4$, $p < 0.0001$]. Relative to controls, lower startle-responding to the 110 dB tone was observed in both *Homer1a*$^{+/-}$ and *Homer1a*$^{-/-}$ mice during the habituation phase (hab110), when it was presented alone (st110) or when it was preceded by either the 74 or the 90 dB pre-pulse (p's < 0.007; LSD *post hoc* tests). Moreover, both *Homer1a*$^{+/-}$ and *Homer1a*$^{-/-}$ mice exhibited lower spontaneous activity than *Homer1a*$^{+/+}$ animals in the absence of any acoustic stimuli (st0; $p = 0.006$).

3.2.2 PPI

While the 74 dB pre-pulse failed to inhibit acoustic startle in any genotype ($p > 0.25$), a genotypic difference in PPI was observed in the presence of the 90 dB pre-pulse (**Figure 2B**) [Genotype effect: $F(2,79) = 4.59$, $p = 0.01$]. Further, a significant impairment in PPI was observed at the 90 dB prepulse in *Homer1a*$^{-/-}$ mice, relative to controls ($p = 0.003$), with a modest impairment observed in *Homer1a*$^{+/-}$ mice ($p = 0.09$).

3.2.3 Acoustic Acuity

Although the tone-dependency of the startle response exhibited by *Homer1a*$^{+/-}$ and *Homer1a*$^{-/-}$ mice during PPI testing (**Figure 2A**) argued that *Homer1a* deletion does not impair the ability to discriminate between certain acoustic stimuli, the unexpected blunting of the startle-responsiveness of the mutant mice prompted a test for a primary hearing deficit. For this, male *Homer1a*$^{+/+}$, *Homer1a*$^{+/-}$, and *Homer1a*$^{-/-}$ mice were randomly presented with acoustic stimuli ranging in intensity from 0 to 125 dB. In this cohort, startle amplitude was also intensity-dependent (**Figure 2C**) [Tone effect: $F(8,152) = 82.29$, $p < 0.0001$]; however, although the results of the statistical

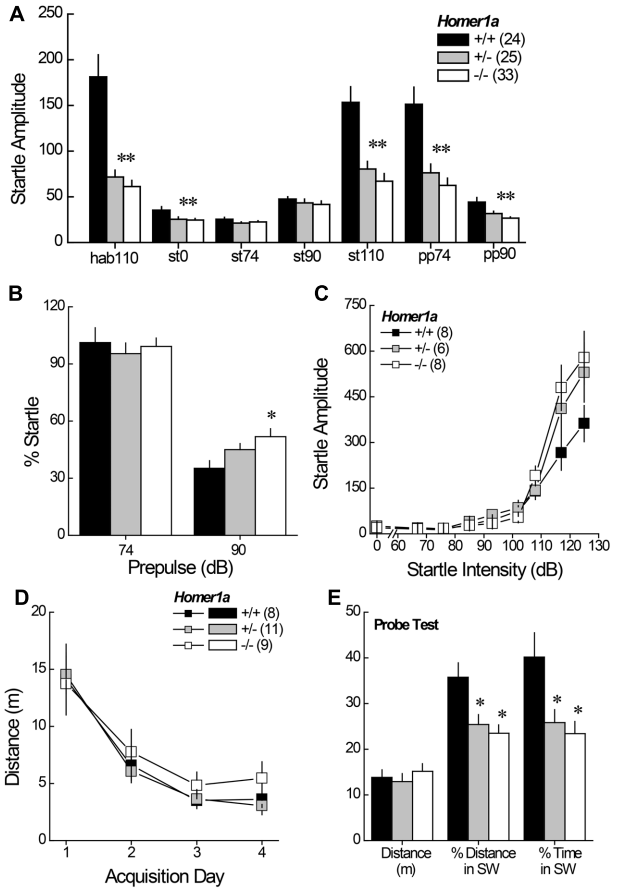

FIGURE 2 | Sensorimotor processing and spatial memory in *Homer1a*$^{+/+}$, *Homer1a*$^{+/-}$, and *Homer1a*$^{-/-}$ mice. **(A)** Both *Homer1a*$^{+/-}$ and *Homer1a*$^{-/-}$ mice exhibited reduced startle amplitude during testing PPI testing. **(B)** Only *Homer1a*$^{-/-}$ mice exhibited impaired PPI at the 90 dB pre-pulse. **(C)** No overt genotypic differences were apparent in acoustic acuity across a range of auditory stimuli. **(D)** No overt genotypic differences were observed over 4 days of training in the acquisition of a Morris water maze task. **(E)** However, *Homer1a*$^{+/-}$ and *Homer1a*$^{-/-}$ mice were impaired in their ability to recall the platform location, 24 h later. The data represent the mean ± SEM of the number of mice indicated in parentheses . *$p < 0.05$ vs. +/+ (LSD *post hoc* tests).

analyses indicated a significant interaction between genotype and tone [$F(8,152) = 2.36$, $p = 0.004$], *post hoc* comparisons at each dB level failed to reveal genotypic differences in startle responsiveness.

3.3 Spatial Learning and Memory

Pan-*Homer1* deletion markedly impairs learning and memory on several assays (Lominac et al., 2005; Szumlinski et al., 2005a; Gerstein et al., 2012) and intracranial manipulations of Homer1a expression affect spatial memory (e.g., Klugmann et al., 2005; Celikel et al., 2007; Gerstein et al., 2012, p. 213). Thus, we trained male mice to locate a hidden platform in a Morris water maze task and then assessed for long-term spatial memory in a probe trial, conducted 24 h following the 4th acquisition session. Surprisingly, genotypic differences in behavior were not detected during the acquisition phase of testing, either across trials within days, or across days of training (**Figure 2D**) [distance to locate platform, Trial effect: $F(3,225) = 5.18$, $p = 0.003$; Day effect: $F(3,225) = 33.50$, $p < 0.0001$; no main effect of, or interactions with the Genotype factor; data not shown for time taken to locate platform, Trial effect: $F(3,225) = 7.87$, $p < 0.0001$; Day effect: $F(3,225) = 34.46$, $p < 0.0001$; no main effect of, or interaction with, the Genotype factor]. In contrast to the data for maze acquisition, marked genotypic differences were observed regarding recall of the platform location, when tested 24 h later (**Figure 2E**), with both *Homer1a*$^{+/-}$ and *Homer1a*$^{-/-}$ mice exhibiting impaired recall, as indicated by lower percent total distance and percent time spent in the SW quadrant, which formerly contained the hidden platform [for % Distance: Genotype effect: $F(2,27) = 6.79$, $p = 0.004$; for % Time: Genotype effect: $F(2,27) = 5.82$, $p = 0.008$; *post hoc* tests]. No genotypic difference in the distance swam (**Figure 2E**; $p = 0.65$) argues that the recall impairment exhibited by Homer1a mutants was unrelated to swimming ability.

3.4 Cocaine-Conditioned Place-Preference

Pan-*Homer1* deletion increases sensitivity to the conditioned rewarding properties of cocaine (Szumlinski et al., 2004; Lominac et al., 2005). Thus, we characterized the dose-response function for cocaine-induced place-conditioning in male and female *Homer1a*$^{+/+,+/-,-/-}$ mice. No sex differences were observed for cocaine-induced place-conditioning [Side effect: $F(1,167) = 223.67$, $p < 0.000$; Dose × Side: $F(2,167) = 11.36$, $p < 0.0001$; Sex effect and interactions, p's > 0.10] and so, the data were collapsed across sex for presentation. Despite the large sample sizes employed in this experiment, we did not detect an effect of *Homer1a* deletion upon the dose-response function for cocaine-induced place-preference (**Figure 3**; no Genotype effect or Genotype × Side interactions, p's > 0.35). These data argue little role for Homer1a induction in either the associative learning/memory or incentive motivational processes involved in the development and expression of cocaine-conditioned reward.

3.5 DHPG-Induced Glutamate Release

Although it appeared as if *Homer1a* deletion elevated baseline glutamate in the study of DHPG-induced glutamate release (**Figure 4A**; DHPG = 0 μM), the genotypic difference was not significant (*t*-test: $p = 0.19$). However, *Homer1a* deletion prevented the capacity of DHPG to stimulate glutamate release within NAC (**Figure 4A**) [Genotype × Dose: $F(3,36) = 26.02$, $p < 0.0001$]. DHPG dose-dependently elevated glutamate in *Homer1a*$^{+/+}$ mice [DHPG effect: $F(3,15) = 68.31$, $p < 0.0001$], while no DHPG-induced rise was apparent in *Homer1a*$^{-/-}$ mice (DHGP effect: $p = 0.70$). In contrast to *Homer1a* deletion, a comparison of baseline glutamate indicated lower glutamate levels in *Homer1*$^{-/-}$ versus *Homer1*$^{+/+}$ mice [$t(16) = 2.36$, $p = 0.02$]. Further, pan-*Homer1* deletion blunted, but did not block, the capacity of DHPG to increase NAC glutamate (**Figure 4B**) [Genotype × DHPG: $F(3,51) = 4.65$, $p = 0.006$], as evidenced by a significant DHPG-induced rise in glutamate in

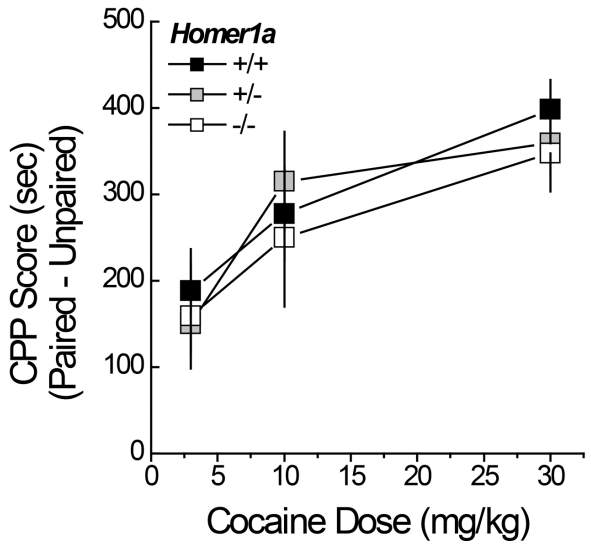

FIGURE 3 | Cocaine-induced place-conditioning in *Homer1a*$^{+/+}$, *Homer1a*$^{+/-}$, and *Homer1a*$^{-/-}$ mice. When tested in a cocaine-free state, genotypic differences were not observed in the dose-response function for cocaine-induced place-preference exhibited by male and female mice. Occupancy difference = time spent on the cocaine-paired minus -unpaired side in seconds). The data represent the mean ± SEM of the number of mice indicated in parentheses.

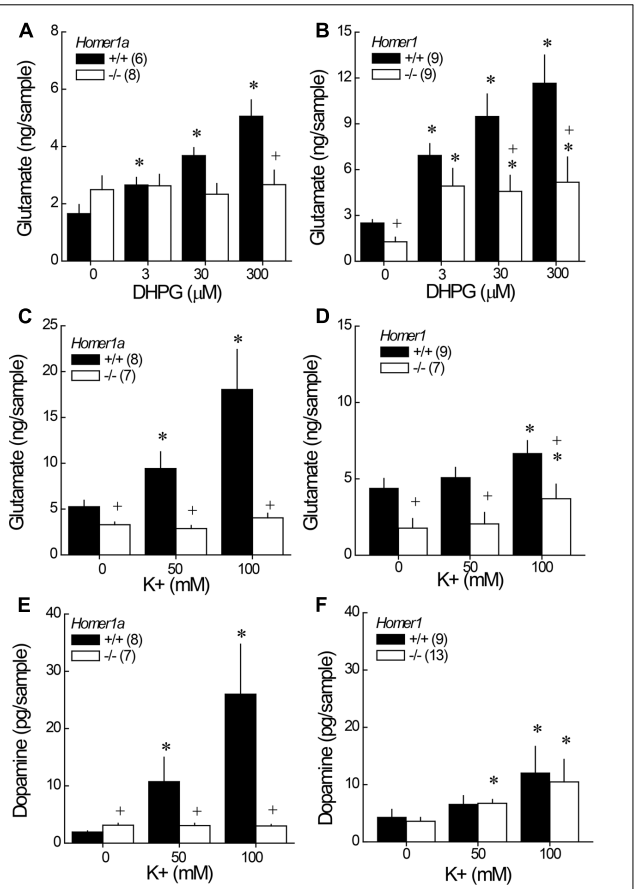

FIGURE 4 | Comparison of the effects of *Homer1a* versus pan-*Homer1* deletion upon the regulation of NAC glutamate and dopamine. Intra-NAC perfusion with increasing concentrations of the mGlu1/5 agonist DHPG failed to increase extracellular glutamate in *Homer1a*$^{-/-}$ mice **(A)**, while *Homer1*$^{-/-}$ mice exhibited a moderate, but significant, DHPG-stimulated rise in NAC glutamate **(B)**. Intra-NAC perfusion with high K^+ solutions elevated NAC glutamate levels and this effect was markedly blunted in *Homer1a*$^{-/-}$ mice **(C)**, but not in *Homer1*$^{-/-}$ mice, although *Homer1*$^{-/-}$ mice exhibited lower glutamate levels, overall **(D)**. *Homer1a*$^{-/-}$ mice failed to exhibit K^+-stimulated dopamine release **(E)**, while dopamine release was unaffected in *Homer1*$^{-/-}$ mice **(F)**. The data represent the mean ± SEM of the number of mice indicated in parentheses. *$p < 0.05$ vs. 0 μM; +$p < 0.05$ vs. *Homer1a*$^{+/+}$ or *Homer1*$^{+/+}$.

both genotypes [DHPG effect, for *Homer1*$^{+/+}$: $F(3,24) = 15.00$, $p < 0.0001$; for *Homer1*$^{-/-}$: $F(3,27) = 4.37$, $p < 0.01$]. These data provide evidence for distinct effects of *Homer1a* and pan-*Homer1* deletion upon mGlu1/5-dependent regulation of extracellular glutamate *in vivo*.

3.6 Depolarization-Induced NAC Glutamate and Dopamine Release

In this study, both *Homer1a*$^{-/-}$ (**Figure 4C**) and pan-*Homer1*$^{-/-}$ (**Figure 4D**) mice exhibited significantly lower baseline glutamate levels than their wild-type controls [for *Homer1a*: $t(13) = 2.39$, $p = 0.03$; for pan-*Homer1*: $t(14) = 3.02$, $p = 0.009$]. In contrast, *Homer1a*$^{-/-}$ KO mice exhibited a modest, but statistically significant elevation, in basal dopamine levels (**Figure 4E**) [$t(13) = 2.05$, $p = 0.045$], whereas differences in basal dopamine were not apparent between *Homer1*$^{+/+}$ and *Homer1*$^{-/-}$ mice (**Figure 4F**; *t*-test, $p > 0.35$).

Despite employing the same perfusate through the course of study, the effects of depolarization were more pronounced in the *Homer1a* versus the *Homer1* study, which might reflect differences in the genetic background of the two mutant lines. Nevertheless, a marked genotypic difference in K^+-stimulated glutamate release was apparent in the study of *Homer1a* mice [Genotype × K^+: $F(2,26) = 7.93$, $p = 0.002$]; while, K^+ stimulated a rise in glutamate in both genotypes (**Figure 4C**) [for *Homer1a*$^{+/+}$: $F(2,14) = 10.70$, $p = 0.002$; for *Homer1a*$^{-/-}$: $F(2,12) = 6.01$, $p = 0.02$], the magnitude of the rise observed in *Homer1a*$^{-/-}$ animals was considerably less than that observed in their controls. Although, *Homer1*$^{-/-}$ mice exhibited lower glutamate levels than *Homer1*$^{+/+}$ animals, irrespective of the concentration of K^+ perfused (**Figure 4D**) [Genotype effect: $F(1,14) = 9.67$, $p = 0.008$], *Homer1* deletion did not alter the capacity of K^+ to stimulate glutamate release within the NAC [K^+ effect: $F(2,28) = 13.08$, $p < 0.0001$; Genotype × K^+: $p = 0.11$].

In the case of dopamine, *Homer1a* deletion prevented K^+-stimulated dopamine release within the NAC (**Figure 4E**) [Genotype × K^+: $F(2,38) = 4.57$, $p = 0.02$; one-way ANOVAs: for *Homer1a*$^{+/+}$, $F(2,26) = 9.32$, $p = 0.001$; for *Homer1a*$^{-/-}$: $p = 0.78$]. In contrast, pan-*Homer1* deletion had no impact upon K^+-stimulated dopamine release in this region [K^+ effect: $F(2,40) = 6.86$, $p = 0.003$; Genotype effect and interaction, *p*'s > 0.70]. These data provide novel evidence that the selective deletion of *Homer1a*, but not the entire *Homer1* gene, reduces

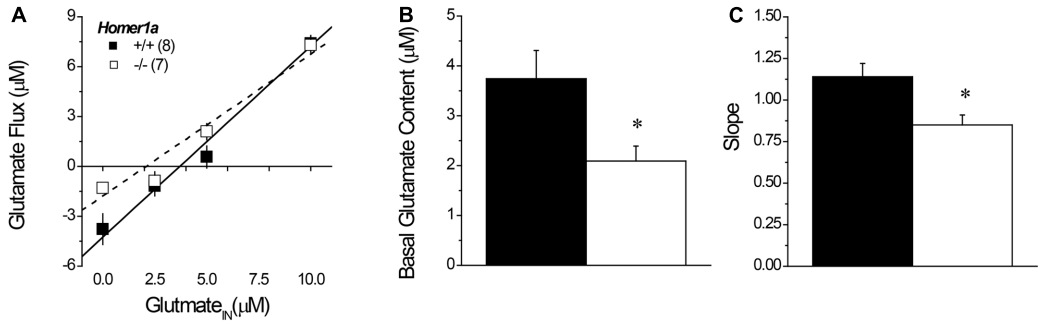

FIGURE 5 | Basal extracellular glutamate content and release/clearance in *Homer1a*$^{+/+}$ and *Homer1a*$^{-/-}$ mice. Summary of the results of the linear regression analyses of the data from the no net-flux study of extracellular glutamate **(A)**, highlighting **(B)** the lower x-intercept (an estimate of basal glutamate content) and **(C)** reduced slope (an index of reuptake/release) in *Homer1a*$^{-/-}$ versus *Homer1a*$^{+/+}$ animals. The data represent the mean ± SEM of the number of mice indicated in parentheses in **(A)**. $^{*}p < 0.05$ vs. +/+.

the excitability of both dopamine and glutamate terminals within the NAC.

3.7 Glutamate No Net-Flux

Linear regression analyses of the plots of the no net-flux study (**Figure 5A**) confirmed lower NAC extracellular glutamate content in *Homer1a*$^{-/-}$ versus *Homer1a*$^{+/+}$ mice (**Figure 5B**) [$t(13) = 5.85$, $p = 0.03$]. Linear analysis also indicated a significant reduction in the *in vivo* recovery of glutamate in *Homer1a*$^{-/-}$ mice relative to *Homer1a*$^{+/+}$ controls, as indicated by differences in the slopes of the linear regressions (**Figure 5C**) [$t(13) = 5.51$, $p = 0.04$].

3.8 AAV-Homer1a Effects upon Genotypic Differences in Behavior in Drug-Naïve Animals

To examine an active role for nucleus accumbens Homer1a in the behavioral phenotype of *Homer1a*$^{-/-}$ mice, AAV constructs carrying either Homer1a cDNA (AAV-Homer1a) or a scrambled DNA control were infused into the nucleus accumbens of *Homer1a*$^{+/+}$ and *Homer1a*$^{-/-}$ mice.

3.8.1 Acoustic Startle and PPI

We first employed Tukey tests for planned comparisons to compare between *Homer1a*$^{+/+}$ and *Homer1a*$^{-/-}$ mice infused with the control AVV to confirm replication of our prior genotypic difference in startle amplitude. Indeed, we replicated lower startle responsiveness in *Homer1a*$^{-/-}$ mice, specifically during habituation to the 110 dB stimulus ($p = 0.04$), in response to the 90 dB tone ($p = 0.02$), the 110 dB tone ($p = 0.04$) and in response to the 110 dB tone when preceded by the 74 or the 90 dB prepulse (both p's $= 0.05$). We did not observe a genotypic difference between AAV-controls at the 0 or 74 dB stimuli or when the 110 dB stimulation was preceded by the 90 dB prepulse (p's > 0.90). Planned comparisons were then conducted between AAV-control- and AAV-Homer1a-infused animals within each genotype. Despite what appeared to be a relatively selective effect of *Homer1a* over-expression upon the startle responsiveness of *Homer1a*$^{-/-}$ mice (**Figure 6A**), this statistical approach yielded

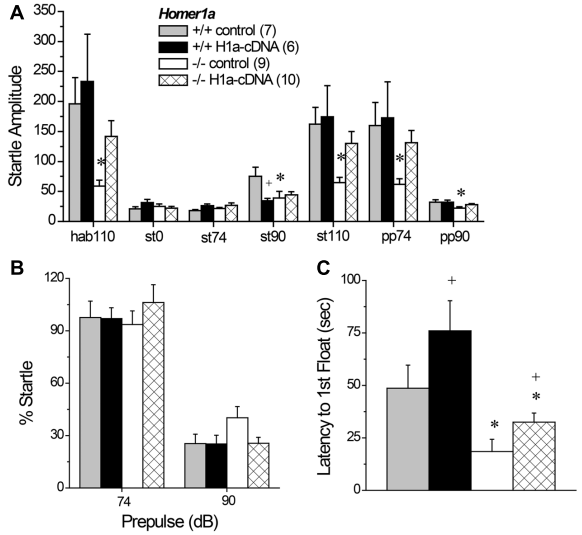

FIGURE 6 | The effects of AAV-mediated restoration of Homer1a expression in the NAC of *Homer1a*$^{-/-}$ mice upon sensorimotor function and depressive-like behavior. **(A)** Genotypic differences in acoustic startle were apparent only between *Homer1a*$^{+/+}$ and *Homer1a*$^{-/-}$ mice infused with AAV-control (con). **(B)** Group differences were not observed for PPI. **(C)** *Homer1a*$^{-/-}$ mice exhibited a shorter latency to float in a 15-min Porsolt swim test and AAV-Homer1a (H1a) increased the latency to float in both genotypes. The data represent the mean ± SEM of the number of mice indicated in parentheses. $^{*}p < 0.05$ vs. respective +/+; $^{+}p < 0.05$ vs. respective AAV control.

no AAV-Homer1a effects in either genotype at any of the stimulus intensities (p's > 0.20), with the exception of a significant reduction in the response exhibited by *Homer1a*$^{+/+}$ mice to the 90 dB tone ($p = 0.02$; st90 in **Figure 6A**). However, a comparison of the startle amplitude exhibited between *Homer1a*$^{+/+}$ and *Homer1a*$^{-/-}$ mice infused intra-NAC with AAV-Homer1a indicated no significant genotypic differences at any of the stimulus intensities (p's > 0.40), arguing that AAV-Homer1a infusion into the NAC was sufficient to reverse the blunted sensorimotor-processing induced by *Homer1a* deletion.

A comparable analysis of PPI (**Figure 6B**) detected no group differences at the 74 dB prepulse (for all comparisons, p's > 0.70) or at the 110 dB prepulse (for all comparisons, p's > 0.10). Thus, we failed to replicate the PPI deficit observed in AAV-naïve mice (**Figure 2B**), nor did we find evidence to support an active role for NAC Homer1a in regulating the magnitude of PPI.

3.8.2 Porsolt Swim Test

Replicating the data in **Figure 1E**, *Homer1a* deletion reduced the latency to first exhibit floating [Genotype effect: $F(1,29) = 15.93$, $p < 0.0001$]; however, intra-NAC AAV-Homer1a infusion increased the latency first float, irrespective of genotype (**Figure 6C**) [AAV effect; $F(1,29) = 5.00$, $p = 0.03$; interaction: $p = 0.48$], with AAV-Homer1a partially reversing the behavior of *Homer1*$^{-/-}$ mice. In contrast, we did not replicate the genotypic difference in total floating nor did we observed any AAV effect upon total floating in this experiment (data not shown; Genotype × AAV ANOVA, all p's > 0.35). Further, AAV infusion did not impact the distance traveled during swim testing (data now shown; Genotype × AAV ANOVA, all p's > 0.55).

3.9 AAV-Homer1a Effects upon Genotypic Differences in Cocaine-Induced Behavior

Although we failed to detect an effect of *Homer1a* deletion upon cocaine-conditioned reward (**Figure 3**), *Homer1a* deletion prevents the development of cocaine-induced behavioral sensitization (Park et al., 2013) and the effect of Homer1a over-expression within the NAC is not known. Thus, we employed our cocaine-induced place-conditioning procedures to determine the effects of intra-NAC AAV-Homer1a infusion upon spontaneous and cocaine-induced changes in locomotor activity, as well as place-conditioning, in *Homer1a*$^{+/+}$ and *Homer1a*$^{-/-}$ mice.

3.9.1 Locomotor Reactivity to Novelty and Habituation

A significant Genotype by AAV interaction was observed for the locomotor hyperactivity expressed when mice were allowed to explore the place-conditioning apparatus on the PreTest (**Figure 7A**) [Genotype × AAV: $F(1,31) = 4.45$, $p = 0.04$]. This interaction reflected genotypic differences between *Homer1a*$^{+/+}$ and *Homer1a*$^{-/-}$ mice infused with AAV-control ($p = 0.02$), which was not apparent in mice infused with AAV-Homer1a ($p = 0.70$). When compared to their respective AAV-control, there was no detectable effect of AAV-Homer1a upon the novelty-induced locomotion of either *Homer1a*$^{+/+}$ ($p = 0.12$) or *Homer1a*$^{-/-}$ mice ($p = 0.18$). No group differences were observed with respect to either the locomotor response to an acute saline injection (Genotype × AAV ANOVA, all p's > 0.65) or the change in saline-induced locomotion across the four saline-conditioning sessions (data not shown) [Saline Injection effect: $F(3,84) = 4.69$, $p = 0.04$; all other p's > 0.07].

3.9.2 Cocaine-Induced Locomotion and Sensitization

Interestingly, the group differences in the acute locomotor response to an acute injection of 30 mg/kg cocaine was inverse of that observed for novelty-induced locomotion (**Figure 7A** vs. **7B**) [Genotype × AAV: $F(1,28) = 5.30$, $p = 0.03$], with significant genotypic differences observed between *Homer1a*$^{+/+}$ and *Homer1a*$^{-/-}$ mice infused with AAV-control ($p = 0.03$), but not mice infused with AAV-Homer1a ($p = 0.38$). Further, we observed no effect of AAV-Homer1a upon acute cocaine-induced locomotion in either *Homer1a*$^{+/+}$ ($p = 0.13$) or *Homer1a*$^{-/-}$ mice ($p = 0.11$).

In contrast to the locomotor response to acute cocaine, AAV-Homer1a infusion completely reversed the effects of *Homer1a* deletion upon the development of cocaine-induced locomotor sensitization [Genotype × AAV × Cocaine Injection: $F(3,84) = 4.94$, $p = 0.003$]. Consistent with prior results for AAV-naïve mice (Park et al., 2013), *Homer1a*$^{+/+}$ mice infused with AAV-control exhibited robust sensitization across the four cocaine injections [$F(3,21) = 15.23$, $p < 0.0001$], as did *Homer1a*$^{+/+}$ mice infused with AAV-Homer1a [$F(3,21) = 12.37$, $p < 0.0001$]. While cocaine-induced locomotion did vary as a function of repeated cocaine treatment in *Homer1a*$^{-/-}$ infused with AAV-control [$F(3,21) = 4.33$, $p = 0.02$], this reflected a decline, rather than an increase, in activity with repeated cocaine treatment. In contrast, *Homer1a*$^{-/-}$ mice infused with AAV-Homer1a exhibited an injection-dependent increase in cocaine-induced locomotion [$F(3,21) = 8.36$, $p = 0.001$], indicative of restored sensitization.

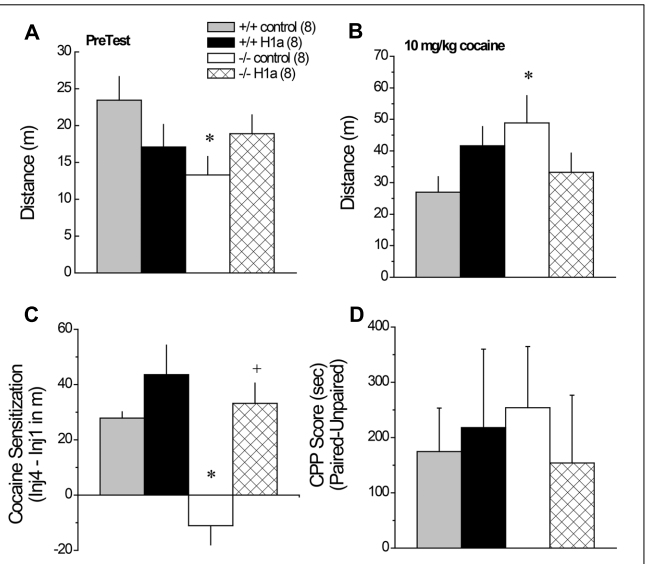

FIGURE 7 | The effects of AAV-mediated restoration of Homer1a expression in the NAC of *Homer1a*$^{-/-}$ mice upon behavior in a cocaine-conditioned place-preference assay. Genotypic differences were observed only in mice infused with AAV-control for both the total distance traveled during the 15-min PreTest **(A)** and the distance traveled in response to an acute injection of 10 mg/kg cocaine **(B)**. **(C)** *Homer1a*$^{-/-}$ controls failed to exhibit cocaine-induced sensitization and this phenotype was reversed by AAV-Homer1a infusion. **(D)** Group differences were not observed for the magnitude of a conditioned place-preference elicited by four pairings of 10 mg/kg cocaine. The data represent the mean ± SEM of the number of mice indicated in parentheses. *$p < 0.05$ vs. respective +/+; +$p < 0.05$ vs. respective AAV control.

To facilitate visualization of the group differences in cocaine-induced sensitization, the data are presented as difference in locomotor activity from injection 1 to 4 of repeated treatment (**Figure 7C**). Analysis of these data further indicates a reversal of the cocaine-sensitization phenotype of *Homer1a*$^{-/-}$ mice [Genotype × AAV: $F(1,31) = 4.21$, $p = 0.05$], with *Homer1a*$^{-/-}$ AAV-control exhibiting a locomotor phenotype distinct from the other groups tested (p's ≤ 0.001). Although AAV-Homer1a infusion increased the magnitude of the cocaine-sensitized response in both genotypes, the AAV effect was significant only in *Homer1a*$^{-/-}$ mice (*Homer1a*$^{+/+}$: $p > 0.20$; *Homer1a*$^{-/-}$: $p < 0.0001$).

3.9.3 Cocaine-Conditioned Place-Preference

Relative to their sensitized locomotor response to cocaine (**Figure 7C**), the magnitude of a cocaine-conditioned place-preference was highly variable (**Figure 7D**) and we detected no effect of genotype or AAV treatment upon the CPP Score exhibited by the mice when tested for compartment preference in a cocaine-free state (**Figure 8D**; Genotype × AAV ANOVA on CPP Score, p's > 0.45).

3.10 AAV-Homer1a Effects on Basal and Cocaine-Stimulated Neurotransmission

3.10.1 Cocaine-Induced Glutamate Sensitization

An analysis of the average baseline glutamate levels obtained using conventional *in vivo* microdialysis procedures failed to detect any group differences (data not shown; Genotype × AAV × Injection Number ANOVA, p's > 0.15). As expected, the capacity of cocaine to increase NAC glutamate was injection-dependent [Injection Number effect: $F(1,67) = 16.04$, $p < 0.0001$]. However, the magnitude of the cocaine-sensitized glutamate response varied as a function of both genotype and AAV treatment [Genotype × AAV × Injection Number: $F(1,67) = 8.36$, $p = 0.005$]. Neither genotype nor AAV infusion influenced the glutamate response to acute cocaine (Genotype × AAV ANOVA: all p's > 0.45), while AAV-Homer1a infusion augmented the capacity of the 6th cocaine injection to increase glutamate response only in *Homer1a*$^{-/-}$ mice [Genotype × AAV: $F(1,32) = -10.58$, $p = 0.003$]. Indeed, all groups but the *Homer1a*$^{-/-}$ controls exhibited greater cocaine-induced glutamate release on the 6th versus the 1st cocaine injection (for *Homer1a*$^{-/-}$ AAV-control: $p = 0.23$; for all other groups, p's < 0.04).

3.10.2 Cocaine-Induced Dopamine Sensitization

As observed for glutamate, there were no group differences in baseline levels of dopamine (data not shown; Genotype × AAV × Injection Number ANOVA, all p's > 0.06) and repeated cocaine treatment elicited a robust increase in dopamine that varied as a function of Genotype [Injection Number effect: $F(1,67) = 21.76$, $p < 0.0001$; Genotype × Injection Number: $F(1,67) = 9.00$, $p = 0.004$], but not AAV infusion (AAV × Injection Number: $p = 0.15$). Although the magnitude of the dopamine response to cocaine was greater following repeated treatment, the effects of AAV infusion upon the genotypic differences in dopamine responsiveness were similar across the microdialysis sessions (**Figure 8B**) [Genotype × AAV: $F(1,67) = 7.46$, $p = 0.008$; three-way interaction: $p = 0.21$], with *Homer1a*$^{-/-}$ controls exhibiting a blunted dopamine response to cocaine, relative to their *Homer1*$^{+/+}$ counterparts (p) and AAV-Homer1a augmenting the cocaine-induced rise in dopamine only in Homer1a$^{-/-}$ animals (for *Homer1a*$^{+/+}$: $p = 0.57$; *Homer1a*$^{-/-}$: $p < 0.0001$).

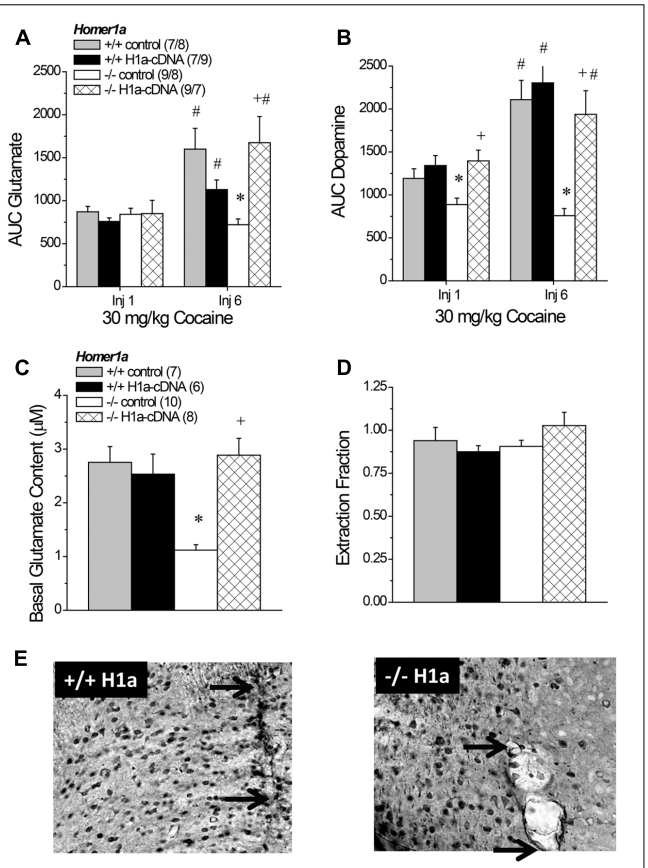

FIGURE 8 | The effects of AAV-mediated restoration of Homer1a expression in *Homer1a*$^{-/-}$ mice upon basal and cocaine-stimulated neurotransmission. **(A)** Under conventional *in vivo* microdialysis procedures, repeated treatment with 30 mg/kg cocaine sensitized cocaine-induced glutamate release in both *Homer1a*$^{+/+}$ infused intra-NAC with AAV, but only in *Homer1a*$^{-/-}$ mice infused with AAV-Homer1a (H1a). **(B)** Intra-NAC AAV-Homer1a infusion increased the magnitude of the cocaine-elicited rise in dopamine in *Homer1a*$^{-/-}$ mice and facilitated the expression of dopamine sensitization with repeated cocaine treatment. The sample sizes in **(B)** are presented in **(A)**. When determined using glutamate no net-flux procedures, intra-NAC AAV-Homer1a reversed the low basal glutamate phenotype of *Homer1a*$^{-/-}$ mice **(C)**, but did not alter the slope of linear regressions in either genotype **(D)**. The samples sizes in **(D)** are presented in **(C)**. The data in **(A–D)** represent the mean ± SEM of the number of mice indicated in parentheses. *$p < 0.05$ vs. respective +/+; +$p < 0.05$ vs. respective AAV control; #$p < 0.05$ vs. Injection 1 (sensitization). **(E)** Representative images of HA-staining within the NAC of *Homer1a*$^{+/+}$ (left) and *Homer1a*$^{-/-}$ mice (right) infused with AAV-Homer1a (20X magnification), illustrating comparable neuronal transduction around the site of microinjection/microdialysis probe insertion. Arrows in **(E)** represent the extent of the microdialysis probe membranes.

3.10.3 No Net-Flux Glutamate

A planned comparison of the genotypic difference in the x-intercept for mice infused with AAV-control replicated lower basal glutamate content in *Homer1a*$^{-/-}$ mice ($p < 0.0001$). We also detected a significant Genotype × AAV interaction for the x-intercept (**Figure 8C**) [$F(1,30) = 14.08$, $p = 0.001$] that reflected an AAV-Homer1a-induced increase in basal glutamate content in *Homer1a*$^{-/-}$ ($p < 0.0001$), but not in *Homer1a*$^{+/+}$ animals ($p = 0.65$). A comparable analysis of the slope of the linear regressions indicated that we did not replicate the genotypic difference in the extraction fraction in AAV-controls (**Figure 8D**; $p = 0.67$). Further, we did not observe any effect of AAV infusion upon this measure of glutamate release/reuptake in the NAC (Genotype × AAV: all p's > 0.10). Thus, Homer1a actively regulates basal extracellular glutamate content with in the NAC without influencing release/reuptake.

4 DISCUSSION

The induction of the IEG Homer isoform Homer1a is theorized to play a critical role in synaptic rearrangement and neuroplasticity relevant to various cognitive and behavioral processes, as well as pathophysiology in mental disease (cf. Shiraishi-Yamaguchi and Furuichi, 2007; Szumlinski et al., 2008a; Fagni, 2012; Marton et al., 2015). Despite its initial characterization over 20 years ago (Brakeman et al., 1997; Kato et al., 1998), we still know relatively little regarding the functional relevance of Homer1a induction *in vivo*. Herein, we employed an AAV strategy and extend prior results for *Homer1a*$^{-/-}$ mice (Park et al., 2013) by demonstrating that Homer1a within the NAC actively regulates cocaine-induced behavior, glutamate and dopamine sensitization, as well as basal extracellular glutamate levels in this region. Moreover, we demonstrate that NAC Homer1a expression actively regulates sensorimotor processing and sensorimotor-gating. We show also that Homer1a induction is necessary for normal recall of spatial memory and serves to inhibit depressive-like behavior in certain behavioral paradigms, but is not required to manifest anxiety-like behavior or cocaine-conditioned reward. Lastly, we demonstrate the importance of Homer1a induction for mGlu1/5 function within the NAC, as well as the excitability of both glutamate and dopamine terminals therein, and provide novel *in vivo* evidence that the NAC neurochemical phenotype produced by *Homer1a* deletion is distinct from that produced by deletion of the entire *Homer1* gene.

Our current knowledge concerning the behavioral relevance of distinct Homer1 isoforms can be derived from a relatively short-list of behavioral phenotyping studies of: pan-*Homer1*$^{-/-}$ mice (Szumlinski et al., 2004, 2005a; Lominac et al., 2005; Jaubert et al., 2007; Gerstein et al., 2012; Wagner et al., 2014, 2015), transgenic mice over-expressing Homer1a within the striatum (Tappe and Kuner, 2006) and transgenic mice incapable of Homer1a induction (Inoue et al., 2009; Park et al., 2013; present study). This line of interrogation is complemented and augmented by a number of reports describing the effects of site-specific manipulations of different Homer1 isoforms in the brain of wild-type rodents, as well as Homer-related mutants (Ghasemzadeh et al., 2003; Klugmann et al., 2005; Lominac et al., 2005; Szumlinski et al., 2006; Ary et al., 2013; Gerstein et al., 2013; Wagner et al., 2013, 2014, 2015; Banerjee et al., 2016; **Figure 7**). However marked procedural variations across these studies render it difficult to compare outcomes directly. This being said, the behavioral testing procedures employed in the present study of *Homer1a*$^{-/-}$ mice were highly comparable, and sometimes identical, to those used in our earlier behavioral characterization of pan-*Homer1*$^{-/-}$ mice (Szumlinski et al., 2004, 2005a; Lominac et al., 2005), facilitating interpretation of disparities and similarities in behavioral phenotypes across the two mutant lines. A side-by-side comparison of the effects of *Homer1a* versus pan-*Homer1* deletion upon spontaneous behaviors is provided in **Table 1**. As is apparent from this table and the extant literature, pan-*Homer1*$^{-/-}$ mice exhibit robust and complex behavioral anomalies (Szumlinski et al., 2004, 2005a; Lominac et al., 2005; Jaubert et al., 2007; Gerstein et al., 2012; Wagner et al., 2013, 2014, 2015). In contrast, the behavioral phenotype of *Homer1a*$^{-/-}$ mice is surprisingly similar to their wild-type controls in a variety of assays (Inoue et al., 2009; Park et al., 2013, **Table 1**), although some behavioral anomalies were observed in the present study that are discussed below.

4.1 Preventing Homer1a Induction Produces an Affective Phenotype That Is Distinct from That Produced by *Homer1* Deletion

Pan-*Homer1*$^{-/-}$ mice exhibit hyper-emotionality across a variety of paradigms (Szumlinski et al., 2004, 2005a; Jaubert et al., 2007; Wagner et al., 2014, 2015). In stark contrast, the anxiety-like behavior exhibited by *Homer1a*$^{-/-}$ herein was indistinguishable from that of wild-type mice, although *Homer1*$^{+/-}$ mutants did exhibit signs of *hypo*-anxiety on the elevated plus maze (**Figures 1A,B**). The present results are in line with the results of a study by Inoue et al. (2009), in which the *Homer1a* deletion did not alter anxiety-like behavior on the elevated plus-maze, open field or light–dark box tests (Inoue et al., 2009). These data for Homer1a-null mutants are interesting in light of evidence that transgenic mice over-expressing Homer1a within striatum increases anxiety signs in mice, which could be reversed upon conditional deletion of *Homer1a* (Tappe and Kuner, 2006). Taken together, the above findings from constitutive mutant mice argue that Homer1a over-expression is sufficient, but not necessary, for the manifestation of anxiety-like behavior.

However, other data in the literature argue that Homer1a induction exerts an "anxiolytic" or "anti-depressant" effect. For example, AAV-mediated Homer1a over-expression within the basolateral amygdala reduces the manifestation of fear-conditioning, as well as social interaction (Banerjee et al., 2016), while AAV-mediated restoration of Homer1a to the prefrontal cortex of pan-*Homer1*$^{-/-}$ mice reverses their hyper-anxious and depressive-like phenotype (Lominac et al., 2005). Consistent

TABLE 1 | Comparison of the behavioral phenotypes observed in *Homer1a*$^{-/-}$ versus pan-*Homer1*$^{-/-}$ mice.

Paradigm	Dependent variable	*Homer1a*$^{-/-}$	Pan-*Homer1*$^{-/-}$
Anxiety-like behavior:			
Elevated plus-maze	Open arm entries	WT = KO[Section 3.1.1]	WT = KO[1]
	Open arm time	WT = KO[Section 3.1.1]	WT = KO[1]
Novel object test	Latency to contact	WT = KO[Section 3.1.2]	
	No. Ccontacts	WT = KO[Section 3.1.2]	WT > KO[2]
	Time in contact	WT = KO[Section 3.1.2]	WT > KO[2]
Novel environment test	Distance	WT = KO[Section 3.1.4]	WT ≤ KO[2,3]
	Latency to center	WT = KO[Section 3.1.4]	
	Center entries	WT = KO[Section 3.1.4]	
Habituation to Nnovel Eenvironment	Distance	WT = KO[Section 3.1.4]	WT > KO[2,3]
	Latency to center	WT = KO[Section 3.1.4]	
	Center entries	WT = KO[Section 3.1.4]	
Depressive-like behavior:			
Porsolt swim test	Latency to float	WT > KO[Section 3.1.3]	WT > KO[2,3]
	No. floats	WT = KO[Section 3.1.3]	WT > KO[2,3]
Learning and memory:			
Morris water maze	Training: latency to platform	WT = KO[Section 3.3]	WT < KO[1]
	Training: distance to platform	WT = KO[Section 3.3]	WT < KO[1]
	Test: time in quadrant	WT > KO[Section 3.3]	WT = KO[1]; WT > KO (rotation)[1]
Sensorimotor processing:			
Acoustic startle	Basal startle (0 dB)	WT > KO[Section 3.2.1]	WT = KO[1–3]
	Startle amplitude w/o prepulse	WT > KO[Section 3.2.1]; WT = KO[Section 3.2.3]	WT = KO[1–3]
PPI	74 dB prepulse	WT = KO[Section 3.2.2]	WT = KO[1–3]
	90 dB prepulse	WT > KO[Section 3.2.2]	WT = KO[1–3]

Summary of the results of comparable studies examining the effects of Homer1a or Homer1 deletion (KO) upon behavior. > Denotes greater behavioral response in wild-type (WT) than KO; < denotes less behavioral response in WT than KO; = denotes no genotypic difference; ≥denotes inconsistencies in study outcomes, with reports of either no genotypic difference or greater behavioral response in WT than KO mice; ≤ denotes inconsistences in study outcomes, with reports of either no genotypic difference or less behavioral response in WT than KO. Highlighted rows indicate phenotypic similarities between Homer1a$^{-/-}$ *and Homer1*$^{-/-}$ *mice. [1]Jaubert et al. (2007); [2]Szumlinski et al. (2005a); [3]Lominac et al. (2005); [4]Park et al. (2013).*

with these latter data, both *Homer1a*$^{+/-}$ and *Homer1a*$^{-/-}$ mice exhibited a reduced latency to float in the Porsolt swim test in the present study (**Figure 1E**). In fact, this particular response to a stressor was the only emotion-related phenotype expressed in common by *Homer1a*$^{-/-}$ and pan-*Homer1*$^{-/-}$ mice (see **Table 1**). Moreover, AAV-mediated Homer1a overexpression within the NAC increased the float latency in both *Homer1a*$^{+/+}$ and *Homer1a*$^{-/-}$ mice (**Figure 6C**), arguing that Homer1a induction within both the cell body and terminal regions of the corticoaccumbens projections actively gates a depressive-like phenotype – a finding in line with associations between variants in the *Homer1* gene and major depressive disorder (e.g., Rietschel et al., 2010; Strauss et al., 2012; Rao et al., 2017). However, it is noteworthy that *Homer1a* deletion did not produce a "pro-depressive" effect in our saccharin preference test. In fact, a review of the extant literature suggests that site-specific Homer1a manipulations induce more robust and consistent effects upon negative affect (Lominac et al., 2005; Tappe and Kuner, 2006; Banerjee et al., 2016) than *Homer1a* deletion (Inoue et al., 2009; **Figure 1** and **Table 1**). This raises the possibility that Homer1a induction within distinct brain regions may exert opposing roles in regulating affective responding that are masked under constitutive inhibition of IEG induction.

4.2 Prevention of Homer1a Induction Produces a Cognitive Phenotype That Is Distinct from That Produced by *Homer1* Deletion

Homer1a induction is theorized to facilitate the rearrangement of the synaptic architecture of excitatory glutamate synapses necessary for metaplasticity and learning (e.g., Hu et al., 2010; Fagni, 2012; Marton et al., 2015). Although associative learning is intact in pan-*Homer1*$^{-/-}$ mice (Szumlinski et al., 2004), this mutant exhibits deficits several other forms of learning and memory, including simple habituation (Lominac et al., 2005; Szumlinski et al., 2005a), spatial learning/memory (Lominac et al., 2005; Szumlinski et al., 2005a; Jaubert et al., 2007), and instrumental learning (Szumlinski et al., 2005a; Wagner et al., 2014). Moreover, pan-*Homer1*$^{-/-}$ mice are deficient in long-term depression (Ronesi and Huber, 2008) and long-term potentiation (Gerstein et al., 2012), while *Homer1a*$^{-/-}$ mice fail to exhibit homeostatic AMPA receptor scaling within cortex (Hu et al., 2010). In contrast to pan-*Homer1*$^{-/-}$ mice (Szumlinski et al., 2005a), *Homer1a*-null mutants exhibited no observable deficits in the acquisition phase of a Morris water maze (**Figure 2D**), arguing that the induction of Homer1a is not necessary for the consolidation

or recall of the information pertaining to the platform location.

However, akin to pan-*Homer1*$^{-/-}$ mice (Szumlinski et al., 2005a), both *Homer1a*$^{+/-}$ and *Homer1a*$^{-/-}$ animals spent less time in the quadrant of the pool that formerly contained the hidden platform on the probe test (**Figure 2E**). While this result might reflect impaired spatial memory recall, such an interpretation is inconsistent with their intact learning of the maze location during the acquisition phase of this experiment. As no genotypic differences were observed in the amount of floating behavior during Porsolt swim testing (**Figure 1F**) or in the total distance traveled during the probe trial (**Figure 2E**), it is unlikely that *Homer1a* deletion altered swimming capacity during the memory probe test. Alternatively, the reduced time spent in the platform quadrant might reflect the deployment of an alternative platform search or escape strategy in *Homer1a* mutant mice. Indeed, pan-*Homer1*$^{-/-}$ mice employ a "chaining" strategy to successfully navigate a water-version of the radial arm maze (Lominac et al., 2005; Szumlinski et al., 2005a) and AAV-mediated restoration of Homer1c, not Homer1a, to the PFC of pan-*Homer1*$^{-/-}$ mice reverse their deficits in spatial working memory (Lominac et al., 2005). Further, hippocampal restoration of Homer1c reverses the spatial memory impairments exhibited by pan-*Homer1*$^{-/-}$ (Gerstein et al., 2012), as well as aged mice (Gerstein et al., 2013) and hippocampal over-expression of Homer1a impairs the acquisition and/or recall of spatial memories (Klugmann et al., 2005; Celikel et al., 2007). Thus, it is clear that a more concerted research effort is required to understand more precisely how Homer1a induction within specific neurocircuits contributes to spatial and non-spatial learning of relevance to the etiology and treatment of attentional, learning and memory disorders (Wells et al., 2015).

4.3 Preventing Homer1a Induction Profoundly Impairs *in Vivo* Regulation of NAC Neurotransmitter Levels

Since their initial discovery, the glutamatergic consequences of *Homer1* vs. *Homer1a* deletion have been studied extensively *in vitro*, with a focus on the regulation of post-synaptic signal transduction mechanisms (cf. Shiraishi-Yamaguchi and Furuichi, 2007; Worley et al., 2007; Fagni, 2012; Marton et al., 2015). However, transfection of hippocampal neuronal cultures with constitutively expressed Homer isoforms can augment, while transfection with Homer1a reduces, presynaptic neuronal activity (Sala et al., 2001) and a more recent study demonstrated opposing roles for CC- and IEG-Homer1 isoforms in regulating mGlu1/5-stimulated Ca^{2+} entry and glutamate release from astrocytes both *in vitro* and *in situ* (Buscemi et al., 2017). Further, CC- and IEG-Homer1 isoforms exert opposing effects upon mGlu1/5-dependent generation of anandamide (Jung et al., 2007), which is well-characterized to inhibit glutamate release via activation of CB1 receptors (e.g., Johnson and Lovinger, 2016). Indeed, we have reported several anomalies with respect to *in vivo* measures of glutamate function in the brains of pan-*Homer1*$^{-/-}$ mice, notably, reduced NAC and elevated PFC basal extracellular glutamate content (Szumlinski et al., 2004, 2005a), and an altered capacity of acute cocaine to elevate extracellular glutamate levels within both the NAC (increased in pan-*Homer1*$^{-/-}$; **Table 2**) (Szumlinski et al., 2004) and PFC (decreased in pan-*Homer1*$^{-/-}$) (Lominac et al., 2005; Szumlinski et al., 2005a) that suggest a critical role for Homer1 proteins in regulating presynaptic aspects of glutamate transmission.

Herein, we report that, akin to *Homer2*$^{-/-}$ mice (Szumlinski et al., 2004), pan-*Homer1*$^{-/-}$ mice also exhibit a blunted capacity of the Group1 mGlu receptor agonist DHPG to stimulate glutamate release within the NAC (**Figure 4B**), which might reflect a "de-scaffolding" of mGlu1/5 receptors within neurons and/or astrocytes in this region (Brakeman et al., 1997; Tu et al., 1998, 1999; Buscemi et al., 2017). This being said, *Homer1a*$^{-/-}$ mice also exhibited blunted DHPG-stimulated glutamate release (**Figure 4A**), as well as reduced basal extracellular glutamate content within the NAC (**Figure 5B**). These results were rather unexpected given that prior transgenic work argued a more critical role for CC-Homer1 versus Homer1a expression in regulating extracellular glutamate levels *in vivo* (Lominac et al., 2005), as well as glutamate release *in vitro* (Buscemi et al., 2017) and *Homer1a*$^{-/-}$ mice exhibit *increased* cell surface expression of mGlu5 *in situ* and *in vitro* (Hu et al., 2010). While the source of the extracellular glutamate remains to be determined (i.e., neuronal, glial, or both), the capacity of DHPG to augment NAC glutamate levels in freely moving animals can be blocked by co-infusion of an mGlu1, but not mGlu5, inhibitor (Swanson et al., 2001). By extension then, the failure of both pan-*Homer1*$^{-/-}$ and *Homer1a*$^{-/-}$ mice to exhibit a DHPG-induced rise in NAC extracellular glutamate likely relates to a deficit in mGlu1-dependent, rather than mGlu5-dependent, signaling, with the DHPG phenotype of pan-*Homer1*$^{-/-}$ mice likely reflecting incapacity to induce Homer1a.

DHPG-induced glutamate release within the NAC is also activity-dependent *in vivo* (Swanson et al., 2001). However, while *Homer1* deletion does not alter the extraction fraction index of glutamate clearance/reuptake within the NAC (Szumlinski et al., 2005a), the extraction fraction was lower in *Homer1a*$^{-/-}$ mice than wild-type controls (**Figure 5C**). This difference in extraction fraction was driven primarily by genotypic differences in glutamate flux at concentrations below $y = 0$ (**Figure 5C**), suggesting reduced glutamate release, rather than increased glutamate reuptake in *Homer1a*$^{-/-}$ animals (Parsons and Justice, 1992). Although *Homer1a*$^{-/-}$ mice do not exhibit changes in the frequency of miniature excitatory post-synaptic potentials within cortical pyramidal neurons (Hu et al., 2010), *Homer1a*$^{-/-}$ mice exhibited robust deficits in high K^+-stimulated release of both dopamine and glutamate within the NAC (**Figures 4C,E**), which is a finding in-line with their blunted dopamine response to acute cocaine and their failure to exhibit both dopamine and glutamate sensitization with repeated cocaine treatment (Park et al., 2013; **Figures 8A,B**). In contrast, depolarization-induced glutamate or dopamine release was intact within the NAC of pan-*Homer1*$^{-/-}$ mice, despite their lower extracellular glutamate levels (**Figures 4D,F** and **Table 2**).

TABLE 2 | Comparison of the cocaine phenotype of *Homer1a*$^{-/-}$ versus pan-*Homer1*$^{-/-}$ mice.

Paradigm	Dependent variable	*Homer1a*$^{-/-}$	Pan-*Homer1*$^{-/-}$
Behavior:			
Activity monitoring	Acute: total distance	WT < KO[4,Section 3.9.2]	WT < KO[1]
	Locomotor sensitization	WT > KO[4,Section 3.9.2]	n.d.
Conditioned place-preference	Time on paired side	WT = KO[Section 3.4,3.9.3]	WT (KO1
Neurochemistry:			
Conventional microdialysis	Acute cocaine: dopamine	WT ≥ KO[4,Section 3.10.2]	WT = KO[1]
	Dopamine sensitization	WT > KO[4,Section 3.10.2]	n.d.
	Acute cocaine: glutamate	WT = KO[4,Section 3.10.1]	WT < KO[1]
	Glutamate sensitization	WT > KO[4,Section 3.10.1]	n.d.

Summary of the results of comparable studies examining the effects of Homer1a or Homer1 deletion (KO) upon cocaine-induced changes in behavior and neurochemistry. > Denotes greater response in wild-type (WT) than KO; < denotes less response in WT than KO; = denotes no genotypic difference; ≥ denotes inconsistencies in study outcomes, with reports of either no genotypic difference or greater response in WT than KO mice. Highlighted rows indicate phenotypic similarities between Homer1a$^{-/-}$ *and Homer1*$^{-/-}$ *mice. [1]Szumlinski et al. (2004).*

This result indicates that pan-*Homer1* deletion does not grossly impair the excitability of either dopamine or glutamate terminals within this region – a finding consistent with the fact that cocaine can elevate the NAC levels of both neurotransmitters in *Homer1*$^{-/-}$ mice (Szumlinski et al., 2004). Importantly, AAV-mediated restoration of Homer1a to the NAC of *Homer1a*$^{-/-}$ mice reversed both the anomalies in basal glutamate, as well as the blunted cocaine responsiveness of dopamine and glutamate observed in *Homer1a*$^{-/-}$ animals, while NAC Homer1a over-expression in *Homer1*$^{+/+}$ mice was without any overt effect on our neurochemical measures (**Figure 8**). It remains to be determined whether or not virally mediated Homer1a expression incorporates into the post-synaptic density in a manner akin to endogenous protein to influence neurotransmission. Nevertheless, the present neurochemical data argue that Homer1a induction within the NAC actively regulates, but is not sufficient to augment, basal extracellular glutamate, as well as dopamine and glutamate responsiveness within this region. These data provide confirmatory evidence of a novel role for this IEG Homer1 isoform in regulating at least two neurotransmitters systems within a brain region highly implicated in incentive motivational and attentional processing.

4.4 NAC Homer1a Induction Actively Regulates Cocaine-Induced Behavioral Sensitization But Not Conditioned Reward

As reported previously (Park et al., 2013), the failure of *Homer1a*$^{-/-}$ mice to develop cocaine-induced neurochemical sensitization within the NAC (**Figures 8A,B**) is associated with a lack of locomotor sensitization in these animals (**Figure 7C**). Herein, we extend this prior work by demonstrating that AAV-mediated restoration of NAC Homer1a reversed the sensitization phenotype of *Homer1a*$^{-/-}$ mice, without significantly affecting spontaneous or cocaine-induced changes in locomotor activity within *Homer1a*$^{+/+}$ controls (**Figure 7**). While we previously observed no overt effects of *Homer1a* deletion upon spontaneous locomotion or acute cocaine-induced locomotor-activity (Park et al., 2013), *Homer1a*$^{-/-}$ GFP-controls exhibited blunted novelty-induced locomotion (**Figure 7A**), but greater locomotor activity in response to an acute cocaine injection (**Figure 7B**), which could relate to surgical/AAV procedures employed herein. Nevertheless, the striking parallels between the effects of manipulating Homer1a induction upon behavioral and neurochemical sensitization within the NAC observed to date argue a facilitatory role for Homer1a induction in the development of dopamine/glutamate plasticity that sensitizes drug-induced psychomotor activity.

The capacity of cocaine to augment NAC Homer1a expression (Brakeman et al., 1997; Ghasemzadeh et al., 2009b; Park et al., 2013) is reported to develop tolerance with repeated drug experience (Ghasemzadeh et al., 2009b). However, repeated cocaine exposure also reduces the NAC expression of constitutively expressed Homer1 and Homer2 proteins (e.g., Swanson et al., 2001; Ary and Szumlinski, 2007; Ben-Shahar et al., 2009; Ghasemzadeh et al., 2009a, 2011; Knackstedt et al., 2010; Loweth et al., 2014). A cocaine-induced reduction in the relative expression of CC-Homers is predicted to bias intracellular signaling within the NAC in favor of the dominant negative actions of the IEG isoforms, which we have now confirmed, herein, to be necessary for the development of drug-sensitized behavior. Consistent with this notion, pan-*Homer1* deletion (Szumlinski et al., 2004; Lominac et al., 2005) or anti-sense oligonucleotide-mediated knock-down of Homer1 expression within the NAC augments the acute locomotor-stimulatory effects of cocaine (Ghasemzadeh et al., 2003). Conversely, restoration of the CC-Homer2b isoform to the NAC of cocaine *Homer2*$^{-/-}$ mice (Szumlinski et al., 2004) or over-expression of either Homer1c or Homer2b within the NAC of cocaine-sensitized rats (Szumlinski et al., 2006) prevents their sensitized locomotor and glutamate responses to cocaine. Given the association between polymorphisms in the human *Homer1* with disorders characterized by preservative behaviors (e.g., addiction, autism, and schizophrenia) (Norton et al., 2003; Dahl et al., 2005; Kelleher et al., 2012), it will be important to understand more precisely the mechanisms through which Homer1a modulates the capacity of cocaine, as well as other

TABLE 3 | Comparison of the NAC neurochemical phenotype of *Homer1a*$^{-/-}$ versus pan-*Homer1*$^{-/-}$ mice.

Paradigm	Dependent variable	*Homer1a*$^{-/-}$	Pan-*Homer1*$^{-/-}$
Glutamate no net-flux	x-intercept (basal glutamate)	WT > KO[Section 3.7,3.10.3]	WT > KO[1]
	Slope (release/reuptake)	WT ≥ KO[Section 3.7,3.10.3]	WT = KO[1]
Reverse dialysis	DHPG-stimulated glutamate release	WT > KO[Section 3.5]	WT > KO[Section 3.5]
	K^+-stimulated glutamate release	WT > KO[Section 3.6]	WT = KO[Section 3.6]
	K(-stimulated dopamine release	WT > KO[Section 3.6]	WT = KO[Section 3.6]

Summary of the results of comparable studies examining the effects of Homer1a or Homer1 deletion (KO) upon in vivo measures of basal glutamate function. > Denotes greater response in wild-type (WT) than KO; < denotes less response in WT than KO; = denotes no genotypic difference; ≥ denotes inconsistencies in study outcomes, with reports of either no genotypic difference or greater response in WT than KO mice. Highlighted rows indicate phenotypic similarities between Homer1a$^{-/-}$ *and Homer1*$^{-/-}$ *mice. [1]Szumlinski et al. (2005a); [2]Szumlinski et al. (2004).*

psychotomimetic drugs, to induce neuroplasticity within corticostriatal circuits that underpin enduring psychomotor hyperactivity.

Our prior studies of pan-*Homer1*$^{-/-}$ and *Homer2*$^{-/-}$ mice also demonstrated active and necessary roles for CC-Homer isoform expression within the NAC in regulating more complex forms of drug-induced associative and instrumental learning (Szumlinski et al., 2004, 2005b, 2008b; Lominac et al., 2005; **Table 3**). Indeed, disruption of Homer1-dependent binding within the NAC core subregion prevents both cue- and cocaine-primed reinstatement of drug-seeking (Wang et al., 2013), although AAV-mediated over-expression of either Homer1c or Homer2b within this subregion does not prevent the incubation of cue-elicited cocaine-seeking in rat models of drug self-administration (Loweth et al., 2014). Given the robust effects of *Homer1a* deletion upon cocaine-induced behavioral and neurochemical sensitization within the NAC, we were very surprised by the absolute lack of genotypic differences upon the capacity of cocaine to elicit a conditioned place-preference in mice (**Figures 3**, **7D**). The cocaine doses and place-conditioning procedures employed in this study are sensitive to the effects of other transgenic manipulations of Homer and/or mGlu5 function (Szumlinski et al., 2004; Ary et al., 2013; Park et al., 2013). In fact, the dose-response function for cocaine-induced place-conditioning in both pan-*Homer1*$^{-/-}$, as well as *Homer2*$^{-/-}$, mice is shifted to the left of wild-type controls, indicating that deletion of either CC-Homer isoform increases behavioral sensitivity to cocaine-conditioned reward (Szumlinski et al., 2004). Important for data interpretation, the negative results for place-conditioning were derived from the same *Homer1a*$^{+/+}$ and *Homer1a*$^{-/-}$ that exhibit blunted cocaine-induced behavioral and neurochemical sensitization (AAV-naïve, Park et al., 2013; AAV-infused, **Figures 7**, **8**) and thus, the disparate findings across the different dependent measures cannot reflect cohort effects.

Although both transgenic and pharmacological evidence indicates that a cocaine-conditioned place-preference can develop independent of a dopamine-sensitized state within the NAC (e.g., Szumlinski et al., 2004, 2007), in our experience and to the best of our knowledge, the *Homer1a*$^{-/-}$ mouse is the first example in which the magnitude of drug-conditioned reward in mice is independent of a glutamate-sensitized state within the NAC (e.g., Szumlinski et al., 2005a,b, 2008b, 2017; Penzner et al., 2008). Thus, while increasing NAC glutamate is sufficient to augment the magnitude of drug-induced place-conditioning (e.g., Szumlinski et al., 2017), NAC glutamate hypersensitivity does not appear to be required for this form of drug-related learning. Whether or not *Homer1a* deletion impacts more complex drug-related instrumental learning remains to be determined.

The NAC receives major glutamate input from the PFC and local manipulations of both CC- and IEG-Homer expression can regulate extracellular glutamate, as well as the cocaine responsiveness of glutamate within the PFC (Lominac et al., 2005; Szumlinski et al., 2005a; Ary et al., 2013). In fact, mimicking cocaine-induced changes in the expression of different Homer isoforms within PFC (Ary and Szumlinski, 2007; Ben-Shahar et al., 2009; Gould et al., 2015), by increasing Homer2 expression or reducing Homer1c expression, produces a number of glutamatergic adaptations within the NAC of cocaine-naïve mice that resemble those observed in cocaine-experience rodents (Ary et al., 2013). Importantly, mimicking the cocaine-induced imbalance in CC-Homer isoforms within PFC is also sufficient to potentiate a cocaine-conditioned place-preference (Ary et al., 2013). Conversely, Homer1c over-expression within ventral PFC prevents cocaine-primed reinstatement in a rat self-administration model (Gould et al., 2015). As withdrawal from repeated cocaine augments the glutamate responsiveness to drug-associated cues (Shin et al., 2016) and prior work indicates that *Homer1a* induction is required for normal homeostatic scaling of glutamatergic transmission within cortex (Hu et al., 2010), it will be important in future work to characterize the importance of Homer1a induction for the gating of corticofugal glutamate output from PFC subregions and the executive control the PFC exerts over subcortical structures regulating incentive motivation, affect and learning. It is clear that Homer1a function is complex, strengthening or weakening synaptic connectivity in manner that depends upon on-ongoing cellular activity and the specific intracellular signaling pathways engaged (e.g., Park et al., 2013; Marton et al., 2015). Given the interpretational difficulties associated with studying a constitutive mutant model, new animal models are required that are capable of acute up- and down-regulation of this isoform within specific neuronal and glial populations, to gain a deeper understanding of how Homer1a induction bi-directionally influences synaptic strength of relevance to the etiology of human neuropsychiatric disease.

AUTHOR CONTRIBUTIONS

MCD, MW, CMR, KDL, and KKS conducted the experiments. MW, KL, and KKS analyzed the data. GvJ and MK supplied the AAVs. J-HH and PFW supplied the KO mice. MCD, PFW, and KKS composed the manuscript. All authors edited the manuscript.

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REVIEW
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Immediate-Early Genes Modulation by Antipsychotics: Translational Implications for a Putative Gateway to Drug-Induced Long-Term Brain Changes

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An increasing amount of research aims at recognizing the molecular mechanisms involved in long-lasting brain architectural changes induced by antipsychotic treatments. Although both structural and functional modifications have been identified following acute antipsychotic administration in humans, currently there is scarce knowledge on the enduring consequences of these acute changes. New insights in immediate-early genes (IEGs) modulation following acute or chronic antipsychotic administration may help to fill the gap between primary molecular response and putative long-term changes. Moreover, a critical appraisal of the spatial and temporal patterns of IEGs expression may shed light on the functional "signature" of antipsychotics, such as the propensity to induce motor side effects, the potential neurobiological mechanisms underlying the differences between antipsychotics beyond D2 dopamine receptor affinity, as well as the relevant effects of brain region-specificity in their mechanisms of action. The interest for brain IEGs modulation after antipsychotic treatments has been revitalized by breakthrough findings such as the role of early genes in schizophrenia pathophysiology, the involvement of IEGs in epigenetic mechanisms relevant for cognition, and in neuronal mapping by means of IEGs expression profiling. Here we critically review the evidence on the differential modulation of IEGs by antipsychotics, highlighting the association between IEGs expression and neuroplasticity changes in brain regions impacted by antipsychotics, trying to elucidate the molecular mechanisms underpinning the effects of this class of drugs on psychotic, cognitive and behavioral symptoms.

Keywords: Arc, BDNF, Homer1a, clozapine, haloperidol, schizophrenia, bipolar disorders, cognition

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INTRODUCTION

There is growing interest in unraveling the cellular mechanisms putatively involved in long-term changes in brain architecture and function following antipsychotic administration (Ahmed et al., 2008; Ho et al., 2011; Cannon et al., 2015; Vita et al., 2015; Yue et al., 2016; Emsley et al., 2017). *In vivo* human studies have pointed out that volumetric and functional changes may be detected after

acute antipsychotic treatments (Emsley et al., 2015, 2017). However, the long-term consequences of these acute changes remain still elusive.

Immediate-early genes (IEGs) may represent a significant candidate to explore how acute antipsychotics administration may set the molecular scenario for long-term changes.

New insights in IEGs expression following acute or chronic antipsychotic administration in preclinical models may help to fill the gap between primary molecular responses to antipsychotic administration and putative long-term synaptic changes (**Figure 1**). Recent observations are opening new avenues in our understanding of how antipsychotics work and strongly challenge the old idea that significant changes in synaptic plasticity may be caused by prolonged treatments. Indeed, multiple lines of evidence demonstrated that *in vivo* antipsychotic treatment may significantly impact the architecture of the synapse, as well as the re-arrangement of gene expression of scaffolding and adaptor proteins after acute exposure to the drugs. In line with this view, haloperidol acute administration has been shown to reduce dendritic spines size, possibly through a beta-adducin-mediated mechanism (Engmann et al., 2016). Moreover, the acute administration of typical and atypical antipsychotics has been demonstrated to re-arrange the topography of *Homer1a* gene expression in cortical and subcortical brain regions (Buonaguro et al., 2017b).

Acute antipsychotics administration has been demonstrated to impact signal-transduction pathways in specific brain regions with significant implications for long-term treatment (De Bartolomeis et al., 2013b, 2015a). For instance, acute i.v. infusion at therapeutic doses of haloperidol may trigger changes in the volume of the striatum (Tost et al., 2010), this effect being consistent with the rapid and transient IEGs induction by acute dopamine D2 receptors (D2Rs) blockade.

IEGs activation after antipsychotics acute administration could be pivotal to dissect primary molecular and cellular events that may prime the long-term effects of antipsychotic treatment. Similarly, multiple studies have pointed out the significant functional changes in cortical and subcortical networks after acute administration of antipsychotics (Emsley et al., 2015, 2017).

At the same time, new exciting discoveries in early gene functions have reinvigorated the research on the role of IEGs in the brain, thanks also to novel techniques, such as the following: serial two-photon tomography (STP) for automated whole-brain histology using fluorescent reporters (Ragan et al., 2012); light sheet fluorescence microscopy (LSFM) coupled with tissue clearing for imaging IEG expression in the intact brain (Renier et al., 2016); optogenetics for selectively activate target neurons (Bepari et al., 2012).

Despite the relevance of the issue, the role of IEGs in antipsychotics action has not been reviewed recently and a comprehensive analysis is still lacking.

Herein, starting from the major IEGs proven to be induced by antipsychotics and from their involvement in brain functions believed to be translationally relevant for schizophrenia as well as for antipsychotic mechanism of action, we will review the following issues:

1) IEGs regulation with focus on dopamine-related mechanisms relevant for or related to antipsychotics action;
2) IEGs expression in psychosis and differential modulation by antipsychotics.

Moreover, we will consider:

1) How IEGs induction may impact directly or indirectly synaptic architecture;
2) How IEGs are differentially affected by acute and chronic antipsychotic treatment;
3) How antipsychotics with different receptor profile or the same antipsychotic at different doses may affect the expression of different IEGs with regard of brain topography.

BACKGROUND: ANTIPSYCHOTICS, IEGs, AND BRAIN CHANGES

IEGs Relevance for Synaptic Plasticity

IEGs are a heterogeneous class of genes that are rapidly and transiently activated by a large number of stimuli, including environmental (i.e., light/dark phase changes, exposure to behavioral stressors such as intruder animals, learning session during acquisition tasks), pharmacological, and physical stimuli (Perez-Cadahia et al., 2011; Sauvage et al., 2013). IEGs represent a primary response to cellular perturbation, which is a standing process that is activated at the transcriptional level and occurs in the absence of *de novo* protein synthesis. IEGs are dynamically regulated by different forms of synaptic activity underlying information processing and storage, therefore they are excellent candidates involved in both Hebbian and homeostatic plasticity (Hu et al., 2010; Hayashi et al., 2012; Shin et al., 2012). Several studies demonstrated, indeed, that long-term forms of synaptic plasticity—such as long-term potentiation (LTP)—require new production of intracellular macromolecules, whereas short-term synaptic plasticity processes do not (Kandel, 2001; Hayashi et al., 2012). IEGs expression occurring promptly after stimuli is considered a fundamental step for the establishment of synaptic plasticity, since synaptic plasticity changes may be prevented when mRNA synthesis is blocked early after the induction of a stimulus (Lanahan and Worley, 1998). Thus, IEGs may be considered as "gateway" genes controlling synaptic plasticity and may underlie processes like learning and memory formation.

IEGs encode a large number of proteins with different functions, such as transcription factors (e.g., c-Fos, Egr1, NGFI-B), postsynaptic proteins (e.g., Norbin, Homer 1a, Arc) and signaling molecules (e.g., RSG2, CaMKII).

The induction of an IEG is one of the earliest intracellular mechanism mediating the cellular response to external stimuli (Lanahan and Worley, 1998). According to these view, IEGs induction may be considered a recent activity marker, and its assessment may be used to determine when specific neural populations are activated, making possible to assess the extent of antipsychotics spatial and temporal impact on neural plasticity in different brain areas.

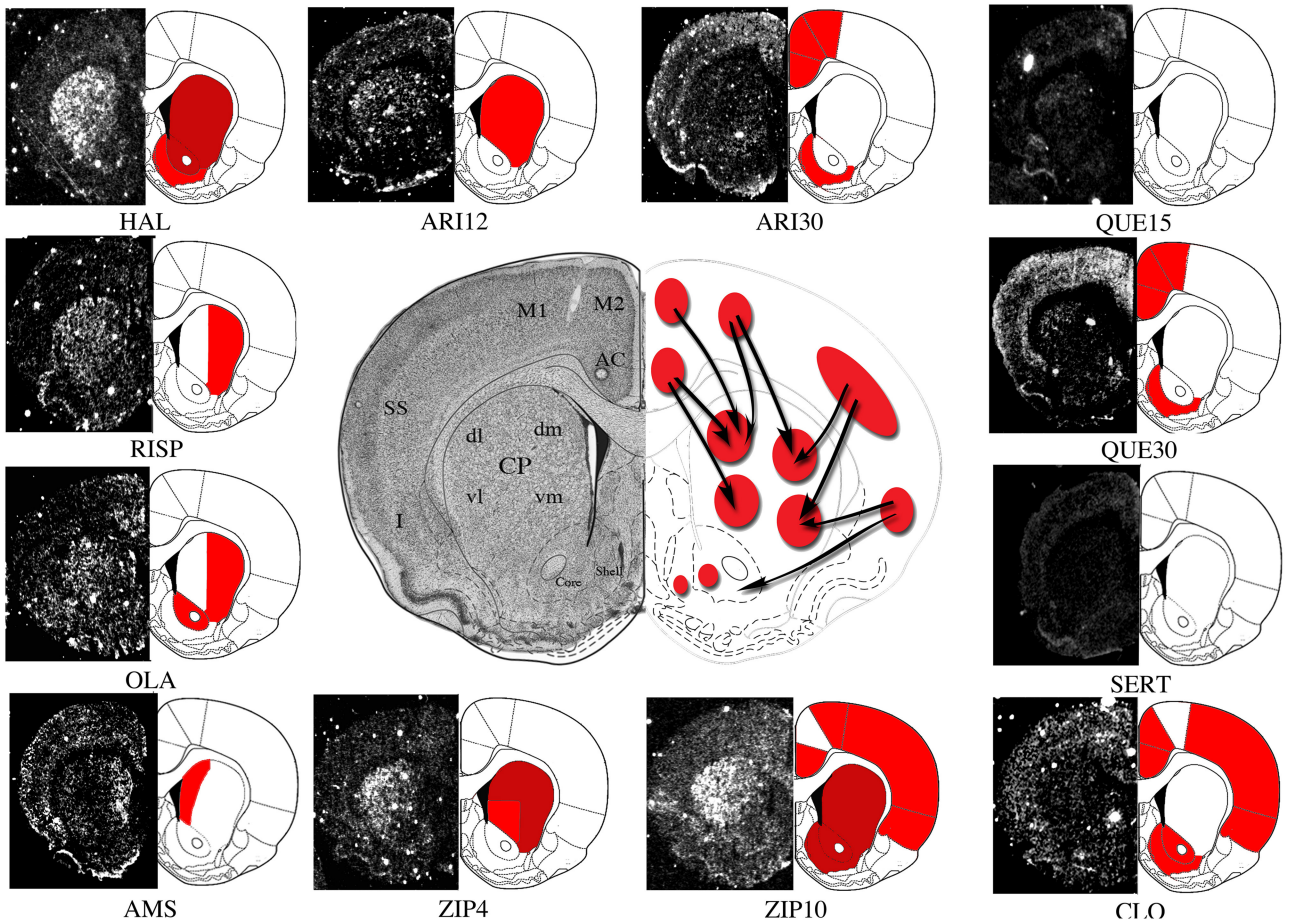

FIGURE 1 | Representative molecular imaging of *Homer1a* IEG expression by acute antipsychotics administration. Molecular imaging of IEGs expression may represent a tool to investigate topographic distribution of antipsychotic-mediated acute and long-term molecular effects within brain Regions of Interest (ROIs). Here we show a representative depiction of *Homer1a* IEG expression by different antipsychotics acutely administered in rodents. The autoradiographic film images of *Homer1a* mRNA detected by means of *in situ* hybridization histochemistry in coronal brain sections have been extracted from different studies carried on by our laboratory (Polese et al., 2002; Ambesi-Impiombato et al., 2007; Tomasetti et al., 2007, 2011; Iasevoli et al., 2009, 2010a,b, 2011; De Bartolomeis et al., 2015a) and representatively placed side by side in order to outline gene expression topography after treatments with haloperidol (HAL), olanzapine (OLA), sertindole (SERT), amisulpiride (AMS), risperidone (RISP), clozapine (CLO), and different doses of ziprasidone (4 mg/kg, ZIP4; 10 mg/kg, ZIP10), aripriprazole (12 mg/kg, ARI12; 30 mg/kg, ARI30), quetiapine (15 mg/kg, QUE15; 30 mg/kg, QUE30). *Homer1a* is a postsynaptic effector of plastic synaptic changes mainly mediated by dopamine and glutamate-dependent signaling pathways. Therefore, in this case, IEG molecular imaging may also provide putative information on antipsychotic-triggered changes in synaptic plasticity. ROIs: AC, Anterior Cingulate Cortex; M2, Medial Agranular Cortex; M1, Motor Cortex; SS, Somatosensory Cortex; I, Insular Cortex; dmCP, Dorso Medial Caudate-Putamen; dlCP, Dorso Lateral Caudate-Putamen; vlCP, Ventro Lateral Caudate-Putamen; vmCP, Ventro Medial Caudate-Putamen; Core, Nucleus Accumbens; Core, Shell, Nucleus Accumbens, Shell. Red, significant gene induction as compared to the respective control ($p < 0.05$); Dark red, significant gene induction as compared to the respective control ($p < 0.001$).

IEGs: A Putative Gateway for Antipsychotic-Induced Brain Changes

Antipsychotic drugs are the mainstay of pharmacological treatment for schizophrenia, and their use has been expanded for the treatment of bipolar disorder and, in some cases, for pervasive disorders of the autistic spectrum (Geddes and Miklowitz, 2013). All antipsychotics share a variable degree of antagonism, or partial agonism, at D2 dopamine receptors (D2Rs) and both therapeutic and motor side effects of either typical or atypical antipsychotic drugs have been proven to directly depend on the occupancy of D2Rs (Seeman, 2002; Ginovart and Kapur, 2012). However, besides the dynamics of D2Rs binding by different antipsychotics, emerging evidence demonstrates that the study of the downstream signaling elicited by these compounds may help to better understand the mechanisms of action implicated in their clinical effects (De Bartolomeis et al., 2013a; Iasevoli et al., 2013). Additionally, dissecting the molecular basis of antipsychotic actions may shed light on new avenues of investigation to bypass the critical issues related to receptor pharmacodynamics, such as D2Rs down- or up-regulation and D2Rs supersensitivity, which have been considered among the potential reasons of antipsychotic treatment resistance (Seeman, 2002; Nnadi and Malhotra, 2007; Seeman and Seeman, 2014; Oda et al., 2015).

In the last decades, several studies on the effects of typical and atypical antipsychotics on brain IEGs expression have been carried out, trying to unravel the bases of regional neuronal response to pharmacological stimuli (Dragunow, 1990; Miller, 1990; Young et al., 1998; Semba et al., 1999; Beaudry et al., 2000; Kovacs et al., 2001; Cochran et al., 2002), as well as to shed light on the molecular mechanisms implicated in antipsychotic actions (Deutch et al., 1991, 1995). Transcriptional fingerprint of IEGs, and their functionally related molecules, has progressively emerged as a potential methodology to explore temporal and functional brain regions recruitment by antipsychotics and psychotomimetic compounds (Gonzalez-Maeso et al., 2003; Tomasetti et al., 2007; Sakuma et al., 2015). Moreover, IEGs have been found to be modulated also by other psychotropic drugs, such as antidepressants (De Foubert et al., 2004; Alme et al., 2007; Molteni et al., 2008; Calabrese et al., 2011), mood stabilizers (De Bartolomeis et al., 2012), as well as by the combination of antipsychotics and antidepressants or mood stabilizers, which has been demonstrated to differentially induce IEGs patterns of expression as compared to the compounds when individually administered (Dell'aversano et al., 2009; Tomasetti et al., 2011).

LITERATURE RESEARCH METHODOLOGY

As a first step, we carried out multiple searches on Pubmed, Scopus, and ISI Web of Knowledge using as a reference the following keywords (we reported in parentheses the results obtained on Pubmed for the search conducted on May 2017): Immediate Early Genes AND brain (2335); Immediate Early Genes AND antipsychotics (84); Immediate early genes AND antipsychotics AND brain (74). Successively, we searched by the name of each single IEG or related gene of interest, together with the keywords "brain" AND/OR "antipsychotics." The name of the IEGs or related genes searched were: Arc/Arg, BDNF, c-fos, fos, c-Jun, jun, Egr1, Delta-fos, Narp1, NPAS-4, Homer1, Homer2, Homer3, Nor1, Nurr, Nurr1, NGFI-B/Nur77, Nerve Growth Factor Inducible-B, NR4A.

A "parallel search" was conducted using as key words the combination the following ones: antipsychotics AND acute effects, antipsychotics AND brain volume, antipsychotics AND cortical thickness, antipsychotics AND acute AND PET, antipsychotics AND acute AND fMRI, antipsychotics AND brain changes.

For the above-mentioned first search (keywords: Immediate Early Genes AND brain), each abstract retrieved was considered for coherence of the subject with the content of the review by two independent co-authors. If the text of the abstract was coherent with the review, the full text was considered and the references double-checked for potential new articles of relevance. All the articles retrieved with the second search (Immediate-early genes AND antipsychotics) and the third search (Immediate-early genes AND antipsychotics AND brain), as well as the articles retrieved by the search for single early gene name, were considered for the full text. The results of the first search where then compared with the result of the other searches and with the one of the "parallel search."

C-*fos*, Δ*FosB*, C-*Jun*: MAPPING THE NEURAL ACTIVITY IN RESPONSE TO ANTIPSYCHOTICS, OLD AND NEW FINDINGS

Synaptic plasticity processes occurring in response to neural activity are mediated by complex programs of gene expression controlled by transcription factors (TFs; Beckervordersandforth et al., 2015; Ortega-Martinez, 2015; Ehrlich and Josselyn, 2016).

Antipsychotics have been demonstrated to differentially impact IEGs encoding neural TFs, thus inducing a significant reprogramming in the expression of genes involved in synaptic plasticity (**Figure 2**; **Table 1**).

C-fos: The Prototypical IEG

C-*fos* Regulation by Dopamine

C-fos is a proto-oncogene encoding for a TF that is induced in response to multiple stimuli, included neural activity (Durchdewald et al., 2009). In resting conditions, the product of the *c-fos* gene, the Fos protein, is expressed in small amounts in the brain.

C-fos transcription may be activated in response to many different extracellular signals, including growth factors and neurotransmitters, such as dopamine. Several early studies demonstrated that transcriptional regulation after dopaminergic stimuli, such as amphetamine/cocaine administration, is a pivotal mechanism by which neurons may respond to environmental adaptations (De Bartolomeis et al., 2013b). The phosphorylation of the cAMP response element binding protein (CREB) is crucial to couple dopamine stimulation to the IEG transcription. Indeed, signals starting at dopamine receptors may promote CREB phosphorylation, which in turn regulates *c-fos* transcription. When translated, the Fos protein may dimerize with members of Jun family in order to start the formation of the Activator Protein-1 heterocomplex (AP-1), which in turn may trigger the expression of genes involved in cell proliferation and differentiation, as well as in activity-stimulated synaptic rearrangements (Herrera et al., 1990).

Based on its fast induction dynamics, *c-fos* expression has been widely used to characterize the different topographic patterns of neural activation following treatments with different antipsychotics (Nguyen et al., 1992; Merchant et al., 1994).

C-*fos* in Schizophrenia and Its Modulation by Antipsychotics

Recent human studies have pointed out that specific polymorphisms of *c-fos* gene may be either negatively or positively associated to schizophrenia, since decreased Fos protein blood levels may be found in schizophrenia patients (Boyajyan et al., 2015), thus reinforcing the possibility of an implication of this IEG in schizophrenia pathophysiology and, possibly, in its treatment.

Early studies observed that typical and atypical antipsychotics may induce different patterns of *c-fos* activation in cortical and subcortical brain regions. Indeed, typical antipsychotics, such as haloperidol, may induce *c-fos* expression in the dorso-lateral

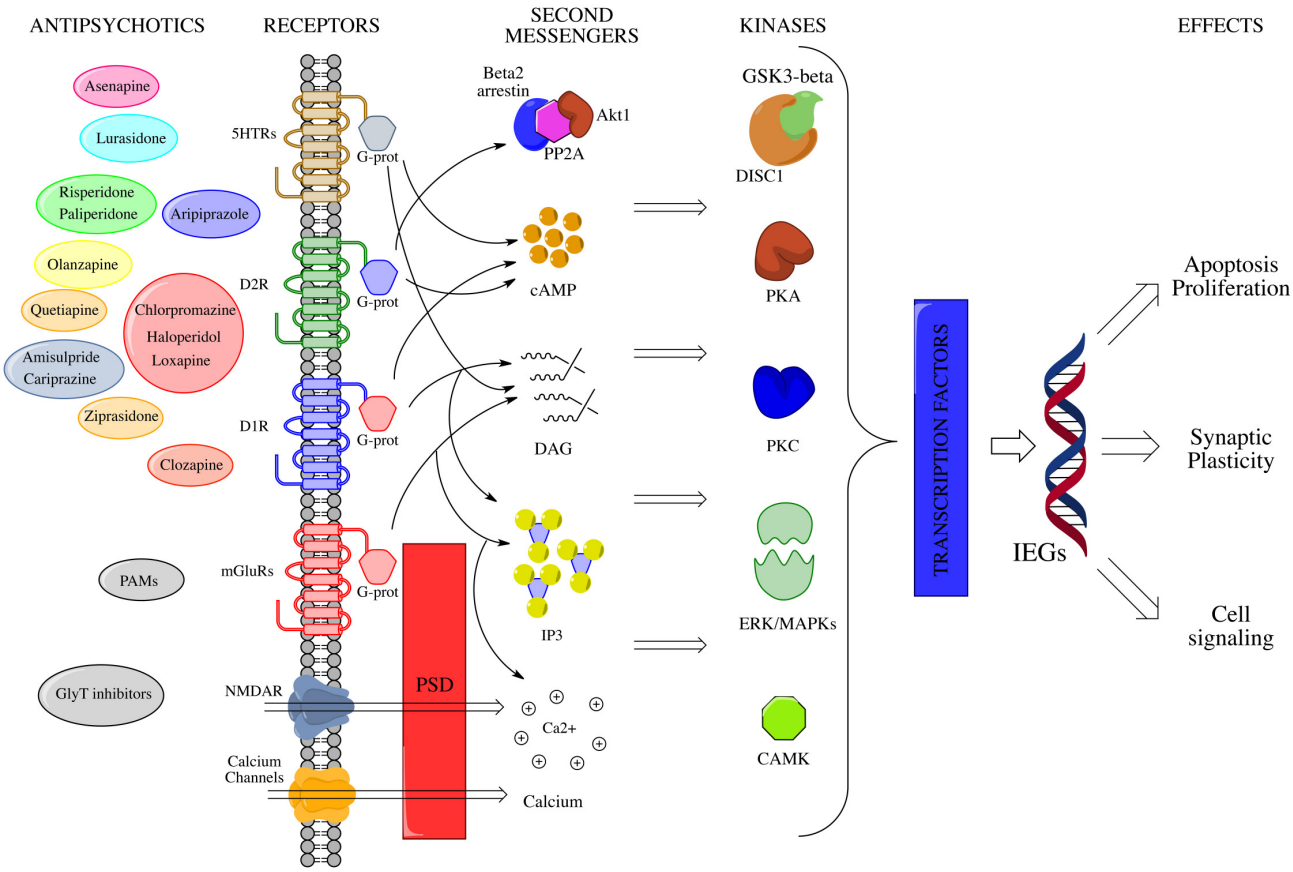

FIGURE 2 | IEGs modulation by antipsychotic drugs. Membrane receptors activate multiple signal transduction pathways, which interact at several sites through the mediation of a large number of second messengers. A crucial role in the post-membrane interaction is played by the PSD, which serves as a physical connection among ionotropic and metabotropic glutamate receptors, and links them to intracellular calcium stores. All these pathways converge in the end to appropriate nuclear targets (i.e., transcription factors, TFs) via specific effectors, largely kinases (e.g., CAMK, MAPKs, PKA etc.), in order to fine modulate long-term activity dependent neuronal rearrangements through changes in IEGs expression levels. On the left side of the picture, antipsychotic compounds are depicted near to the membrane receptors to which they show maximum affinity. NMDAR, N-methyl-D-aspartate glutamate receptor; mGluR1a/5, metabotropic glutamate receptor type 1a/5; D1, dopamine receptor D1; D2, dopamine receptor D2; DAG, diacylglycerol; IP3, inositol 1,4,5-trisphosphate; Akt1, RAC-alpha serine/threonine-protein kinase; PP2A, protein phosphatase 2A; GSK3b, Glycogen synthase kinase 3 beta; DISC1, Disrupted in schizophrenia 1; CAMK, Ca^{2+}/calmodulin-dependent protein kinase; cAMP, cyclic adenosine monophosphate; PKC, protein kinase C; PKA, protein kinase A; MAPKs, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinase; CREB-P, cAMP response element-binding protein; IEGs, immediate early genes; PSD, post-synaptic density; PAMs, positive allosteric modulators of mGluRs; GlyT, glycine transporter.

regions of the striatum, as well as in the nucleus accumbens and in the lateral septum. Atypical antipsychotics, such as clozapine, were found to induce *c-fos* expression in prefrontal cortex and medial striatum (Robertson et al., 1994). Since the dorso-lateral striatum has been implicated in motor control (Balleine and O'doherty, 2010), it has been suggested that the liability of an antipsychotic drug to induce extrapyramidal side-effects (EPSEs) might be predicted by its propensity to induce *c-fos* expression in the motor circuits of the striatum (Robertson and Fibiger, 1996). On the other hand, the induction of *c-fos* expression in prefrontal cortex and limbic striatum by atypical antipsychotics (e.g., clozapine; Robertson et al., 1994) has been potentially correlated with the ability of these compounds to impact, at least in part, brain circuitry implicated in the pathophysiology of negative symptoms of schizophrenia, based on the hypothesis explaining negative symptoms with a potential hypo-frontality in schizophrenia patients (Weinberger and Berman, 1996). However, this observation should nowadays be discussed with caution, considering recent advances in molecular characterization of old and novel antipsychotics, as well as the latest results on the real effect size of atypical antipsychotics on negative symptoms (Kantrowitz, 2017).

Typical and atypical antipsychotics differentially enhance *c-fos* expression in the two histological compartments of the striatum, striosome, and matrix. Indeed, typical antipsychotics induce *c-fos* at a similar extent in the striosome and in the matrix, while most atypical antipsychotics preferentially induce *c-fos* in the striosome (Hiroi and Graybiel, 1996; Bubser and Deutch, 2002). It is noteworthy that no difference in the striosome/matrix ratio (SMR) has been found for typical antipsychotics between dorso-lateral caudate-putamen and dorso-medial caudate-putamen, while clozapine showed a

TABLE 1 | Detection of IEGs expression evaluation in rodents after antipsychotics administration.

Gene	Drug	Effect on gene expression	Brain region	References
c-fos	Acute **Amisulpride**	↑	Medial Striatum	De Bartolomeis et al., 2013b
	Acute High dose **Asenapine**	↑	Striatum, NAc	De Bartolomeis et al., 2015a
	Acute **Clozapine**	↑	NAc, Thalamus, Striatum	Robbins et al., 2008
	Acute **Clozapine**	↑	NAc Shell	Werme et al., 2000; Polese et al., 2002
	Acute **Haloperidol**	↑	NAc Shell and Core, Medial, and Lateral Caudate Putamen, Lateral septum	Robertson et al., 1994; Werme et al., 2000 Robbins et al., 2008 Polese et al., 2002 De Bartolomeis et al., 2015a
	Acute **Haloperidol** or acute **Clozapine**	↑	Anteroventral Thalamus	Cochran et al., 2002
	Acute but not chronic **Risperidone**	↑	Striatum	Robinet et al., 2001
	Transient treatment with **Haloperidol** but not continuous treatment	↑	Striatum	Samaha et al., 2008
	Chronic **Clozapine** (after 6 days of washout)	↑	PFC, FC, NAc Core	Kontkanen et al., 2002
c-jun	Acute **Clozapine**	↑ ↓	NAc Hippocampus	Robbins et al., 2008
	Acute **Haloperidol**	↓	NAc	Robbins et al., 2008
	Chronic **Clozapine**	↑	FC, NAc Shell	Kontkanen et al., 2002
***Nur* family**	Acute **Typical antipsychotics** on Nurr1, Nur77 and Nor-1	↑	Striatum	Maheux et al., 2005
	Acute **Clozapine** on Nor1 and Nurr77	↑	NAc Shell	Werme et al., 2000
	Acute **Haloperidol** On Nor1 and Nurr77	↑	NAc Shell and Core, medial, and lateral Caudate Putamen.	Werme et al., 2000
	Acute **Clozapine** or **Haloperidol** on Nurr77	↑	PFC, cingulate cortex and NAc Shell	Beaudry et al., 2000
	Acute and chronic **Haloperidol** on Nurr77	↑	Lateral striatum	
	Chronic **Haloperidol** or **Clozapine** on Nurr77	↓	Primary Somato-sensory cortex	Langlois et al., 2001
Egr1	Acute **Haloperidol** or **Clozapine**	↑	Striatum, NAc	Nguyen et al., 1992; MacGibbon et al., 1994
	Acute **Haloperidol, Asenapine or Olanzapine**	↑	Striatum, NAc Shell	De Bartolomeis et al., 2015a
	Acute **Lurasidone**	↑	Striatum	Luoni et al., 2014b
	Chronic **Haloperidol**	↑	PFC	Verma et al., 2007
	Chronic low dose **Lurasidone**	↑	PFC, Striatum	Luoni et al., 2014b
	Chronic **Olanzapine**	↓	PFC, Locus Coeruleus	Verma et al., 2006, 2007
Arc	Acute **Asenapine**	↓	PFC	De Bartolomeis et al., 2015a
	Acute high dose **Asenapine**	↑	Striatum, NAc Core	De Bartolomeis et al., 2015a
	Acute **Clozapine**	↓	Thalamus, mPFC, Cingulate cortex	Robbins et al., 2008
	Acute **Haloperidol** or **Olanzapine** or High dose **Amisulpiride**	↑	Striatum	Robbins et al., 2008; Fumagalli et al., 2009; Iasevoli et al., 2010b; De Bartolomeis et al., 2013b
	Acute **Haloperidol**	↑	Striatum, NAc Core and Shell	Polese et al., 2002; Dell'aversano et al., 2009; Iasevoli et al., 2010a,b, 2011; De Bartolomeis et al., 2015a
	Acute **Haloperidol** or **Olanzapine**	↓	PFC	Fumagalli et al., 2009
	Chronic **Haloperidol** or **Olanzapine**	↓	Striatum	Fumagalli et al., 2009
	Acute **Lurasidone**	↑	Hippocampus, Striatum	Luoni et al., 2014b
	Chronic **Aripiprazole**	↑	PFC, Striatum, Hippocampus	Luoni et al., 2014a
	Chronic **Asenapine or Olanzapine or Haloperidol**	↓	PFC	Buonaguro et al., 2017a
	Chronic **Lurasidone**	↑	PFC, Hippocampus, and Striatum	Luoni et al., 2014b
Homer1a	Acute **Asenapine or Olanzapine**	↑	PFC, Lateral Striatum, NAc	Iasevoli et al., 2010a; De Bartolomeis et al., 2015a

(Continued)

TABLE 1 | Continued

Gene	Drug	Effect on gene expression	Brain region	References
	Acute **Clozapine**	↑	NA	Polese et al., 2002
	Acute **Haloperidol**, but not **Clozapine**,	↑	Lateral Striatum	Cochran et al., 2002
	Acute **Risperidone**	↑	Lateral Striatum, NAc	Iasevoli et al., 2010a
	Acute **Sertindole**	↑	PFC	Iasevoli et al., 2010b
	Acute **Ziprasidone**	↑	Striatum	Iasevoli et al., 2011
	Sub-chronic **Amisulpiride**	↑	PFC, Striatum	De Bartolomeis et al., 2016
	Chronic **Haloperidol**	↑	Striatum	Iasevoli et al., 2010b; Buonaguro et al., 2017a
	Chronic **Olanzapine** or high dose **Asenapine**	↑	Striatum	Buonaguro et al., 2017a
BDNF	Acute **Haloperidol**	↓	Thalamus	Robbins et al., 2008
	Acute or chronic **Clozapine**	*nc*	Cortex, Hippocsmpus	Linden et al., 2000
	Acute and chronic **Clozapine** or **Haloperidol**	↓	Hippocampus	Lipska et al., 2001
	Chronic **Aripiprazole**	↓	Hippocampus	Luoni et al., 2014a
	Chronic **Clozapine**	↑	Whole rat brain	Kim et al., 2012; Rizig et al., 2012
	Chronic **Haloperidol** or high-dose **Risperidone**	↓	Hippocampus	Chlan-Fourney et al., 2002; Parikh et al., 2004
	Chronic **Lurasidone**	↑	PFC, Hippocampus	Fumagalli et al., 2012
	Chronic **Olanzapine** or **Clozapine**	↑	Hippocampus	Bai et al., 2003
	Chronic **Quetiapine**	↑	Hippocampus	Park et al., 2006
Npas4	Acute **Lurasidone**	↓	Hippocampus	Luoni et al., 2014b
	Chronic **Aripiprazole**	↑	Dorsal Hippocampus	Luoni et al., 2014a
Narp	Acute **Clozapine**	↓	Striatum	Robbins et al., 2008
	Acute **Haloperidol**	↓	Thalamus	

↑, *Gene expression is up-regulated;* ↓, *Gene expression is down-regulated; NAc, Nucleus accumbens; PFC, Prefrontal cortex, FC Frontal cortex.*

significantly higher SMR in dorso-lateral than in the dorso-medial region of the caudate-putamen (Bubser and Deutch, 2002). Numerous studies have correlated the matrix with motor behavior and stimulus-response memory consolidation, while the striosome has been related to reward mechanisms (White and Hiroi, 1998). Thus, the striosome/matrix architecture of the striatum has been proposed as a morphological substrate for a modular reinforcement-learning model (Amemori et al., 2011). Moreover, it has been suggested that the striosome may be linked to cognition, since it receives prominent inputs from association cortex (Bubser and Deutch, 2002).

Differences in the putative clinical profile of typical and atypical antipsychotics may be inferred by their specific spatial pattern of *c-fos* induction.

D2Rs blockade by antipsychotics has been demonstrated to relieve the inhibition of adenylyl cyclase and activate the PKA, which in turn is responsible for the phosphorylation of CREB. Phospho-CREB may then interact with the cAMP response element (CRE) site in the promoter region the *c-fos* gene (Benito and Barco, 2015). Considering that all the antipsychotics, with few exceptions, are multireceptor-binding drugs, it is conceivable that other receptors beyond D2Rs could be responsible and/or contribute to *c-fos* activation.

Nevertheless, mapping *c-fos* expression to unravel antipsychotics differential functional impact on brain areas has some limitations. In fact, although antipsychotics show specific topographical patterns of *c-fos* induction according to their typical/atypical characteristics, the expression of *c-fos* in neurons has been described to be coupled to multiple different extracellular stimuli, hence it is difficult to attribute a specific *c-fos* "fingerprint profile" to each antipsychotic compound. *C-fos* induction may help to detect where and when a brain area is activated by a certain compound, but it gives little information on which is the specific intracellular pathway stimulated by this compound. Thus, the data obtained by *c-fos* induction in response to antipsychotics need to be integrated with by other IEGs induction with a more direct function in the synapse.

Δ*FosB:* An IEG with a Dual Function

Δ*FosB* is a splicing variant of the *FosB* gene, a member of the FRA family (Fos Related Antigens). Depending on its expression kinetics, the role of ΔFosB can be either of transcription activator or repressor, with lower levels leading to short-term gene repression and higher levels leading to long-term gene activation (Nestler, 2015).

Among the main target genes of Δ*FosB* there are: *metabotropic Glutamate Receptors subtype 2 (mGluR2), Dynorphin,* the *nuclear factor kB (NFkB)* and *c-fos*.

Δ*FosB* gene is rapidly induced in the dorsal striatum and nucleus accumbens dynorphin-expressing Medium-sized Spiny Neurons (MSNs)—two neural populations closely involved into reward and addiction—in response to addictive drugs such as cocaine and amphetamines (Maze and Russo, 2010). Δ*FosB* induction is reported to not undergo tolerance, thus its accumulation in those brain regions is quite stable after repeated administrations. Moreover, unlike other IEGs, Δ*FosB* levels in striatum and nucleus accumbens are quite stable across the time, and remain up-regulated for weeks after the initial stimulus (Nestler, 2005).

Δ*FosB* Modulation by Antipsychotics

Δ*FosB* expression has been also studied in response to antipsychotic drugs. Indeed, chronic treatment with haloperidol may enhance the expression of Δ*FosB* in the ventral, medial, and dorso-lateral aspects of the striatum (Rodriguez et al., 2001). Clozapine, on the other hand, may induce ΔFosB-like immunoreactivity not only in the ventral striatum but also in the prefrontal cortex and lateral septum, with a weaker impact on dorso-lateral striatum, whereas risperidone and olanzapine only weakly induce Δ*FosB* in striatum (Vahid-Ansari et al., 1996; Atkins et al., 1999). Given the long-term standing of Δ*FosB* activation in response to dopaminergic drugs, this IEG has been proposed as a specific marker to identify neurons undergoing prolonged activation in chronic paradigms (Dietz et al., 2014).

C-Jun

C-Jun Regulation and Schizophrenia Preclinical Modeling

C-Jun is a TF that dimerizes with Fos family members to form the AP-1 complex. As an IEG, c-*Jun* plays a pivotal role in neuronal apoptosis and neurons survival (Jochum et al., 2001). Various extracellular stimuli may activate the JNK (c-jun kinase)/C-Jun cascade, including stress, ischemia, and stroke, seizures, learning and memory, axonal injury (Raivich and Behrens, 2006). Interestingly, a potential indirect role for *c-Jun* in a preclinical model of schizophrenia pathophysiology has been recently highlighted by investigating attentive function in mice haploinsufficient for *Map2k7* (*Map2k7*$^{+/-}$ mice). Specifically, *Map2k7* encodes for MKK7 (MAP kinase kinase 7), which is responsible for the activation of JNK. The reduction of *Map2k7* function has been found to be associated to cognitive deficits in mice (Openshaw et al., 2017).

Moreover, putative links between *c-Jun* and schizophrenia are suggested in preclinical models by several findings showing that both psychotomimetic and antipsychotic drugs modulate *c-Jun* levels in brain regions implicated in schizophrenia. Indeed, *c-Jun* expression is affected by N-methyl-D-aspartate (NMDA) receptor antagonists such as MK-801 (Gerlach et al., 2002) mimicking a preclinical model of schizophrenia.

C-Jun Modulation by Antipsychotics

C-Jun expression has been demonstrated to be modulated by antipsychotics. Chronic treatment with clozapine or haloperidol induces long-lasting *c-Jun* expression in the rat forebrain and basal ganglia even after a washout period (Kontkanen et al., 2002). A recent proteomic quantification analysis has demonstrated that chronic haloperidol administration in rodents may modulate the expression of 216 proteins in hippocampus, including c-Jun N-terminal kinase signaling (Schubert et al., 2016). In contrast, acute haloperidol or clozapine treatment have been shown to exert no effects on *c-Jun* expression, although both these treatments produce clear changes in the expression of several other IEGs, including *c-fos* and other Jun-family members (MacGibbon et al., 1994). These data suggest that antipsychotic drugs may play different roles in modulating apoptosis-related molecules, and are coherent with the findings suggesting that typical and atypical antipsychotics may differentially affect putative neuroprotection (Jarskog, 2006; Nandra and Agius, 2012).

Thus, taken together, the findings reviewed until now may suggest that:

1) *c-fos* activation could represent a valuable tool to understand how antipsychotics recruit different brain regions;
2) *c-fos* activation mirrors, with acceptable approximation, the involvement by antipsychotics of motor vs. limbic brain regions based on the different receptor profile of the antipsychotic taken into account;
3) Based on the emerging role of *c-fos* polymorphisms in schizophrenia, it will be of interest to investigate whether and how the association with *c-fos* and related genes may have any causative role in the pathophysiology of the disorder;
4) Δ*FosB* expression is probably of higher interest to explore the long-term effects of antipsychotics action;
5) Despite being less investigated compared to other IEGs, *c-Jun* stands by itself for the recent proteome findings linking its signaling pathway to antipsychotics action in a network fashion.

NUCLEAR RECEPTORS (NUR) SUPERFAMILY: ANTIPSYCHOTICS MODULATION OF DOPAMINERGIC NEURODEVELOPMENTAL FACTORS

The Nur Superfamily: Role in Dopamine System Development and Modulation by Antipsychotics

Nurr1, NGFI-B/Nur77 (Nerve Growth Factor Inducible-B) and Nor1 (neuron-derived orphan receptor-1) are members of the NR4A (Nuclear Receptors 4A) subgroup of nuclear orphan receptors superfamily, which includes a wide variety of TFs, such as retinoid hormone receptor, steroid, and thyroid hormone receptor (Law et al., 1992; Maxwell and Muscat, 2006). All the three Nur members share overlapping sequences and play essential roles in the development of the dopaminergic system. The three members of NR4A subgroup have been described to

respond to several physiological and physical stimuli, such as prostaglandins, stress, hormones, neurotransmitters, membrane depolarization, and magnetic fields (Katagiri et al., 1997; Tetradis et al., 2001; Kagaya et al., 2005) in an IEG-like fashion. Thus, the immediate-early response of *NR4A* genes to environmental stimuli is an essential feature of these nuclear receptors, which has been extensively studied with regard to their correlation with dopaminergic system (Campos-Melo et al., 2013).

Nurr1 is mainly expressed in the central nervous system, especially in midbrain dopaminergic neurons of the substantia nigra and the ventral-tegmental area (VTA; Backman et al., 1999). Several studies have reported that *Nurr1* plays an essential role in the development and differentiation of dopaminergic neurons of the midbrain. Given its role in dopamine neurons development, *Nurr1* has been implicated in neuropsychiatric disorders in which dopamine system is dysfunctional, such as schizophrenia. Recent studies reported that *Nurr1* gene may be considered as a possible candidate to explore the dysfunctional gene-environment interaction that is considered to be at the basis of these disorders. Indeed, two missense mutations in the gene have been found in schizophrenia patients (Buervenich et al., 2000) and may be directly related to their impaired cognitive performances (Ancin et al., 2013).

Heterozygous deletion of *Nurr1* gene in mice has been recently considered as a possible animal model of schizophrenia, since these animal display elevated dopamine levels in basal ganglia (Moore et al., 2008), and characteristic dysfunctional behaviors resembling psychotic symptoms in humans (Rojas et al., 2007).

Similarly, also N*ur77* has been implicated in the pathophysiology of schizophrenia. Indeed, reduced levels of *Nur77* have been detected in prefrontal cortex of post-mortem schizophrenia patients (Xing et al., 2006). Moreover, single nucleotide polymorphisms of *Nur77* gene have been associated with elevated risk of tardive dyskinesia (TD) in schizophrenia patients (Novak et al., 2010).

Given the direct correlation between *Nur* IEGs and the development of dopaminergic system, a large body of evidence has been set up on the regulation of *NR4A* IEGs in response to dopamine manipulation.

Nur IEGs Modulation by Antipsychotics

The first studies with antipsychotics demonstrated that *Nur* IEGs response to these drugs may resemble that of *c-fos* in rat brain. For instance, typical and atypical antipsychotics induce differential patterns of *Nur* IEGs expression. Indeed, acute haloperidol administration pronouncedly increases *Nur77* expression in dorso-lateral striatum, whereas clozapine induces this gene preferentially in prefrontal cortex and in the shell of the nucleus accumbens (Beaudry et al., 2000). Moreover, haloperidol selectively increases *Nur77* dorsolateral striatal expression in enkephalin-containing neurons, which are MSN neurons mostly expressing D2Rs, whose up-regulation has been correlated to extrapyramidal symptoms induced by neuroleptics. In addition, the same report showed that chronic haloperidol administration provokes a further increase in *Nur77* dorso-lateral striatal expression, whereas chronic clozapine reduces *Nur77* gene expression below basal values in prefrontal and accumbal areas. To confirm the role of *Nur77* in acute neuroleptic-induced EPSEs, later studies demonstrated that in *Nur77*-deficient mice haloperidol-induced acute catalepsy was completely abolished, as well as the *Nur77* mRNA overexpression in enkephalin-positive neurons (Ethier et al., 2004). Therefore, similarly to *c-fos* expression, *Nur* IEGs modulation by antipsychotics may be used as a tool to dissect the propensity of a neuroleptic drug to induce extrapyramidal side effects.

Some other significant similarities with *c-fos* have been shown in the regulation of *NR4A* members expression by antipsychotics. Indeed, Maheux and coworkers demonstrated that *Nur* IEGs may be induced by typical antipsychotics selectively in striatal areas that control motor functions, whereas atypical antipsychotics induced *Nur* IEGs expression in limbic areas (Maheux et al., 2005). This induction pattern tightly correlates with D2Rs affinity by each antipsychotic in striatum and with D2/D3Rs affinity in the nucleus accumbens. The same research group further demonstrated that selective serotonergic and adrenergic drugs may modulate haloperidol-induced *Nur* IEGs expression, suggesting that also serotonin neurotransmission may take part into the differential patterns of regulation of these genes by typical and atypical antipsychotics (Maheux et al., 2012).

There are substantial differences in response to antipsychotics between the different members of *Nur* family: both *Nur77* and *Nor1* are *de novo* induced in dopamine neurons and striatal areas, whereas *Nurr1* is basally expressed in VTA and substantia nigra and its expression is enhanced by antipsychotics (Eells et al., 2012).

Regarding the mechanisms involved in *Nur* regulation by antipsychotics drugs and D2Rs antagonists, it has been demonstrated that, at least for *Nur-77* and *Nor-1*, the induction/increase in mRNA are depending by both mitogen-associated and extracellular signal-regulated kinases (MEK) and Protein Kinase C (PKC) in the case of *Nurr-77* and by PKC only in the case of *Nor-1* (Bourhis et al., 2008).

Hence, *Nur* IEGs modulation by antipsychotics appears to provide a complementary information as compared to *c-fos* expression patterns, thereby contributing to shed further light on the impact of these drugs not only on brain areas that are targeted by dopamine neurons, but also on areas in which dopamine neurons localize. Thus, although still elusive, the analysis of *Nur* IEGs modulation by antipsychotic drugs may be a further tool to dissect the mechanisms of action of these compounds on dopamine systems, as well as it may help to further clarify the molecular mechanisms by which these drugs alter locomotor activity in animal models and in humans.

MODULATION BY ANTIPSYCHOTICS OF IEGs INVOLVED IN SYNAPTIC PLASTICITY: PUTATIVE TARGETS FOR COGNITIVE DEFICITS IN PSYCHOSIS

Abnormal synaptic plasticity may account for several cognitive and behavioral processes that are dysfunctional in schizophrenia. Cognitive impairment, indeed, is a striking clinical aspect of psychotic illnesses, is detectable before the onset of other

symptoms, and it is considered among the best predictors of long-term lifetime functioning (Green, 1996).

Since long-term neural plasticity requires protein synthesis, IEGs expression could be considered as a necessary step in synaptic architecture remodeling. For several IEGs involved in these processes, a role in the pathophysiology of psychosis has been proposed. In the following section, we will summarize how antipsychotic therapy may impact these genes expression.

Egr1: How Antipsychotics Impact Synaptic Processes Underpinning Memory and Learning

Egr1 Regulation and Involvement in Synaptic Plasticity

The Egr-family consists of four highly homologous zinc-finger TFs: *Egr1* (Early growth response gene 1, also named NGFI-A, zif-268, Krox 24), *Egr2* (Krox 20), *Egr3* (PILOT), and *Egr4* (NGFI-C).

Egr1 is an immediate-early gene (IEG) coding for a TF and is constitutively expressed in the cortex, amygdala, striatum, nucleus accumbens, hippocampus, and cerebellum (Beckmann and Wilce, 1997). Several stimuli have been demonstrated to induce *Egr1* overexpression in these areas, such as seizures, ischemia, stress, and drug administration (Hughes and Dragunow, 1995). Many of these stimuli share the common feature of elevating intracellular calcium (Ca2+; Ghosh et al., 1994). Pharmacological stimuli provoking massive Ca2+ influx in neurons have been described to increase *Egr1* expression (Shirayama et al., 1999; Zhou et al., 2009; Gangarossa et al., 2011). *Egr1* activity has been related to the transcription of other IEGs involved in synaptic plasticity, above of all *Arc* (Penke et al., 2011). In turn, *Egr1* expression may be under the control of other genes, such as the *Brain-Derived Neurotrophic Factor* (*BDNF*; Robinet and Pellerin, 2011) and other proteins involved in intracellular signaling (Lam et al., 2009).

Egr1 synaptic action has been related to neural plasticity, in particular to synaptic processes leading to memory consolidation and behavioral adaptations (Davis et al., 2003; Okada et al., 2015) Intriguingly, dysfunctions in the postsynaptic machinery deputed to control memory and learning processes have been recently related to cognitive impairment in major neuropsychiatric disorders, such as schizophrenia (Grant, 2012). In paradigms of instrumental learning, *Egr1* is markedly induced in frontal and cingulate cortices (Hernandez et al., 2006; Snyder et al., 2012). Furthermore, *Egr1* plays a pivotal role in maintaining the late phase of LTP in hippocampus, in dorsal caudate-putamen (Gill et al., 2007), and in the retrosplenial cortex (Amin et al., 2006). *Egr1*, along with *BDNF*, also appears to play a role in retrieval-dependent plasticity, a mechanism accounting for the modification of previously consolidated memories being recalled (Lee, 2010). Moreover, it has been shown that *Egr1*, together with *BDNF* (Barnes et al., 2012), *Homer1a* and *Arc*, has a relevant role in the mechanisms of the initial consolidation, reconsolidation and extinction of fear- and anxiety-related memory (Lonergan et al., 2010; Maddox et al., 2011; Cheval et al., 2012).

Dopamine and glutamate systems have been implicated in synaptic processes involved in memory consolidation [e.g., long-term Potentiation (LTP) and long-term depression (LTD)], and several studies reported that *Egr1* expression may be modulated by stimuli affecting either glutamate or dopamine neurotransmission (Li et al., 2016).

Egr1 Modulation by Antipsychotics

Several antipsychotics (e.g., clozapine) modulate synaptic proteins related to memory formation in hippocampus and improve cognitive tasks in animal models of pharmacological NMDA receptor hypofunction (Ozdemir et al., 2012). With regard to dopamine, *Egr1* gene and protein expression are modulated by both acute and chronic antipsychotic treatments in preclinical settings (Wheeler et al., 2014; De Bartolomeis et al., 2015a).

Clinical studies have reported an abnormal regulation of *Egr1* in schizophrenia patients compared to normal controls. Specifically, post-mortem gene expression studies and *in vivo* plasma detection have demonstrated that *Egr1* is down-regulated in prefrontal cortex of schizophrenia patients in a fashion that is directly correlated with decrease in GAD1 (glutamate decarboxylase 1, the enzyme that is responsible for GABA production), and plasma levels were reduced, therefore supporting the view that *Egr1* may be a potential biomarker of the disease (Kimoto et al., 2014). Interestingly, an association of SNPs in *Egr3* and *Arc* with schizophrenia has been proposed as a biological pathway of environmentally responsive, synaptic plasticity-related, schizophrenia risk genes (Huentelman et al., 2015).

Given the potential role of this IEG in schizophrenia pathophysiology and treatment, it is to mention that early studies have also compared *Egr1* modulation by antipsychotics to the modulation of the other well-known IEG *c-fos*, showing that these two IEGs, although similarly impacted by typical and atypical antipsychotics in cortex and striatum, displayed some substantial differences. Haloperidol has been reported to induce both *c-fos* and *Egr1* expression in striatum, whereas clozapine may induce *Egr1* but not c-*fos* expression in the same region. Both antipsychotics may induce the expression of both these genes in nucleus accumbens (Nguyen et al., 1992; MacGibbon et al., 1994).. Subsequent findings further demonstrated a differential response of *Egr1* from *c-fos* also in chronic antipsychotics administration paradigms. *Egr1*, indeed, is robustly down-regulated in locus coeruleus and prefrontal cortex of olanzapine chronically administered rats, whereas *c-fos* expression remains up-regulated (Verma et al., 2007). Moreover, chronic haloperidol may increase cortical *Egr1* expression, whereas it decreases *c-fos* expression in this area (Verma et al., 2007).

Targeted experiments have demonstrated that *Egr1* modulation by antipsychotic drugs may be directly related to the synaptic functions of drug-associated memory consolidation. For instance, high D2R-blocking antipsychotics (e.g., sulpiride) may prevent the increase in *Egr1* expression induced by acute cocaine administration in striatum, but not in the cortex (Daunais and McGinty, 1996). The modulation of *Egr1* and other activity-regulated genes such as *Arc* and *Npas4* has been studied

in rodents after the administration of the novel antipsychotic lurasidone, which is characterized by a multi-receptor profile and particularly by a potent 5-HT7 receptor antagonism, considered beneficial for mood and cognition (Luoni et al., 2014b).

Finally, *Egr1* levels of expression have been recently investigated in preclinical settings exploring new therapeutic strategies in schizophrenia beyond current antipsychotic drugs (Gentzel et al., 2015).

However, it should be considered that the dopaminergic regulation of *Erg1* is even more complicated by the action of multiple pathways in reciprocal interplay with dopaminergic system. Recent evidence, indeed, demonstrated that *mu*-opioid receptors have also a major implication in psychostimulant-induced sensitization (Shen et al., 2010) and antipsychotic drugs seem unable to prevent methamphetamine-induced striatal overexpression of *Egr1* in *mu*-opioid receptors knock-out animals (Tien et al., 2010).

Putting together the findings reviewed therein, the role of *Egr1* in antipsychotics action appears relevant mainly because it is directly implicated in specific signaling and neuronal plasticity programs related to memory, cognition and executive-like functions in preclinical models. This is a major point, since there is a need to explore new compounds in schizophrenia that may address dysfunctions in the domains of cognitive and negative symptoms, which are poorly affected by currently available antipsychotic agents.

Arc/Arg3.1: Modulation of Long-Term Activity-Dependent Synaptic Efficacy by Antipsychotic Treatments

Arc Regulation and Involvement in Synaptic Plasticity: Relevance for Schizophrenia

As other IEGs, *Arc* (activity-regulated cytoskeletal-associated protein) also referred to as *Arg3.1* (activity-regulated gene homolog 3.1), is expressed at low levels in neurons, especially in the hippocampus. However, *Arc* levels are relatively higher in cortex, and are directly linked to NMDA receptor activation (Link et al., 1995). *Arc* shows unique features, since its mRNA may be induced together with other IEGs by neural activation (i.e., single seizures), but differently from other mRNAs—such as *Egr1*—that remain in the neuron soma, *Arc* is rapidly translocated to dendritic spines (Wallace et al., 1998). The activity-dependent translocation of *Arc* requires NMDA receptor activation (Steward and Worley, 2001). Moreover, Arc protein is selectively produced in dendritic spines near the activation site, even in the presence of protein-synthesis inhibitors, thereby indicating that *Arc* mRNA owns a unique intrinsic signal that permits the activity-related targeting to stimulated dendrites (Bramham et al., 2010; Steward et al., 2014). The activity-dependent regulation of *Arc* expression has been extensively studied, thus leading to several findings reporting its involvement in synaptic plasticity and its implication in memory and learning processes, which have been demonstrated to be altered in schizophrenia. Experience-related stimuli may potently increase *Arc* expression in brain areas involved in memory consolidation (Lyford et al., 1995). Indeed, the exploration of new environments strongly induces *Arc* expression in hippocampus and cortex (Vazdarjanova et al., 2002). Consistently, *Arc* knock-out animals fail to form long-term memories for learning tasks, displaying impaired LTP and LTD, but unaltered short-term memory (Plath et al., 2006). A role for *Arc* in synaptic scaling, a form of homeostatic synaptic plasticity, has been postulated (Gao et al., 2010).

Hence, *Arc* functions seem to be required for synaptic plasticity processes starting at multiple neurotransmitter receptors, such as alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), NMDA, dopamine, serotonin, acetylcholine, and adrenaline receptors (Chowdhury et al., 2006; Rial Verde et al., 2006).

Dopamine and glutamate are considered among the principal neurotransmitters implicated in memory and learning processes, as well as in schizophrenia pathophysiology in which aberrant salience has been reported. Dopamine has been demonstrated to be pivotal for working memory in rodents and in non-human primates (Castner et al., 2004; Rinaldi et al., 2007). *Arc* response to memory and learning stimuli may account for a direct involvement of this IEG into dopaminergic-dependent mechanisms of memory consolidation, as well as for its implication in dopamine-glutamate subcellular interactions that control synaptic plasticity processes thought to be dysfunctional in the pathophysiology of schizophrenia (Eastwood, 2004; Grant, 2012; Yin et al., 2012). To confirm the role of *Arc* in synaptic processes of dopamine-dependent memory formation, it has been demonstrated that amphetamines may modulate *Arc* learning-induced expression in hippocampus (Wiig et al., 2009). Bloomer and coworkers have reported a combined effect of NMDA receptors and D1Rs on *Arc* expression in hippocampal neurons (Bloomer et al., 2008), as well as a dramatic reduction in *Arc* expression by dopamine agonists when NMDA receptor blockers are concurrently administrated, thus confirming the role of dopamine-glutamate correct interaction in memory consolidation and the crucial functions of *Arc* in this process. *Arc* gene has been reported to be affected by *de novo* mutations in schizophrenia patients and it is part of an enriched gene set characterized by rare disruptive mutations contributing to the genetic risk for schizophrenia (Fromer et al., 2014; Purcell et al., 2014). Further evidence of an implication of *Arc* in schizophrenia pathophysiology comes from preclinical studies (Manago and Papaleo, 2017). Disruption of *Arc* produces deficits in sensorimotor gating, cognitive functions, social behaviors, and amphetamine-induced psychomotor responses in mice that are reminiscent of some features of psychosis (Manago et al., 2016).

Arc Modulation by Antipsychotics

Arc expression in response to antipsychotic challenges has been extensively investigated, in order to shed further light on the molecular mechanisms involved in antipsychotic-mediated modulation of the synaptic plasticity processes putatively disrupted in schizophrenia.

Early works demonstrated that Phencyclidine (PCP)-induced *Arc* overexpression in prefrontal cortex and nucleus accumbens may be inhibited by pretreatment with clozapine, olanzapine, and risperidone, but not by haloperidol (Nakahara et al., 2000), thus suggesting that *Arc* modulation may be useful to dissect

typical from atypical antipsychotics impact on psychotomimetic drug-induced synaptic dysfunctions. Subsequently, acute administration of both typical and atypical antipsychotics was demonstrated to induce *Arc* gene expression in striatum, with haloperidol showing a more prolonged effect on *Arc* induction than olanzapine (Fumagalli et al., 2009). These results have been directly correlated to the degree of D2R blockade induced by each antipsychotic, since a selective D2Rs antagonist, such as raclopride, may induce *Arc* striatal expression and reduce its cortical expression, whereas a selective D2Rs agonist, such as quinpirole, may reduce *Arc* striatal expression and has no effects on its cortical expression. Notably, Fumagalli et al. (2009) further demonstrated that a prolonged treatment with haloperidol and olanzapine markedly reduced *Arc* striatal expression, as well as only olanzapine may reduce the expression of the gene in the cortex.

With regard to cortical and subcortical expression of *Arc*, work from our laboratory demonstrated that *Arc* gene expression may be induced by haloperidol, but not by sertindole in the striatum, thus further suggesting that *Arc* modulation may be tightly related to the tuning of dopamine neurotransmission exerted by each antipsychotic (Iasevoli et al., 2010b). Sertindole shows a milder D2Rs impact than haloperidol in the striatum, with a quite absent blockade of D2 autoreceptors (Valenti and Grace, 2010). The involvement of serotonin 5-HT2A receptors has been demonstrated in *Arc* cortical modulation, with NMDA receptor function being relevant in these effects, thereby suggesting a crucial role of *Arc* in synaptic rearrangements induced by combined serotonin-dopamine-glutamate stimuli in the cortex by antipsychotics (Pei et al., 2004).

Arc has been shown to be responsive to acute (with significant increase of the transcript in striatum) and chronic (with prevalent gene expression increases in prefrontal cortex and hippocampus and decreases in striatum) lurasidone treatment, suggesting a region-specific fingerprint of *Arc* induction (Luoni et al., 2014b). An intra-striatal specificity of *Arc* activation was detected after acute administration of amisulpride (35 mg/kg) with prevalent increase of the transcript in the medial caudate-putamen compared to more pronounced induction in dorsal caudate-putamen by haloperidol (0.8 mg/kg; De Bartolomeis et al., 2013b). Again, a region-specific induction of Arc protein was detected in the shell of the nucleus accumbens by clozapine (20 mg/kg) compared to haloperidol (1 mg/kg; Collins et al., 2014). Finally, *Arc* has been instrumental also for exploring the brain region effect of innovative treatment approaches in schizophrenia, such as augmentation strategies to antipsychotics (i.e., minocycline in combination with haloperidol; Buonaguro et al., 2017b).

Direct evidence exist that *Arc* induction can be responsible for increasing the density and for reducing the width of dendritic spine possibly by a mechanism involving AMPA endocytosis (Peebles et al., 2010).

These data globally suggest that *Arc* is involved in the neural plasticity mechanisms induced by antipsychotics in distinct brain regions. *Arc* modulation may be demonstrated to occur before the timing necessary to observe the therapeutic-like effects commonly observed during antipsychotic therapies, possibly suggesting that *Arc* is potentially relevant in establishing the correct synaptic rearrangements underlying antipsychotic effects. Finally, the recent discovery of *Arc* subdomains similar to the domain of HIV capsid and its involvement in rapid synaptic functions (possibly derived from the ancestral viral origin) deranged in schizophrenia (Zhang et al., 2015), make this IEG of relevant interest for studying specifically "fast" synaptic changes during antipsychotic treatment.

Homer1a: IEG-Mediated Activity-Dependent Postsynaptic and Architecture Rearrangements in Response to Antipsychotic Treatment

The *Homer* Family and Its Regulation by Dopamine-Glutamate Interaction

Homer genes encode a family of scaffolding proteins (Homer1, Homer2, Homer3) localized mainly at the glutamatergic postsynaptic density (PSD) of dendritic spines, where they act as multifunctional adaptors among multiple transduction pathways. *Homer1* gene encodes both constitutively expressed long transcripts (*Homer1b/c*) and for a short isoform named *Homer1a*, which is induced in an IEG-like fashion (Bottai et al., 2002). Within the PSD, Homer proteins couple to metabotropic and indirectly ionotropic glutamatergic receptors, bridging both to intracellular receptors, such as the inositol 1,4,5-trisphosphate receptor (IP3Rs), the ryanodine receptor (RyR), and to other PSD scaffolding proteins, such as Shank (De Bartolomeis and Iasevoli, 2003; Gao et al., 2013). When induced, Homer1a protein disassembles constitutive Homers clusters by acting as a "dominant negative," thus modifying synaptic architecture and Ca2+ homeostasis (Shiraishi-Yamaguchi and Furuichi, 2007). Several studies demonstrated a pivotal role of *Homer1a* in modulating the crosstalk between PSD proteins involved in mechanisms underlying synaptic plasticity, such as receptor localization, distribution and internalization (Iasevoli et al., 2013). For example, it has been observed that *Homer1a* is rapidly up-regulated during enhancement of network activity and promotes the agonist-independent signaling of group I mGluRs that may in turn scale down the expression of AMPA receptors (Hu et al., 2010). To note, in animal models, impaired homeostatic scaling has been reported in a NMDA receptor-blocking experimental paradigm, which mimics psychotic states (Wang and Gao, 2012). Based on the crucial role of *Homer1a* in synaptic plasticity, dysfunctions in its fine-tuning activity have been closely related to psychiatric disorders (Luo et al., 2012). *Homer1* polymorphisms have been associated with schizophrenia (Spellmann et al., 2011) and cocaine addiction (Dahl et al., 2005) and *Homer1* knock-out mice exhibit a behavioral phenotype resembling psychotic disorders (Szumlinski et al., 2005), as well as Homer2 proteins have been implicated in regulating addiction to cocaine in animal models (Szumlinski et al., 2004). A fine-tuned modulation of *Homer1a* expression has been associated to a number of mechanisms of adaptation to different environmental and pharmacological stressors: for instance, *Homer1a* overexpression in cortical structures may facilitate the ability to cope with stress (Szumlinski et al., 2006). Finally,

Homer1 gene variants have been associated with neuropsychiatric disorders such as psychosis in Parkinson disease (De Luca et al., 2009), major depression pathophysiology (Serchov et al., 2016), and response to lithium treatment (Benedetti et al., 2018).

Homer1a Modulation by Antipsychotics

Dopamine indirect agonists, such as cocaine or amphetamines, may induce *Homer1a* expression in striatum and nucleus accumbens with peculiar patterns of expression (Yano and Steiner, 2005; Zhang et al., 2007). Moreover, although the acute administration of cocaine may induce strong *Homer1a* expression in cortico-striatal circuits, these effects are abolished after 2 or 3 weeks of withdrawal (Ghasemzadeh et al., 2009), suggesting a crucial role of *Homer1a* in cocaine-mediated synaptic plasticity. Specific studies demonstrated that dopamine agonists-dependent *Homer1a* induction is regulated by selective activation of D1Rs but not D2Rs (Yamada et al., 2007). Recent findings by our group have demonstrated that this IEG may be differentially induced by antipsychotics, with a peculiar pattern of expression depending on the degree of D2R blockade by each compound and on the selective brain area in which each antipsychotic exerts its functions (Tomasetti et al., 2007). Haloperidol has been demonstrated to induce *Homer1a* expression specifically in dorso-lateral regions of caudate-putamen and in the core of the nucleus accumbens, a feature that is consisting with the propensity of this compound to provoke EPSE in humans at high dosages (Ambesi-Impiombato et al., 2007). By contrast, atypical antipsychotics (i.e., aripiprazole, clozapine, olanzapine, quetiapine, ziprasidone) preferentially induce *Homer1a* gene expression in ventro-medial regions of caudate-putamen and in the shell of the nucleus accumbens, wh1ich are brain regions implicated in the control of reward and motivated behavior (Tomasetti et al., 2007; Iasevoli et al., 2009). It is worthy to note that each antipsychotic compound has been described to induce a specific pattern of *Homer1a* expression that is tightly related to its degree of D2R blockade, being this latter essential in order to stimulate *Homer1a* induction (Iasevoli et al., 2011). *Homer1a* has been shown to be induced differentially also by the administration of the same antipsychotic at different doses: for example, it has been demonstrated that increasing doses of haloperidol not only increase the intensity (i.e., higher autoradiographic signal level) of gene expression in brain regions originally activated by the same drug at lower doses, but also induce the expression of the IEG in new brain regions (i.e., ventral caudate; De Bartolomeis et al., 2015a). Moreover, *Homer1a* modulation has been described in cortical areas only by antipsychotics that may impact serotonergic neurotransmission (Iasevoli et al., 2010a,b). Recent studies, indeed, demonstrated that cortical *Homer1a* induction by antipsychotics may resemble that by selective serotonergic agents, and when co-administered, haloperidol plus a selective serotonergic reuptake inhibitor antidepressant (SSRI, i.e., citalopram or escitalopram) may induce a pattern of *Homer1a* cortical expression tightly resembling the pattern by atypical antipsychotics (Dell'aversano et al., 2009; Serchov et al., 2016). Further evidence demonstrated that *Homer* genes may be involved also in synaptic rearrangements induced by combined mood-stabilizing/antipsychotic treatment (Tomasetti et al., 2011) and in switching between antipsychotics (De Bartolomeis et al., 2016). In sum, regarding the mechanism by which antipsychotics increase *Homer1a* and in turn may modify dendritic spine, D2Rs antagonism or partial agonism is the major candidate, possibly with a mechanism CRE-related, even if other pathways (i.e., ERK-related) can also be involved. *Homer1a* induction may have a pivotal role in remodeling the dendritic spine, modifying the availability of the constitutive isoform (*Homer1b*/*c*) that is involved in a transient spine increase that is eventually followed by more persistent modification by recruitment of other postsynaptic proteins such as PSD-95 (Meyer et al., 2014).

Altogether, these data confirm the role of *Homers* in the fine modulation of synaptic processes triggered by psychotropic drugs also when co-administered with antidepressant or mood stabilizers, posing the bases for further understanding the molecular correlates of real-world clinical psychopharmacology. The pattern of *Homer* inducible isoform expression may therefore provide a specific "fingerprint" profile of psychopharmacologic treatments, which could be a useful tool for elucidating glutamate-dopamine interactions putatively dysfunctional in schizophrenia pathophysiology.

BDNF: Neurotrophic Control of Synaptic Plasticity by Antipsychotic Treatment

BDNF Activity-Dependent Modulation and Synaptic Plasticity

BDNF belongs to a subfamily of neurotrophins that includes the nerve growth factor (NGF), the neurotrophin-3 (NT3), and the neurotrophins 4 and 5 (NT4/5). Several studies have demonstrated that neuronal activity, or in general stimuli that increase intracellular levels of Ca2+, may induce BDNF expression in neurons (Aicardi et al., 2004; Aid et al., 2007). Specifically, exon IV transcription seems to be directly controlled by neural activation (Chen et al., 2003). Moreover, the rapid activity-dependent increase in *BDNF* mRNA after a stimulus and its independence from the most common TFs (such as AP-1), have suggested that *BDNF* may be rather considered a "secreted IEG," because of its immediate-early response fashion that does not involve new protein synthesis (Lauterborn et al., 1996; Xu et al., 2000; Gartner et al., 2006). The activity-dependent modulation of BDNF, as well as the BDNF-dependent master control of synaptic functions, has increased the attention on this molecule in synaptic plasticity. Several reports, indeed, demonstrated that BDNF plays a crucial role in both early and late phases of hippocampal LTP (Pang and Lu, 2004; Rex et al., 2006; Yano et al., 2006), as well as suggest a pivotal role of BDNF also in long-term memory processes (Lu et al., 2008; Waterhouse and Xu, 2009). *BDNF* exerts also a regulatory role on other IEGs expression: *Arc* has been found to be a key molecular effector of *BDNF* action in synaptic plasticity since its expression is necessary for stable LTP formation after BDNF levels increase in both *in vivo* and *in vitro* experiments (Messaoudi et al., 2007; Wibrand et al., 2012; Panja et al., 2014).

As in the case of the IEGs considered before, several studies reported a direct correlation between dopamine

neurotransmission and *BDNF* functions. Indeed, *BDNF* null mice have been demonstrated to display a reduced number of dopaminergic neurons in the substantia nigra (Baquet et al., 2005). Moreover, *BDNF* is crucial for a correct D3Rs expression in nucleus accumbens, and thereby it seems involved in pathological conditions in which these receptors have been reported as dysfunctional, such as Parkinson's disease or antipsychotic-induced TD (Guillin et al., 2001; Zai et al., 2009).

BDNF, Schizophrenia, and Modulation by Antipsychotics

Given the tight association of *BDNF* functions with activity-dependent dopamine-mediated synaptic plasticity, it is not surprising that several studies have highlighted the role of *BDNF* in the pathophysiology of neuropsychiatric disorders in which dopamine-glutamate interaction is dysfunctional, such as schizophrenia. The rs6265 single nucleotide polymorphism (SNP)—which leads to the Val66Met substitution at codon 66—has been reported to alter the activity-dependent trafficking and release of *BDNF* in neurons (Chen et al., 2004). This SNP has been associated with schizophrenia in Chinese and Caucasian populations (Hong et al., 2003; Neves-Pereira et al., 2005; Chen et al., 2006). Moreover, SNP homozygotes display reduced hippocampal cortex (Takahashi et al., 2008). In post-mortem studies, a decrease in BDNF levels in frontal cortices (Weickert et al., 2003) and an increase in hippocampus have been reported in schizophrenia patients (Iritani et al., 2003). Moreover, in the assessment of 825 patients for Positive and Negative Syndrome Scale in a single marker analysis, the *BDNF* rs10835210 mutant A allele was significantly associated with schizophrenia. Haplotype investigation detected higher frequencies of haplotypes with the mutant A allele of the rs10835210 in schizophrenia patients than in controls (Zhang et al., 2016). In addition, schizophrenia patients showed lower basal serum levels of BDNF as compared to healthy subjects (Grillo et al., 2007; Green et al., 2011; Fernandes and Chari, 2016).

Association studies have further suggested that *BDNF* could be involved in both susceptibility to schizophrenia and in clinical symptom severity. Regarding the role of *BDNF* in the onset and evolution of psychosis, it is of interest that the expression of the two forms of BDNF receptors (active TrkB-FL and inactiveTrkB-T1) in Peripheral Blood Monocyte Cells (PBMCs) of first episode psychotic patients showed modifications according to the trajectory of the disease, with TrkB-FL expression increasing by 1 year after diagnosis and TrkB-T1 expression decreasing. Notably, the TrkB-FL/TrkB-T1 ratio increased in the non-affective psychosis group only (Martinez-Cengotitabengoa et al., 2016). Multiple studies have assessed the effects of antipsychotic treatments on *BDNF* expression in preclinical models, as well as of BDNF serum levels in treated schizophrenia patients. Early studies demonstrated that the acute blockade of NMDA receptors may decrease *BDNF* expression in hippocampus and cortical areas, whereas it may increase its expression in limbic cortex (e.g., entorhinal cortex), and these effects may be not reversed by the administration of haloperidol (Castren et al., 1993). Further studies have confirmed the enhancement of *BDNF* expression in entorhinal cortex by NMDA receptor-blocking drugs (i.e., MK-801), these effects being contrasted by a pre-treatment with haloperidol or clozapine (Linden et al., 2000). However, the sole acute or chronic clozapine treatment did not affect *BDNF* mRNA levels (Linden et al., 2000). Starting from the assumption that antipsychotic treatment could be correlated to neurotrophic actions in brain areas affected in schizophrenia, Angelucci et al. (2000) reported that chronic treatment with haloperidol or risperidone may decrease *BDNF* expression in hippocampus, frontal and occipital cortices, also affecting TrkB expression in these areas.

Typical and atypical antipsychotics have differential impact on *BDNF* expression in distinct brain areas. Chronic haloperidol administration may strongly decrease *BDNF* hippocampal expression, whereas clozapine and olanzapine have been demonstrated to enhance *BDNF* expression in the same areas, probably due to 5HT2A receptor modulation by these atypical antipsychotics (Bai et al., 2003). Moreover, olanzapine has been successively demonstrated to normalize *BDNF* hippocampal levels that were reduced by MK-801 administration (Fumagalli et al., 2003). Switching from haloperidol or chlorpromazine to olanzapine, even after a prolonged treatment, may restore *BDNF* brain level that have been decreased by the previously administered typical antipsychotics (Parikh et al., 2004; Pillai et al., 2006).

Park et al. (2009) reported that ziprasidone, but not haloperidol, may attenuate the decrease in *BDNF* expression induced by immobilization stress in rats. Aripiprazole, a partial agonist at D2/D3Rs and a functional selective antipsychotic (De Bartolomeis et al., 2015b), has been shown to up-regulate *BDNF* compared to haloperidol in cell cultures (Park et al., 2009). In early clinical studies, clozapine-treated schizophrenia patients showed higher serum BDNF levels than risperidone-treated patients (Tan et al., 2005). With regard to clinical translation it has been demonstrated that the increase in BDNF serum levels in olanzapine-treated schizophrenia patients may directly correlate with the progressive reduction in positive symptoms (Gonzalez-Pinto et al., 2010).

Regarding the role of BDNF in dendritic spines modulation, is remarkable that multiple lines of evidence point to a brain and cell region specificity of BDNF action.

Specifically, has been shown that BDNF increase in cortical regions may reduce the density of dendritic spines of pyramidal neurons, whereas an increase has been reported for hippocampal pyramidal neurons (Alonso et al., 2004). This finding, considered in the light of antipsychotics modulation of BDNF, could represent a significant morphological underpinning of the association between antipsychotics and changes in brain architecture.

It is questioned if a common ERK-dependent mechanism is involved in the opposite changes observed in cortex and hippocampus.

In summary, the study in preclinical and clinical settings of *BDNF* response to antipsychotics may help to provide further information on the differential impact of typical vs. atypical antipsychotics on neurons survival and neurogenesis, as well as on putative neurodegenerative mechanisms of dopaminergic systems involved in the pathophysiology of schizophrenia.

BDNF role as an immediate-early-like gene, TF, and growth factor makes this molecule an exceptional candidate for the investigation of long-term antipsychotic effects on brain structure and function, and the study of the regulation of its expression could provide a molecular tool to predict clinical outcomes of antipsychotic response (Nandra and Agius, 2012).

IEGs RELATED TO GLUTAMATE DEPENDENT PLASTICITY: ANTIPSYCHOTIC TREATMENT EFFECTS ON *NPAS* 4 AND *NARP* EXPRESSION

Npas4 and *Narp* are IEGs related to glutamate and γ-aminobutyric acid (GABA) neurotransmission, whose implication in schizophrenia pathophysiology and treatment to date has been explored only by few clinical and preclinical studies.

Npas4: An IEG Selectively Induced by Neuronal Activation

Npas4 is a TF that belongs to the basic helix-loop-helix-PAS protein family (Moser et al., 2004; Shamloo et al., 2006) that is transcribed in response to excitatory synaptic activity induced in both excitatory and inhibitory neurons. *Npas4* is expressed almost exclusively in neurons, it is activated selectively by neuronal activity, and has been demonstrated to control directly the expression of a large number of activity-dependent genes (Coutellier et al., 2012). Recent evidence shows that *Npas4* has a regulatory function on the expression of multiple cortical GABAergic markers and that animals null for *Npas4* show a decrease in GAD67 and parvalbumin, which can be reverted after the administration of valproic acid (Shepard et al., 2017). Moreover, *Npas4* has been involved in stress response and linked to the onset of resistance to L-acetyl carnitine in mice (Bigio et al., 2016). Particularly, this IEG has been shown to regulate the balance between neuronal excitation and inhibition by contributing to the maintenance of the inhibitory pathways. This balance is believed to be pivotal for processing sensory information and for cognitive functioning, while an imbalance between inhibitory and excitatory synapses has been associated with multiple developmental disorders such as schizophrenia. Not surprisingly, it has been recently published the first study that investigated *Npas4* expression after antipsychotic administration in rodents. Indeed, the IEG has been demonstrated to be down-regulated acutely, but not chronically, in the cortex by the novel antipsychotic lurasidone (10 mg/kg) at the dose demonstrated to be effective in animal models of schizophrenia (Luoni et al., 2014b).

Narp: An IEG Secreted by Pyramidal Neurons

Narp (Neuron activated regulated pentatraxin) is an AMPA receptor binding protein with the peculiarity to be secreted by pyramidal neurons onto parvalbumin interneurons and whose gene is rapidly transcribed and regulated by physiological synaptic activity (O'brien et al., 1999, 2002; Chang et al., 2010; Lee et al., 2017). Functional studies suggest that Narp promotes neuronal migration and dendritic outgrowth with a potency comparable to neurotrophins and growth factors (Tsui et al., 1996; Doyle et al., 2010). *Narp* is a direct transcriptional target of BDNF. Intriguingly, acute BDNF withdrawal may promote downregulation of *Narp*, whereas transcription of *Narp* is greatly enhanced by BDNF (Mariga et al., 2015). Furthermore, it has been demonstrated that BDNF directly regulates *Narp* to mediate glutamatergic transmission and mossy fiber plasticity (Mariga et al., 2015). Hence, *Narp* serves as a significant epistatic target of BDNF to regulate synaptic plasticity during periods of dynamic activity. Recently, a close association between *Narp* expression and schizophrenia pathophysiology has been suggested. In a post-mortem study conducted on brain specimens ($n = 206$) from schizophrenia, bipolar disorder, and major depressive disorder patients, *Narp* transcript expression was measured at the level of the dorsolateral prefrontal cortex. A significant 25% reduction of *Narp* mRNA expression was detected in schizophrenia patients compared to normal controls (Kimoto et al., 2015). Moreover, as in the case of *Npas4*, the expression of *Narp* after antipsychotic administration has been explored only in one study to date. Indeed, the IEG has been demonstrated to be differentially regulated by haloperidol (1 mg/kg i.p.) and clozapine (20 mg/kg i.p.) in cortical and subcortical rat brain regions Particularly, it has been shown that clozapine causes a specific decrease of *Narp* in the striatum (Robbins et al., 2008).

In summary, *Npas4* and *Narp* share peculiar IEGs characteristics, the first one being expressed exclusively in neurons, and the second one being specifically secreted onto pyramidal cells. Both are deeply linked to glutamatergic and GABAergic functions. All the above-mentioned features make these early genes potential great players both in schizophrenia pathophysiology and in antipsychotic mechanisms of action at the intracellular level. Therefore, further studies are required in order to better clarify their putative specific roles in the disease development and treatment strategies.

CONCLUSIONS

IEGs May Set the Scenario for Acute and Long-Term Changes Induced by Antipsychotics

Antipsychotic agents are the mainstay of treatment in schizophrenia and in other psychotic disorders. However, despite half a century of research, their ultimate molecular actions and the neurobiological mechanisms beyond D2R occupancy are still elusive. *In vivo* human studies have shown that volumetric and functional changes may occur after chronic antipsychotic treatment and that some changes may be detected even after acute antipsychotic administration. Notably, schizophrenia has been considered a disease of synaptic plasticity and of dendritic spines (Penzes et al., 2011), and it is conceivable that antipsychotics exert their action by triggering a complex set of structural and functional modifications, also at the level of dendritic spines (De Bartolomeis et al., 2014).

Tracking down the initial wave of molecular changes from multiple receptor interactions to dendritic spines modifications is a challenging task. In this context, IEGs may represent ideal candidates to explore how antipsychotics may set the scenario for acute and chronic re-arrangements of genes expression at the synapse.

This essential role of IEGs may be justified by multiple reasons:

1) IEGs control the early molecular processes of rapid synaptic plasticity induced by antipsychotics, which do not always require *de novo* protein synthesis.
2) IEGs are pleiotropic molecules that, beyond the common feature of being activated rapidly by diversified stimuli, are characterized by differential and specific functions spanning from transcription modulation (i.e., *c-fos*), to neurotrophic action (i.e., *BDNF*), to regulation of scaffolding proteins at the PSD (i.e., *Homer1a*), all of which have been demonstrated to be induced by antipsychotics.
3) IEGs are strongly involved in complex higher functions affected by antipsychotics in key brain regions implicated in memory and learning (i.e., prefrontal cortex), as well as in reward and volition (i.e., limbic circuits), all of which have been demonstrated to be altered in schizophrenia (Leber et al., 2017).
4) IEGs expression may vary in response to antipsychotics, with a differential level of expression related to the receptor-binding profile (i.e., antipsychotics with prevalent dopaminergic activity vs. antipsychotics with more complex receptor profiles), to the dose, and to the duration of the treatment.
5) Different antipsychotic compounds have been demonstrated to induce specific patterns of IEGs expression in peculiar brain areas implicated in schizophrenia pathophysiology (Matosin et al., 2016).

Future Steps toward a Further Clarification of IEGs Modulation by Antipsychotics

Despite the lot of findings that highlight the complex modulation of IEGs by antipsychotics, the road for taking full advantage of this class of molecules in better understanding antipsychotics mechanisms of action is still long-lasting.

Here are listed few points that may make the issue progress:

1) It would be relevant to develop a research strategy to track down IEGs as putative blood biomarkers for antipsychotics activity under a diagnostic and therapeutic ("theranostic") approach. A recent preclinical investigation has tried to apply this strategy to antidepressant therapy starting from IEGs comparative analysis in brain regions and blood (Waller et al., 2017).
2) IEGs monitoring after acute or continuous antipsychotic treatments should be carried out in *in vivo* models, in order to better understand the role of this class of molecules in the mechanisms of action of antipsychotics. For example, multiphoton imaging of IEGs signals in cortical circuits has already been successfully used to get information on *Egr1* expression after the exposure to a novel context (Xie et al., 2014).
3) The correlation among IEGs induction, antipsychotics, and epigenetic modulation is an attractive new scenario worth to be explored, which is based on the demonstrated modulation of the methylome by antipsychotics, on the recent findings on epigenetic control of IEGs in brain, and on the relevance of the contextual epigenetic-based mechanisms regulating brain higher functions that are involved in psychosis pathophysiology (Saunderson et al., 2016; Srivas and Thakur, 2016).
4) Under a translational perspective, more pharmacogenomic studies and brain post-mortem imaging investigations evaluating antipsychotics-dependent IEGs induction are needed to address in humans the findings that have already been described in animal models.
5) Finally, and again from a translational perspective, it will be important to start considering the "druggable" potential of some IEGs such as *Homer1a*, in order to search for new putative therapeutic strategies that can reach the core of the synapse and eventually correct the alterations linked to aberrant synaptic plasticity (Dev, 2004; Menard et al., 2015).

In sum, IEGs modulation by antipsychotics may provide a key tool to better understand the brain topography of antipsychotic action, the multiple pathways involved in the acute effects of these therapeutics beyond receptor interactions, as well as the molecular background for long-term changes of synaptic architecture promoted by chronic antipsychotic exposure.

AUTHOR CONTRIBUTIONS

AdB conceived the rationale and the structure of the review, wrote the manuscript and revised the literature search; EB analyzed the literature, contributed to write some sections of the second draft and to revise the final draft; FI, CT, GL, and FM revised and made contribution on the final draft; RR made the initial literature research and contribute to write partially the first draft of the manuscript; CT made the literature search, contributed to write the different drafts of the article.

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frontiers in Behavioral Neuroscience

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EGR3 Immediate Early Gene and the Brain-Derived Neurotrophic Factor in Bipolar Disorder

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Bipolar disorder (BD) is a severe psychiatric illness with a consistent genetic influence, involving complex interactions between numerous genes and environmental factors. Immediate early genes (IEGs) are activated in the brain in response to environmental stimuli, such as stress. The potential to translate environmental stimuli into long-term changes in brain has led to increased interest in a potential role for these genes influencing risk for psychiatric disorders. Our recent finding using network-based approach has shown that the regulatory unit of early growth response gene 3 (*EGR3*) of IEGs family was robustly repressed in postmortem prefrontal cortex of BD patients. As a central transcription factor, EGR3 regulates an array of target genes that mediate critical neurobiological processes such as synaptic plasticity, memory and cognition. Considering that *EGR3* expression is induced by brain-derived neurotrophic factor (BDNF) that has been consistently related to BD pathophysiology, we suggest a link between BDNF and EGR3 and their potential role in BD. A growing body of data from our group and others has shown that peripheral BDNF levels are reduced during mood episodes and also with illness progression. In this same vein, BDNF has been proposed as an important growth factor in the impaired cellular resilience related to BD. Taken together with the fact that EGR3 regulates the expression of the neurotrophin receptor p75NTR and may also indirectly induce BDNF expression, here we propose a feed-forward gene regulatory network involving EGR3 and BDNF and its potential role in BD.

Keywords: immediate early genes, early growth response gene 3 (EGR3), brain-derived neurotrophic factor (BDNF), bipolar disorder, neuroplasticity, regulatory network

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Bipolar disorder (BD) is a chronic and potentially severe and disabling mental illness that affects between 1% and 3% of the population worldwide (Merikangas et al., 2011), and is characterized by episodes of mania and depression. Studies evaluating concordance rates between monozygotic twins indicate that 40%–70% of risk for BD is genetically determined (Kieseppa et al., 2004; Craddock and Sklar, 2013). BD is likely influenced by numerous genes, which may individually contribute only a small risk for the disorder but may interact at the gene-network level and respond to environmental stimuli in a complex interaction.

In addition to the genetic contribution to BD, environment influences (Schmitt et al., 2014; Aldinger and Schulze, 2017) risk through both stressors and protective factors, such as childhood

trauma and level of maternal care (Champagne and Curley, 2009; Jansen et al., 2016; Aldinger and Schulze, 2017). The impact that environmental has on the clinical BD course (Aldinger and Schulze, 2017) suggests a potential role for genes that are involved in the response and adaptation to stress. This capacity of immediate early genes (IEGs) to translate environmental stimuli into long-term alterations in the brain makes this class of genes of great interest to the field of psychiatry.

IMMEDIATE EARLY GENES AND PSYCHIATRY

IEGs are a class of genes rapidly and transiently activated in response to a wide range of environmental stimuli (Senba and Ueyama, 1997). Many IEGs encode transcription factors, which regulate downstream target genes that presumably mediate their roles in neurobiological processes including synaptic plasticity and memory formation (Gallitano-Mendel et al., 2007; Poirier et al., 2008; Pérez-Cadahía et al., 2011). Early growth response (EGR) proteins are a family of IEG-encoded transcription factors: EGR1, EGR2, EGR3 and EGR4 (Beckmann and Wilce, 1997; Pérez-Cadahía et al., 2011). EGRs could translate environmental influence into long-term changes in the brain and thus contribute to neuronal plasticity, which has driven to the hypothesis that dysfunction in EGRs may be implicated in both the genetic and environmental involvement on psychiatric disorders susceptibility (Moises et al., 2002; Hanson and Gottesman, 2005; Gallitano et al., 2012; Huentelman et al., 2015).

Studies investigating the potential role of EGR family genes on risk for psychiatric disorders have focused most on the schizophrenia; the most positive findings have been on early growth response gene 3 (*EGR3*). Single nucleotide polymorphisms (SNPs) in *EGR3* are associated with schizophrenia (Kim S. H. et al., 2010; Zhang et al., 2012; Huentelman et al., 2015), and EGR3 mRNA expression is decreased in the postmortem brains of schizophrenia patients compared with controls (Mexal et al., 2005; Yamada et al., 2007). Furthermore, a bioinformatics analysis of the network of transcription factors and microRNAs associated with schizophrenia indicated *EGR3* as a central gene in this regulatory network (Guo et al., 2010).

Regarding a potential role for EGRs in BD, a study focused on association of genes related to circadian rhythms with BD found a nominally significant association for *EGR3* (Mansour et al., 2009). A family-based association study, although limited by small sample size, also showed a nominal and preliminary association of *EGR3* with risk for BD in children (Gallitano et al., 2012), suggesting this gene as a target for subsequent larger follow-up evaluation. Our recent study using a network-based approach showed that the regulatory unit of *EGR3* was robustly reduced in both of the two independent bipolar gene expression signatures examined from postmortem prefrontal cortex (Pfaffenseller et al., 2016), suggesting the entire network centered on *EGR3* might be dysregulated in BD.

Interestingly, *EGR3*-deficient mice, knockout animals generated by targeted mutagenesis in embryonic stem cells (Tourtellotte and Milbrandt, 1998) and thus lacking functional EGR3 in all cells throughout development, show both physiologic and behavioral changes that corroborate with models in psychiatry. Such alterations involve a heightened stress-reactivity (indicated by both an increased behavioral response and elevated corticosterone release following handling, a mild stressor test), hyperactivity in the locomotor activity test (indicating a psychosis-like phenotype), disrupted habituation to environmental stimuli and social cues and increased aggressive behavior toward an unfamiliar animal (Gallitano-Mendel et al., 2007, 2008). These observations suggest that EGR3 may be involved in biological mechanisms needed to an appropriate response to stress that possibly are dysfunctional in BD. Psychosis-like phenotypes and hyperactivity observed in these *EGR3*-deficient mice could be reversed with antipsychotic drugs used in treatment of psychiatric disorders, providing an additional support for these findings (Gallitano-Mendel et al., 2008; Williams et al., 2012).

In this scenario, a recent study showed that EGR3 seems to play an essential role in the susceptibility to stress since it was related to dendritic atrophy in nucleus accumbens medium spiny neurons in mice susceptible to the social defeat stress model, and *EGR3* knockdown inhibited this dendritic atrophy (Francis et al., 2017). The authors suggest that these alterations in dendritic structure mediated by EGR3 could be responsible for loss in the total number of synapses and consequently reduction in the excitatory transmission observed in these defeated mice. This molecular mechanism mediated by EGR3 could reinforce its role in regulating homeostasis and cellular adaptations possibly underlying stress-induced behavior, which highlight the relevance in studying this transcription factor in psychiatry.

EARLY GROWTH RESPONSE 3 PATHWAY

EGR genes are expressed at basal levels throughout the brain, such as the cortex, the hippocampus and the basal ganglia as observed in animal studies (Senba and Ueyama, 1997). EGR3 expression is rapidly induced at high levels in response to environmental alterations, including stressful stimuli and sleep deprivation (Honkaniemi et al., 2000; Thompson et al., 2010; Maple et al., 2015).

The EGR3 expression in neurons is regulated by synaptic activity and is mediated by MAPK-ERK signaling (O'Donovan et al., 1999; Li et al., 2007). Studies have improved the understanding about the signaling cascade that leads to EGR3 expression. EGR3 is induced downstream of numerous proteins, comprising neuregulin 1 (NRG1), calcineurin (CaN), N-methyl-D-aspartate (NMDA) receptors and neurotrophins such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF; Yamagata et al., 1994; Hippenmeyer et al., 2002; Roberts et al., 2006; Yamada et al., 2007; Eldredge et al., 2008).

As a transcription factor, EGR3 could, in turn, activate numerous downstream targets that participate in processes such as synaptic plasticity, axon and dendrites extension and modulation of receptors. Experimental studies have identified effects of EGR3 on NMDA receptors (NMDAR; Gallitano-Mendel et al., 2007), type A gamma amino butyric acid

(GABA) receptors (Roberts et al., 2006), and NGF receptors (*p75NTR*, Gao et al., 2007). EGR3 also regulates *Arc* (activity regulated cytoskeletal associated gene; Li et al., 2005), which modifies synapses in response to environmental stimuli, and genes involved in the development and branching of axons and dendrites (Quach et al., 2013). Moreover, it may modulate genes involved in microglia dysregulation such as triggering receptor expressed on myeloid cells 1 (*TREM-1*, Weigelt et al., 2011). Thus, requirement for EGR3 in processes of memory, learning and neuroplasticity (Gallitano-Mendel et al., 2007; Li et al., 2007) is presumably to be determined by these and possibly other EGR3 target genes not yet identified (**Figure 1**).

BRAIN-DERIVED NEUROTROPHIC FACTOR AND BIPOLAR DISORDER

BDNF is the most highly expressed neurotrophin in the CNS, including brain regions associated with emotion modulation and cognitive processing such as prefrontal cortex, amygdala and hippocampus (Lu et al., 2005, 2014). In addition to its expression in the brain, BDNF is also expressed in peripheral tissues (Fujimura et al., 2002). BDNF plays a critical role in neuronal survival and differentiation, dendritic arborization, synaptic plasticity and also in complex process such as memory consolidation and learning (Minichiello, 2009; Park and Poo, 2013; Lu et al., 2014). BNDF is one of the most extensively investigated biomarkers in BD (Post, 2007; Grande et al., 2010).

Some studies have associated changes in peripheral BDNF levels with BD state, suggesting that serum BDNF levels may represent a potential biomarker of mood episodes. A study by Cunha et al. (2006) reported that patients experiencing an episode of either mania, hypomania, or depression had reduced serum BDNF levels compared to euthymic patients, who had BDNF levels similar to healthy subjects. Another study also showed decreased BDNF levels in transformed lymphoblasts from BD patients in comparison to controls (Tseng et al., 2008). Subsequently, meta-analyses have supported that patients in either a depressive or a manic state have lower blood levels of BDNF than healthy individuals; and serum BDNF levels in euthymic patients did not differ from those observed in controls (Lin, 2009; Fernandes et al., 2011, 2014). A more recent meta-analysis indicated that peripheral BDNF levels are reduced in patients compared to healthy controls, regardless of mood state (Munkholm et al., 2015). Considering central tissue,

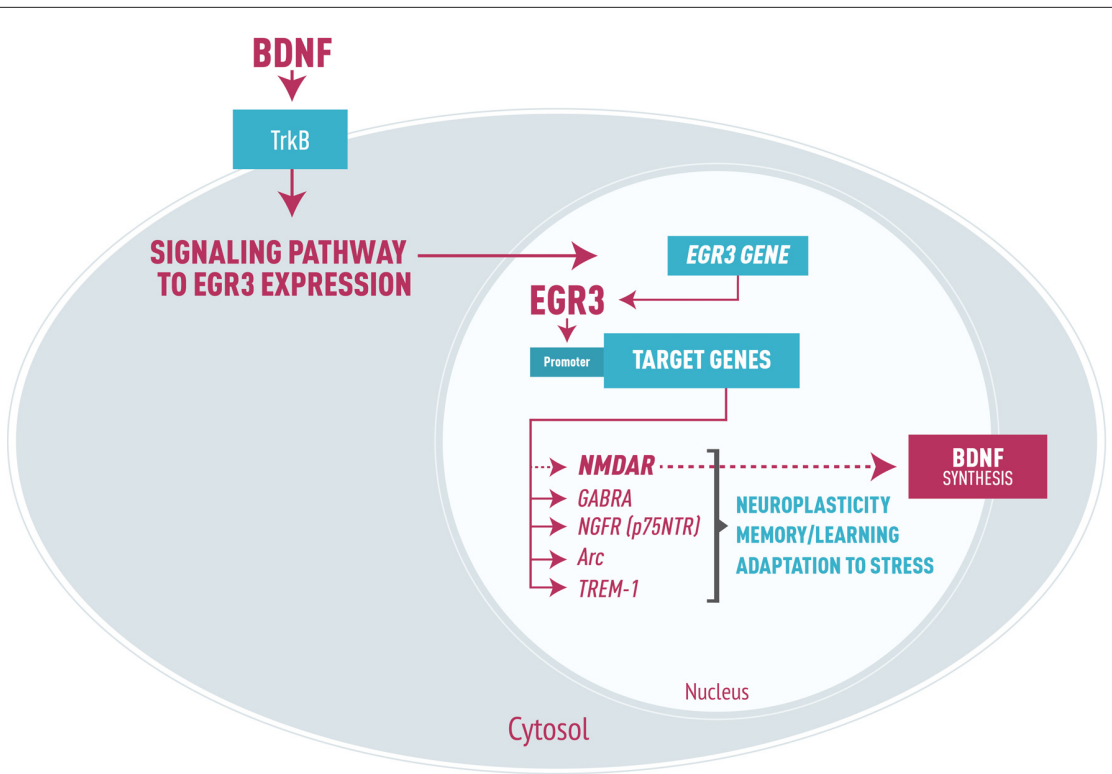

FIGURE 1 | Representation of early growth response gene 3 (EGR3) signaling cascade in neurons, focused on brain-derived neurotrophic factor (BDNF) signaling leading to EGR3 expression. EGR3 is activated downstream of numerous proteins, including BDNF through binding to its receptor TrkB. In turn, EGR3 protein activates numerous downstream target genes. Examples include: type A GABA receptor (GABRA), NGFR (p75NTR) receptor, the activity regulated cytoskeletal associated gene (Arc) and triggering receptor expressed on myeloid cells 1 (TREM-1), as well as, though perhaps indirectly, NMDA receptor (NMDAR). These genes are each involved in critical neurobiological processes such as neuroplasticity, memory and learning and adaptation to stress. It is important to note that EGR3 may indirectly induce BDNF expression via regulation of NMDAR, the activation of which stimulates BDNF synthesis. Thus, we propose a feed-forward regulatory gene network involving EGR3 and BDNF that regulates neuronal gene expression in response to endogenous or environmental stimuli which, when disrupted, may lead to bipolar disorder (BD) pathophysiology.

postmortem studies also have importantly reported alterations in BDNF in BD. For instance, a meta-analysis using postmortem findings from the Stanley Neuropathology Consortium found lower hippocampus BDNF protein levels in BD (Knable et al., 2004). Consistent with this analysis, recent studies have shown that BDNF mRNA was significantly reduced in the hippocampus of BD patients compared to healthy subjects (Thompson Ray et al., 2011; Reinhart et al., 2015). Decreased levels of BDNF have been also found in frontal cortex (protein and mRNA; Kim H. W. et al., 2010) and in inferior and superior temporal gyrus (mRNA) of BD patients (Ray et al., 2014).

Other studies suggest that BDNF may be associated with illness progression. One report showed that BD patients in later stages of the illness show decreased BDNF levels compared to patients in earlier stages, even during euthymic periods (Kauer-Sant'Anna et al., 2009). Moreover, serum BDNF levels are inversely associated with both duration of illness (Kauer-Sant'Anna et al., 2009) and with severity of manic and depressive symptoms (Cunha et al., 2006). Overall the majority of studies indicated that levels of this neurotrophic factor are reduced in bipolar patients compared to controls.

Several studies have been performed to elucidate the mechanisms involved in the presumed reduction in BDNF levels in BD. Some of them have studied polymorphisms in the *BDNF* gene such as the polymorphism involving a methionine substitution for a valine at codon 66 (val66met) in the promoter region of the gene. However, the findings regarding association between the val66met polymorphism and BD are divergent (Sklar et al., 2002; Neves-Pereira et al., 2005), which suggests that this particular variant does not explain the altered BNDF levels in BD (Post, 2007).

The apparent decrease in BDNF levels seen in patients would be expected to result in disruption of the intracellular signaling cascades that are normally regulated by BDNF, such as PLC/PKC, PI3K/Akt and Ras/Erk pathways, interfering with processes regulated by this neurotrophin, such as neuronal differentiation and survival, and synaptic plasticity. In fact, BD has been associated with changes in factors involved in neuroplasticity and resilience pathways, including alterations in apoptotic factors, synaptic markers, neurotrophic and inflammatory factors and oxidative stress markers, as well as in processes related to circadian rhythm, neuronal development and calcium metabolism (Kim H. W. et al., 2010; Frey et al., 2013).

Neuropathological findings in the postmortem brains of BD patients demonstrate the types of abnormalities in neuroplasticity one would expect to see from a deficit in neurotrophic factors. For example, morphometric studies show that patients have enlarged third and lateral ventricles, decreased volume of the orbital and medial prefrontal cortices, ventral striatum and mesotemporal cortex and increased volume of the amygdala compared to controls (Strakowski et al., 2005). Interestingly, such neuroanatomical changes have been found to be more pronounced in patients with multiple mood episodes (Strakowski et al., 2002), suggesting that these abnormalities may increase with severity of the illness (Fornito et al., 2007; Berk et al., 2011). An effective neuroplasticity, considered a cellular and molecular level of adaptation, is likely necessary for the process of resilience, which involves a whole-organism level response to events. Thus, the abnormalities in neuroplasticity possibly translate into reduced resilience related to recurrent mood episodes and illness progression, which could reflect clinically in cognitive impairments in BD patients. In fact, meta-analyses show that most patients exhibit neurocognitive dysfunction, and the most impaired domains are attention, verbal learning, memory and executive functions (Robinson et al., 2006; Bourne et al., 2013; Bortolato et al., 2015).

These findings support the "allostatic load" hypothesis that we have previously described (Kapczinski et al., 2008). This hypothesis asserts that the clinical BD course is determined by a combination of the individual's genetic makeup, history of stressful life events and degree and duration of episodes of mental illness. These factors are connected in a feedback loop that worsens the patient's degree of symptoms or overall life function, leading to a progressive illness course associated with biological and brain changes, and cognitive and functioning impairment—hypothesis of BD neuroprogression (Berk, 2009; Fries et al., 2012). Since stress plays an essential role in both the onset and progression of BD, it is noteworthy that BDNF-related neuroplasticity may be a crucial mediator of the effects of stress on BD. Thus, we could assume that a possible dysfunction in neurotrophin pathway might influence an increased vulnerability of BD patients to stressful conditions.

LINK BETWEEN EARLY GROWTH RESPONSE 3 AND BRAIN-DERIVED NEUROTROPHIC FACTOR IN BIPOLAR DISORDER

This perspective article presents an accumulation of findings indicating that changes in BDNF are a consistent feature of BD, and may contribute to the pathophysiology of this mental illness. Here we summarize the potential molecular links between BDNF and the IEG transcription factor EGR3, two molecules that may each play a critical role in the impaired cellular resilience related to BD (Manji et al., 2003; Berk et al., 2011; Pfaffenseller et al., 2016).

As we have discussed, BDNF is reported to be altered in BD in both peripheral and central tissue, and it is possible that blood BDNF levels correlate positively with brain BDNF levels (Klein et al., 2011). Thus, presuming that the reduced peripheral BDNF levels observed in BD patients accurately reflect levels in the brain, and considering that BDNF may induce EGR3 expression via PKC/MAPK dependent pathway (Roberts et al., 2006), the decreased levels of BDNF may account, at least in part, for the prefrontal cortex EGR3 repression that we identified in BD (Pfaffenseller et al., 2016).

Alternatively, or in addition, EGR3 may indirectly induce BDNF expression via regulation of NMDAR. In mice, EGR3 has been shown to be required for the function of NR2B-containing NMDARs (Gallitano-Mendel et al., 2007). A study has shown that the majority of NMDAR protein levels (NR1 subunits) in rat cortical neurons are regulated by the transcription factors CREB

and EGR3 (Kim et al., 2012), and NMDAR activation stimulates BDNF synthesis (Marini et al., 1998). It is noteworthy that NMDARs have a critical role for memory processes involving long-term potentiation (LTP) and long-term depression (LTD; Bliss and Collingridge, 1993; Collingridge and Bliss, 1995), and it has been demonstrated that BDNF as well as EGR3 participates in LTP and LTD processes. For example, the administration of exogenous BDNF to mice deficient for BDNF restores the impairment in LTP (Patterson et al., 2001). Mice deficient for *EGR3* have deficits in hippocampal LTD and exhibit dysfunction in NMDAR subclasses (NR1/NR2B; Gallitano-Mendel et al., 2007). Thus, dysfunction in signaling pathways involving both BDNF and EGR3 may underlie the cognitive impairment shown by BD patients.

In addition, EGR3 also regulates the expression of NGFR (*p75NTR*, Gao et al., 2007), a receptor for neurotrophins involved in the control of axonal elongation, neuron survival and differentiation (Dechant and Barde, 2002). Neurogenic potential seems to be mediated by p75(NTR) and is greatly enhanced *in vitro* after treatment with BDNF (Young et al., 2007), indicating this EGR3-regulated mechanism integrate a relevant pathway involved in neuroplasticity that likely correspond to changes in BDNF levels in psychiatric conditions.

Taking this into account, we also suggest that EGR3 repression seen in BD patients could be responsible for the reduced BDNF levels associated to this illness. It is most likely to think in a feedback network than in a cause-and-effect relationship considering that EGR3 is responsive to BDNF and regulates NMDAR, which transcription is also mediated by BDNF through activation of the TrKB receptor and in turn induce BDNF synthesis (Kim et al., 2012). Thus, we propose a feed-forward regulatory gene network involving EGR3 and BDNF (**Figure 1**) that may regulate biological mechanisms to change neuronal expression according to endogenous or environmental stimuli, and this process might potentially be related to BD pathophysiology.

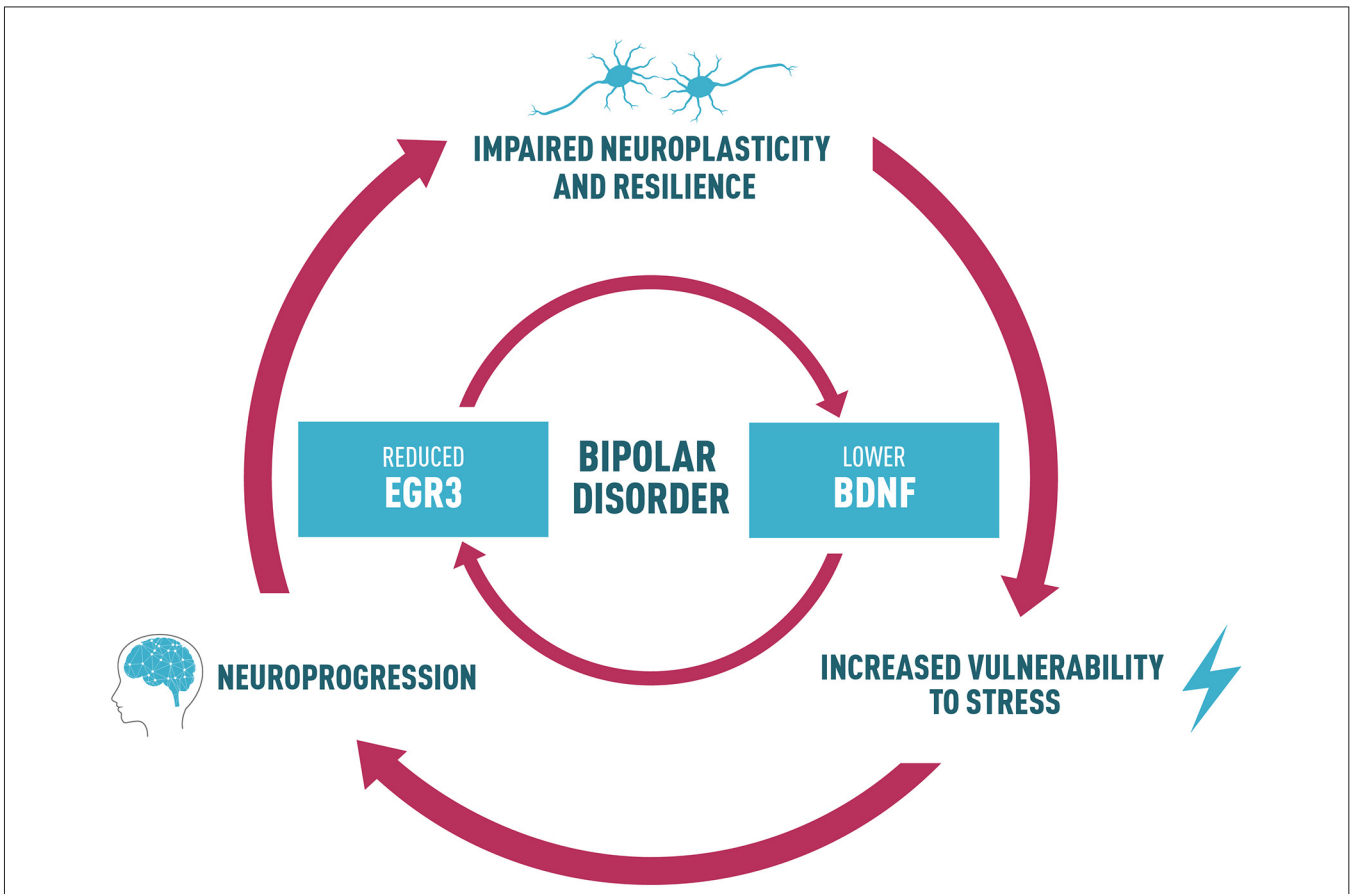

FIGURE 2 | Proposed link between BDNF and EGR3 and their potential role in BD. Lower BDNF levels observed in BD patients may influence the reduced EGR3 levels seen in BD since BDNF regulates EGR3. EGR3 may also indirectly induce BDNF expression via regulation of NMDAR. Thus, we also suggest that reduced EGR3 expression, as we have seen in BD patients in our study, could contribute to lower BDNF levels associated with this illness. Based on these findings, we propose a feedback-loop reinforcing this dysfunctional pathway that could, in turn, impair neuroplasticity and resilience. This process may ultimately lead to increased vulnerability to stress, and could result in alterations in several biological factors that contribute to BD, such as abnormal structural brain changes and the associated cognitive and functional decline (a process called neuroprogression). The neural circuits additionally disrupted in this process could contribute to an impaired neuroplasticity and resilience, increasing vulnerability to stress and mood episodes and reduced responsiveness to pharmacotherapy, thus perpetuating a vicious cycle in illness progression.

Altogether, the findings discussed in this article suggest a potential regulatory pathway that possibly is disrupted in BD. For many years, evidence has pointed to alterations in neurotrophic factors in BD suggesting these changes could contribute to an impaired neuroplasticity and resilience. However, the mechanisms underlying this impairment remain unknown. The identified network focused on *EGR3* thus emerges as a potential central player responsible for some of changes observed in BD such as a reduced neurotrophic support. Taking into consideration that EGR3 translates environmental events into neural long-term alterations, the possible disturbance in molecular pathways involving EGR3 could result in an impaired response and adaptation to stress.

We suggest experimental approaches to test the hypothesis regarding a potential feed-forward mechanism involving BDNF and EGR3 and its role in BD, which might contribute to understanding its pathophysiology. Moreover, as a central transcription factor of a gene network that regulates crucial neurobiological processes, EGR3 may be a promising pharmacological target. Modulation of IEGs as *EGR3* might be beneficial since they could provide a dynamic and fast response to neural activity and thus a sustained adaptation through regulation of an entire regulatory gene network.

With this perspective, we propose that a reduction in EGR3 in BD could contribute to alterations in a neurotrophin cascade in this disorder, which includes reduced BDNF levels. A feedback-loop reinforcing this dysfunctional pathway could, in turn, impair neuroplasticity and resilience (**Figure 2**). This process may ultimately lead to increased vulnerability to stress, underlying the risk to develop the symptoms and BD neuroprogression. Thus, we suggest an interesting link between EGR3 and BDNF in BD, and this shared biological pathway could provide potential targets for follow-up studies to clarify mechanisms responsible for the interaction between environment and genetic factors that influence BD and for the development of novel therapeutics.

AUTHOR CONTRIBUTIONS

All authors provided substantial contributions to the work. BP, FKa and FKl participated in the article design and outline. BP wrote the first draft. ALG revised it critically for relevant scientific content. After a few revisions and editing by all authors, the article was submitted.

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HYPOTHESIS AND THEORY
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Immediate Early Genes Anchor a Biological Pathway of Proteins Required for Memory Formation, Long-Term Depression and Risk for Schizophrenia

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While the causes of myriad medical and infectious illnesses have been identified, the etiologies of neuropsychiatric illnesses remain elusive. This is due to two major obstacles. First, the risk for neuropsychiatric disorders, such as schizophrenia, is determined by both genetic and environmental factors. Second, numerous genes influence susceptibility for these illnesses. Genome-wide association studies have identified at least 108 genomic loci for schizophrenia, and more are expected to be published shortly. In addition, numerous biological processes contribute to the neuropathology underlying schizophrenia. These include immune dysfunction, synaptic and myelination deficits, vascular abnormalities, growth factor disruption, and N-methyl-D-aspartate receptor (NMDAR) hypofunction. However, the field of psychiatric genetics lacks a unifying model to explain how environment may interact with numerous genes to influence these various biological processes and cause schizophrenia. Here we describe a biological cascade of proteins that are activated in response to environmental stimuli such as stress, a schizophrenia risk factor. The central proteins in this pathway are critical mediators of memory formation and a particular form of hippocampal synaptic plasticity, long-term depression (LTD). Each of these proteins is also implicated in schizophrenia risk. In fact, the pathway includes four genes that map to the 108 loci associated with schizophrenia: *GRIN2A*, nuclear factor of activated T-cells (*NFATc3*), early growth response 1 (*EGR1*) and NGFI-A Binding Protein 2 (*NAB2*); each of which contains the "Index single nucleotide polymorphism (SNP)" (most SNP) at its respective locus. Environmental stimuli activate this biological pathway in neurons, resulting in induction of *EGR* immediate early genes: *EGR1*, *EGR3* and *NAB2*. We hypothesize that dysfunction in any of the genes in this pathway disrupts the normal activation of *Egr*s in response to stress. This may result in insufficient electrophysiologic, immunologic, and neuroprotective, processes that these genes normally mediate. Continued adverse environmental experiences, over time, may thereby result in neuropathology that gives rise to the symptoms of schizophrenia. By combining multiple genes associated with schizophrenia susceptibility, in a functional cascade triggered

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by neuronal activity, the proposed biological pathway provides an explanation for both the polygenic and environmental influences that determine the complex etiology of this mental illness.

Keywords: schizophrenia, immediate early gene, long-term depression, EGR3, ARC, NFATC3, EGR1, Nab2

INTRODUCTION

Since the start of the 21st century, tremendous advances have been made in the identification of genes associated with risk for major neuropsychiatric illnesses such as schizophrenia, bipolar disorder and depression. However, the field of psychiatry still lags behind other areas of medicine in identifying even a single gene that has been definitively demonstrated to cause any of these mental illnesses. This is due in large part to the complex genetics that underlie these disorders. Now, 15 years after the landmark publication that identified Neuregulin 1 (NRG1) as a candidate gene for schizophrenia (Stefansson et al., 2002), there remain two unanswered questions at the forefront of the field of psychiatric genetics: (1) how can the polygenic nature of susceptibility to schizophrenia be explained? and (2) how do genes implicated in risk for schizophrenia interact with environmental factors to give rise to the disorder?

To address both of these questions, we have proposed the hypothesis that numerous schizophrenia susceptibility genes form a critical signaling pathway that is responsive to environmental stress. Dysfunction of any of the proteins in this pathway would reduce the normal activation of a key transcription factor, early growth response 3 (*EGR3*), an immediate early gene that is both associated with schizophrenia in humans (Yamada et al., 2007; Kim et al., 2010; Ning et al., 2012; Zhang et al., 2012; Huentelman et al., 2015), and expressed at reduced levels in patients' brains (Mexal et al., 2005; Yamada et al., 2007). As a critical mediator of numerous biological processes disrupted in schizophrenia, such as growth factor signaling, myelination, vascularization, immune function, memory formation and synaptic plasticity (Gallitano-Mendel et al., 2007; Jones et al., 2007; Suehiro et al., 2010; Li et al., 2012; Kurosaka et al., 2017), insufficient activation of *EGR3* may result in neuropathology that gives rise to schizophrenia.

Schizophrenia risk is influenced by many genes in addition to environmental factors. The illness has a prevalence rate of roughly 1% worldwide, and its cause remains unknown. Studies show concordance rates of approximately 50% in monozygotic twins, roughly twice that of dizygotic twins, indicating that there are both genetic and non-genetic determinants of schizophrenia (McGue and Gottesman, 1991). Stressful events are associated with schizophrenia risk. These include prenatal stress such as nutritional deficiency, or exposure to famine, infection (e.g., rubella, influenza, *Toxoplasma gondii* and herpes simplex virus), or maternal stress. Stress during the perinatal period and early life also increase risk for the illness. Examples include obstetric complications and perinatal trauma, and stressful life events such as childhood trauma (Corcoran et al., 2001, 2003; Mittal et al., 2008; van Winkel et al., 2008; Brown and Derkits, 2010; Brown, 2011). Adding to the complex etiology of this illness, the most recent genome-wide association study (GWAS) of single nucleotide polymorphisms (SNPs) identified 108 genomic loci that influence schizophrenia susceptibility (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). To date, there is no consensus on a mechanism to explain how so many genetic variations interact with environmental factors to cause schizophrenia.

IDENTIFYING A PATHWAY

Immediate early genes are a class of genes that are rapidly induced in response to a stimulus, in a manner that is independent of protein synthesis. In the brain, they are expressed within minutes of neuronal activity triggered by environmental stimuli. A large number of immediate early genes encode proteins that function as transcription factors (termed immediate early gene transcription factors (Curran and Morgan, 1995)). These genes are thus poised to translate changes in the environment into long-term changes in the brain through the regulation of their target genes. This presumably underlies the critical role of many immediate early gene transcription factors in memory formation, a process that requires long-term encoding of environmental experiences.

We have hypothesized that this function of immediate early gene transcription factors, as key regulators of the brain's gene-expression response to experience, uniquely positions them to mediate the dual genetic and environmental influences on schizophrenia susceptibility (Gallitano-Mendel et al., 2008). We focus on the *Egr* family of immediate early genes since they are activated in response to changes in the environment (Senba and Ueyama, 1997; Martinez et al., 2002), and regulate fundamental processes in the nervous system that are known to be dysfunctional in schizophrenia. These include myelination, vascularization, learning and memory, and synaptic plasticity (Paulsen et al., 1995; Guzowski et al., 2001; Nagarajan et al., 2001; Bozon et al., 2002, 2003; Flynn et al., 2003; Crabtree and Gogos, 2014). In addition, *Egrs* are activated downstream of N-methyl-D-aspartate receptors (NMDARs; Cole et al., 1989) and growth factors (Schulze et al., 2008; Shin et al., 2010), dysfunction of which have each been hypothesized to contribute to schizophrenia susceptibility (Olney et al., 1999; Moises et al., 2002; Calabrese et al., 2016).

We hypothesize that variations that reduce the normal amount of *Egr* gene expression in response to environmental stimuli would result in lower than normal levels function of these processes. Specifically, this would result in insufficient activation of *Egr* target genes, such as brain-derived neurotrophic factor (BDNF) and activity-regulated cytoskeleton associated protein (*Arc*/*Arg3.1*: hereafter referred to as "*Arc*"; Li et al., 2005;

Maple et al., 2017; Meyers et al., 2017a,b). Lower than normal activation of these genes could account for the reduced levels of white matter, reduced synaptic spine density, and deficiencies in memory and cognitive processing, that are seen in patients with schizophrenia, or hypothesized to underlie its pathogenesis (Ingvar and Franzen, 1974; Saykin et al., 1991; Glantz and Lewis, 2000; Moises et al., 2002; Davis et al., 2003).

Several findings led us to focus specifically on *Egr* family member *Egr3* as we investigated this hypothesis. First, *NRG1* was identified as a schizophrenia candidate gene in a large-scale genetic association study (Stefansson et al., 2002). In mice, *Nrg1* was found to be essential to maintain *Egr3* expression in the peripheral muscle spindle (Hippenmeyer et al., 2002). Subsequently the protein phosphatase calcineurin (CN) was identified as a schizophrenia candidate protein based on the phenotype of $CN^{-/-}$ mice, and the association of the gene encoding one of its subunits (*PPP3CC*) with schizophrenia in a Japanese population (Gerber et al., 2003). CN had also been shown to regulate expression of *Egr3* in the immune system (Mittelstadt and Ashwell, 1998). Together, these findings indicated that *Egr3* was regulated downstream of three proteins independently implicated in schizophrenia risk: NMDARs, NRG1 and CN.

To answer whether *Egr3* may play a role in schizophrenia, we investigated the behavior and physiology of *Egr3*-deficient ($^{-/-}$) mice. Our studies revealed that $Egr3^{-/-}$ mice display behavioral abnormalities consistent with animal models of schizophrenia. These include locomotor hyperactivity, a behavior produced in rodents treated with drugs that cause psychosis in humans, such as NMDAR antagonists—phencyclidine and ketamine, and dopaminergic agents including amphetamines (Lipska and Weinberger, 2000; Tamminga et al., 2003). The fact that this activity is reversible with antipsychotic medications further validates this as a phenotype relevant to schizophrenia (Freed et al., 1984; O'Neill and Shaw, 1999). $Egr3^{-/-}$ mice show locomotor hyperactivity in response to a novel environment that is reversed with antipsychotic treatment (Gallitano-Mendel et al., 2008). Schizophrenia is also characterized by cognitive deficits, which a feature that led to Emil Kraepelin to define the syndrome "Dementia Praecox" (Kraepelin and Robertson, 1919). $Egr3^{-/-}$ mice have deficits in spatial memory as well as defects in hippocampal long-term depression (LTD), a form of synaptic plasticity (Gallitano-Mendel et al., 2007, 2008) normally activated in response to stress and novelty (Manahan-Vaughan and Braunewell, 1999; Kemp and Manahan-Vaughan, 2007). Notably, both NMDARs and CN, proteins that regulate induction of *Egr3*, are also required for memory formation and LTD. This suggested that EGR3 was functioning in a signal transduction cascade of proteins that were activated in the brain in response to novelty and stress, and required for both memory formation and LTD (Gallitano-Mendel et al., 2007).

We subsequently described that numerous of the key proteins in this pathway were associated with risk for schizophrenia (Gallitano-Mendel et al., 2008). This led us to hypothesize that other genes in this pathway that shared the characteristics of regulating memory and hippocampal LTD, such as the EGR3 target gene *ARC*, should also be candidates for a role in schizophrenia susceptibility (Gallitano, 2008). Indeed, numerous genome wide association studies of copy number variations (CNVs), *de novo* mutations and SNPs (Kirov et al., 2012; Fromer et al., 2014; Purcell et al., 2014), as well as our own resequencing study that identified a schizophrenia associated *ARC* SNP (Huentelman et al., 2015), have subsequently supported this hypothesis.

Here we present the key proteins that comprise our proposed biological pathway, shown in **Figure 1**. We begin with a brief review of hippocampal LTD, highlighting its response to stress. We follow with a section devoted to each protein in the pathway. For each protein we: (1) summarize the evidence demonstrating the regulatory relationships that support its position in the pathway; (2) outline the findings indicating its roles in processes that are disrupted in schizophrenia; (3) mention supporting studies that demonstrate its genetic association with risk for the illness (summarized in **Supplementary Table S1**); and (4) if known, altered levels in the brains of patients with schizophrenia. When available, we will also review the role for each in synaptic plasticity, focusing on LTD, and define the relationship of the protein being discussed with EGR3. We will include a brief section on additional proteins for which there is new evidence indicating a potential contribution to this pathway. We briefly review the role of LTD in the neurobiological response to stress and novelty. In the Discussion we propose our hypothesis that this biological pathway mediates protective neurobiological responses to stress. We then present a model to explain how genetic variations in genes of this pathway may produce a predisposition to schizophrenia that results in illness in a manner dependent upon the stress history of an individual.

LONG-TERM DEPRESSION, STRESS AND MEMORY

LTD

LTD is a form of synaptic plasticity in which stimulation results in a prolonged decrease in strength of the synaptic connection (reviewed in Malenka and Bear, 2004; Kemp and Manahan-Vaughan, 2007; Collingridge et al., 2010). One of the most well-investigated forms of LTD is that produced by low frequency stimulation of the hippocampal Schaffer Collateral pathway, the axonal projections from CA3 neurons that synapse onto CA1 pyramidal neurons. This process is essential for spatial memory consolidation, and induces expression of immediate early genes. Moreover, the stimulation that induces the highest levels of immediate early genes also results in the longest duration of LTD (Abraham et al., 1994) suggesting an important functional role for immediate early genes in this form of synaptic plasticity. The low frequency stimulation that induces LTD also requires the function of NMDARs, as well as numerous proteins in our proposed biological pathway for schizophrenia susceptibility.

Compared to its counterpart long-term potentiation (LTP), LTD has received much less investigative attention, and is thus less well understood. NMDAR activation is an essential

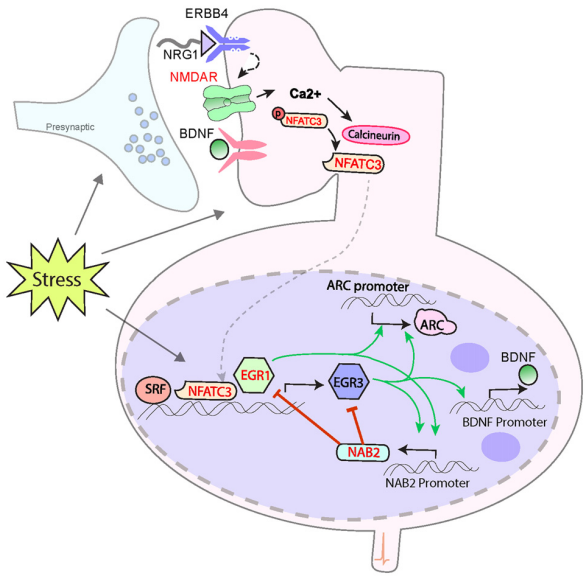

FIGURE 1 | Model for a biological pathway regulating memory, synaptic plasticity and schizophrenia risk. Numerous schizophrenia-risk associated proteins act either upstream or downstream of immediate early genes *EGR3* and *ARC*. Red labels indicate proteins encoded by genes that map to the 108 loci for schizophrenia risk. Both EGR3 and ARC are activated by environmental events, such as stress, via activity-dependent activation of NMDARs. The resulting influx of Ca++ triggers Calcineurin/(CN) nuclear factors of activated T-cell (NFAT) signaling which can induce *EGR3* expression. EGR3 positively regulates, and maintains expression of, *ARC*. NGFI-A Binding Protein 2 (NAB2), which is a transcriptional target of EGR3 and EGR1, binds to EGR proteins (EGR1, 2 and 3) to co-regulate expression of target genes. This action includes feedback inhibition of EGR1 and EGR3, and NAB2 itself, a critical element of the temporal regulation of activity-dependent genes. Additional proteins that interact in the pathway include NRG1 which, via binding to ERBB receptors, maintains expression of EGR3 (in muscle spindles), and has been shown to activate NMDARs. BDNF, via binding to Trk-B receptors, regulates EGR3-mediated expression of NMDAR subunit NR1. EGR3, in turn, is required for activity-dependent expression of BDNF. SRF is a transcription factor required for memory formation and long-term depression (LTD), which regulates expression of EGR3 (and EGR1). Perturbations in any components of this biological pathway are expected to result in deficient induction of EGR3, and its target genes, in response to neuronal activity, including that triggered by stress. Insufficient activation of this pathway in response to stimuli may result in poor memory formation and contribute to cognitive deficits. Deficient activation of the pathway in response to stress may also result in insufficient neuroprotective processes and thereby contribute, over time, to the neuropathology that gives rise to schizophrenia (see **Figure 2**). Note that many interactions represented are taken from literature on studies in immune system and have yet to be validated in the brain. The shared roles of these proteins in the immune system may contribute to association between schizophrenia and immune dysfunction. Abbreviations: NMDAR, N-methyl d-aspartate receptor; NFATC3, nuclear factor of activated T-cells; EGR1, 3, Early growth response 1, 3; ARC, Activity-regulated cytoskeleton-associated protein. Additional potential contributing proteins are indicated in gray include: SRF, serum response factor; BDNF, brain-derived neurotrophic factor; NRG1, neuregulin 1; ErbB4, ErbB2 receptor tyrosine kinase 4, Trk-B neurotrophic receptor tyrosine kinase 2. References: Yamagata et al. (1994); Senba and Ueyama (1997); Mittelstadt and Ashwell (1998); Rengarajan et al. (2000); Hao et al. (2003); Jacobson et al. (2004); Kumbrink et al. (2005, 2010); Li et al. (2005); Ramanan et al. (2005); Etkin et al. (2006); Lindecke et al. (2006); Bjarnadottir et al. (2007); Gallitano-Mendel et al. (2007, 2008); Gallitano (2008); Marrone et al. (2012); Herndon et al. (2013); Ramirez-Amaya et al. (2013); Schizophrenia Working Group of the Psychiatric Genomics Consortium (2014); Maple et al. (2015); Pouget et al. (2016); Meyers et al. (2017a,b); Ramanan (personal communication).

step in establishment of low frequency stimulation-induced hippocampal LTD (Dudek and Bear, 1992; Mulkey and Malenka, 1992). Both *GluN1* and *GluN2B* subunits have also been implicated in mediating this form of LTD (Kutsuwada et al., 1996; Manahan-Vaughan and Braunewell, 1999; Liu et al., 2004). Early studies demonstrated that mice lacking the specific *GluN2B*-containing NMDAR isoform had deficits in LTD (Kutsuwada et al., 1996; Brigman et al., 2010). However, recent work investigating the role of GLUN2B in this form of synaptic plasticity have produced differing results, some of which may be explained by technical differences in study methodologies (Chen and Bear, 2007; Bartlett et al., 2011; Shipton and Paulsen, 2014).

As described in the sections below, numerous downstream effectors of NMDAR activation have also been shown to be critical for activity dependent LTD. These include CN, EGR3, ARC, serum response factor (SRF) and BDNF (Zeng et al., 2001; Etkin et al., 2006; Plath et al., 2006; Gallitano-Mendel et al., 2007; Beazely et al., 2009; Mizui et al., 2015; Novkovic et al., 2015; Bukalo et al., 2016; Kojima and Mizui, 2017). The roles of several of the proteins that comprise our proposed pathway for schizophrenia risk are not limited to this form of synaptic plasticity. For example, NMDARs, BDNF and ARC are also required for LTP. Interestingly, dysfunction in LTP has also been hypothesized to play a role in schizophrenia (Rison and Stanton, 1995). However, the shared role of numerous core pathway genes in the process of LTD stood out to us early on in the recognition of this biological cascade (Gallitano-Mendel et al., 2007, 2008). Since LTD had received so much less emphasis, at that time, this seemed a coincidence worthy of further investigation.

Several features of LTD make this form of synaptic plasticity of particular interest for a potential contribution to the development of schizophrenia. Stress is one of the major environmental risk factors for this schizophrenia (Holtzman et al., 2013). Numerous studies have demonstrated a relationship between stress and LTD (Rowan et al., 1998). For example, while stress inhibits establishment of LTP in the hippocampal CA1 region, it augments LTD (Kim et al., 1996; Xu et al., 1997; Artola et al., 2006). The association of stress with schizophrenia susceptibility suggests the possibility that disruption of the normal LTD response to stress could result in pathology that may increase risk for this, and potentially other, psychiatric illness.

A second intriguing role of LTD is that of encoding long-term plasticity in response to novelty. Studies involving numerous genes in the proposed biological pathway have demonstrated an association between acquisition and retention of novel information and establishment of LTD (Manahan-Vaughan and Braunewell, 1999; Artola et al., 2006; Etkin et al., 2006; Plath et al., 2006; Gallitano-Mendel et al., 2007; Kemp and Manahan-Vaughan, 2007). Exposure to novelty enhances LTD (Manahan-Vaughan and Braunewell, 1999) and also induces

expression of pathway genes such as *Egr3* and *Arc*, the latter of which persists in the hippocampal dentate gyrus for up to 8 h (Marrone et al., 2012; Ramirez-Amaya et al., 2013).

We have recently reported that this persistent expression of *Arc* induced by a brief novel exposure requires *Egr3* (Maple et al., 2017). We have also reported that *Egr3* deficient mice, which have deficits in hippocampal LTD, also demonstrate hyper-reactivity to novel environments. Not only does exploration of a novel location induce LTD, but the reverse is also true; that low frequency stimulation of the hippocampus *in vivo* facilitates the exploratory behavior of rodents (Manahan-Vaughan and Braunewell, 1999). It is intriguing to consider whether dysfunction in these processes in humans may contribute to the cognitive and memory deficits that are a key feature of schizophrenia. These findings suggest it will be of great interest to investigate whether other genes recently identified as candidates for influencing schizophrenia risk may also play a role in this specific form of hippocampal synaptic plasticity.

Response to Stress and Novelty

Stress and exposure to novelty are two of the major stimuli that activate expression of immediate early genes, including *EGR3* and *ARC*, key output proteins in our proposed biological pathway (Senba and Ueyama, 1997; Marrone et al., 2012; Ramirez-Amaya et al., 2013; Maple et al., 2015). These stimuli also facilitate induction of hippocampal LTD, a form of synaptic plasticity associated with the formation and retention of immediate memories of novel experiences (Rowan et al., 1998; Manahan-Vaughan and Braunewell, 1999; Xiong et al., 2003; Artola et al., 2006; Kemp and Manahan-Vaughan, 2007; Collingridge et al., 2010).

Our prior work characterizing the phenotypes of $Egr3^{-/-}$ mice revealed that *Egr3* is required for the normal response to stress and novelty (Gallitano-Mendel et al., 2007). Similar behavioral abnormalities in the stress and novelty responses have been reported in preclinical studies of other genes in the proposed pathway including CN, SRF and ARC (Miyakawa et al., 2003; Etkin et al., 2006; Kozlovsky et al., 2008; Bramham et al., 2010; Weinstock, 2017).

Schizophrenia is also characterized by a heightened sensitivity to stress, abnormal sensorimotor gating in response to stressful stimuli, and abnormalities in habituation to novel and stressful stimuli (Braff et al., 1992; Corcoran et al., 2001; van Os et al., 2010; Holtzman et al., 2013; Kahn et al., 2015). The fact that LTD is facilitated by novelty and stress, and is disrupted when genes of our proposed pathway, numerous of which are associated with schizophrenia risk, are dysfunctional, suggests an intriguing link between this form of synaptic plasticity and the processes that are awry in schizophrenia.

PROTEINS THAT FORM THE PATHWAY

NMDA Receptor

NMDARs are activated in response to neuronal depolarization, which occurs when the brain responds to stimuli in the environment. NMDARs are one of three major classes of ligand-gated ionotropic receptors for glutamate in the brain. They are multi-subunit Ca++ channels, formed by hetero-tetramers of two glycine-binding (GLUN1), and two glutamate-binding (GLUN2), subunits (Papadia and Hardingham, 2007). The GLUN1 subunit, which has eight isoforms, is encoded by the *GRIN1* gene. In contrast, there are four subtypes of GLUN2 subunits (GLUN2A, GLUN2B, GLUN2C and GLUN2D), which are encoded by four different genes (*GRIN2A*, *GRIN2B*, *GRIN2C* and *GRIN2D*, respectively). Each subunit has three distinct domains, an external N-terminal domain, a transmembrane domain that forms the channel pore, and an internal C terminal domain (Carvajal et al., 2016). The NMDAR is activated when glutamate binds to the GLUN2 subunit, coincident with binding of co-agonists magnesium and glycine/D-serine to GLUN1 (Balu, 2016). Depolarization of the neuron is essential for NMDAR function, as this change in charge is required to remove the Mg++ ion that blocks the channel pore region, thereby allowing calcium to enter the neuron.

Evidence for a Role in Schizophrenia

The NMDAR hypofunction model of schizophrenia was proposed in the 1990's (Javitt and Zukin, 1991; Olney et al., 1999) based on studies highlighting the ability of NMDAR antagonists, phencyclidine and ketamine, to cause psychosis and memory impairments in humans. The effect of these drugs in rodents were the basis for subsequently defining "schizophrenia-like" behavioral abnormalities in animal models (Malhotra et al., 1997; Lahti et al., 2001; Amitai and Markou, 2010; van den Buuse, 2010).

Mice deficient for the *GluN1* subunit of the NMDAR show schizophrenia-like behavioral abnormalities that are reversed with antipsychotic treatment (Mohn et al., 1999). Studies employing a conditional knockout of the *GluN1*subunit have been used to identify specific cell types in which disruption of the receptor results in cognitive and behavioral defects. Selective disruption of the *GluN1* gene in excitatory neurons of layer II/III of the prefrontal and sensory cortices of mice causes impairments in short-term memory and prepulse inhibition (Rompala et al., 2013), and *GluN1* gene inactivation in parvalbumin interneurons caused deficits in habituation, working memory and associative learning (Carlén et al., 2012).

Genetic studies also support the importance of NMDAR genes in schizophrenia. Genome wide significant associations with schizophrenia have been reported for at least two of the NMDAR genes- *GRIN2A* and *GRIN2B* (Taylor et al., 2016). *GRIN2A* was recently shown to be associated with schizophrenia in the largest GWAS to date (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Moreover, it is the sole gene at the locus (108 loci-region 82), and thereby also contains the "Index SNP", defined as the SNP with the greatest significance at the disease-associated genomic locus (Please refer to **Supplementary Table S1** for details of all index SNPs found in genes from our biological pathway). Genetic variation in the *GRIN2B* gene has also been reported to be associated with schizophrenia (Awadalla et al., 2010; Demontis et al., 2011; Kenny et al., 2014).

A large exome sequencing study of *de novo* mutations in schizophrenia conducted by Fromer et al. (2014) used DNA from 623 family "trios" (affected individual and both parents) to

identify novel mutations in schizophrenia patients. They found that *de novo* mutations are increased in schizophrenia patients compared to the general population and, that these mutations were enriched in genes encoding proteins forming the NMDAR complex (Fromer et al., 2014), further supporting the hypothesis that dysfunction of NMDARs, or their downstream processes, increases risk for schizophrenia.

Postmortem brain tissue studies have shown region-specific alterations in NMDAR subunits at both the gene and protein levels. Weickert et al. (2013) showed decreased GRIN1 gene and protein expression, and decreased *GRIN2C* gene expression, in the dorsolateral prefrontal cortex regions of schizophrenia patients compared to controls. In a separate study, Gao et al. (2000) demonstrated high levels of *GRIN1* and low levels of *GRIN2C* mRNA in postmortem hippocampal samples from schizophrenia patients compared with healthy controls.

Role in LTD

NMDARs have long been recognized to play critical roles in hippocampal long-term potentiation (LTP) and LTD, forms of synaptic plasticity that correlate with learning and memory (Bear and Malenka, 1994; Malenka and Bear, 2004). LTP is defined as the activity-dependent strengthening of synapses (Scharf et al., 2002), while LTD refers to an activity-dependent decrease in synaptic strength (Mulkey et al., 1994). Although NMDARs influence both forms of synaptic plasticity, a role in LTD appears to be a feature of numerous genes functioning both upstream and downstream of EGR3, and is therefore the focus of this review.

LTD mediated by NMDARs is crucial for consolidation of hippocampal dependent memory (Wong et al., 2007; Brigman et al., 2010; Ge et al., 2010), and alterations in the ratio of GLUN2A:GLUN2B subunits affects levels of LTD induced by a low frequency (3–5 Hz) electrical stimulation (Cui et al., 2013). Mice that lack *GluN2B* in pyramidal neurons in the cortex and CA1 regions of the hippocampus show impaired NMDAR-mediated LTD, and decreased dendritic spine density in CA1, compared to wildtype mice (Brigman et al., 2010).

Relationship to EGR3

The connection between NMDAR activation and *Egr3* gene expression was first shown by Yamagata et al. (1994), who demonstrated that high frequency stimulation of rat hippocampal neurons that induces LTP also activates expression of *Egr3*. Both *Egr3* expression and establishment of LTP are blocked by pretreatment with the NMDAR antagonist MK-801. Thus, this form of activity-dependent expression of *Egr3* in the hippocampus requires NMDAR function.

Interestingly, *Egr3* also influences the function of NMDARs. *Egr3*$^{-/-}$ mice display deficits in the electrophysiologic response of hippocampal neurons to drugs selective for GLUN2B-containing NMDARs (Gallitano-Mendel et al., 2007). However, the fact that quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis did not reveal differences in the levels of *Grin2b* mRNA in *Egr3*$^{-/-}$ mice compared with controls, suggests that EGR3 may not be directly regulating expression of this NMDAR subunit (Gallitano-Mendel et al., 2007). Others have reported that overexpression of *Egr3* (but not *Egr1*) *in vitro* regulates expression of an NMDAR subunit *NR1*-promoter-driven reporter construct, a response that is BDNF dependent (Kim et al., 2012). Thus, *Egr3* expression is induced by neuronal activity in an NMDAR-dependent fashion. In addition, *Egr3* is also required for function of GLUN2B-containing NMDARs, though the mechanisms underlying this regulation have not yet been established.

Calcineurin

A key process triggered by calcium influx through NMDARs is the activation of calcium-dependent phosphatases, including CN (Horne and Dell'Acqua, 2007). CN is a multi-subunit protein consisting of two main subunits: the catalytic subunit calcineurin A, and the calcium binding regulatory subunit calcineurin B (Rusnak and Mertz, 2000). CN plays important roles in both the immune system and the brain. In T-cells, mitogenic stimuli increase levels of intracellular calcium, causing calmodulin-mediated activation of CN. CN then dephosphorylates nuclear factors of activated T-cell (NFAT) in the cytoplasm, causing its translocation to the nucleus where it regulates expression of target genes (Rao et al., 1997). In the nervous system, CN acts in conjunction with NFAT proteins to regulate numerous critical processes—reviewed elegantly by Kipanyula et al. (2016). These range from developmental roles in corticogenesis (Artegiani et al., 2015) and myelination (Kao et al., 2009), to activity dependent synaptogenesis (Ulrich et al., 2012), to glial cell activation, particularly in the context of neuroinflammation (Neria et al., 2013).

Evidence for a Role in Schizophrenia

An increasing body of *in vivo* and genetic evidence suggests a role for CN in the pathogenesis of schizophrenia. Immunosuppressive drugs that block CN, such as cyclosporin A and FK-506 produce side effects reminiscent of the symptoms of schizophrenia. These include memory impairment, auditory and visual hallucinations, paranoia, depression, and flattened affect (Bechstein, 2000; Porteous, 2008; American Psychiatric Association, 2013). Forebrain specific CN knockout mice show cognitive and behavioral abnormalities consistent with animal models of schizophrenia, such as working memory deficits (Zeng et al., 2001), increased locomotor activity and abnormalities in attention, social interaction and nesting behavior (Miyakawa et al., 2003).

Human genetic studies have identified associations between variations in *PPP3CC*, the gene that encodes the α-1 subunit of calcineurin A, and schizophrenia, in both Caucasian (Gerber et al., 2003) and Asian populations (Horiuchi et al., 2007; Liu et al., 2007; Yamada et al., 2007). Yamada et al. (2007) noted that *EGR3* was located near *PPP3CC* (within a 252 kb interval) on the short arm of chromosome 8, and showed through linkage disequilibrium studies that they form distinct regions of schizophrenia susceptibility. Although *PPP3CC* is not listed as a candidate gene at one of the 108 loci associated with schizophrenia, numerous other non-calcineurin protein phosphatase genes do map to the loci identified in that GWAS. These include: *PPP1R13B*, *PPP1R16B*, *PPP2R3A* and *PPP4C*. This suggests the importance of protein-phosphatases in the

etiology of schizophrenia. Notably serine/threonine phosphatase PP1/PP2A is a crucial player in synaptic plasticity and LTD (Mulkey et al., 1994).

Role in LTD

The forebrain specific CN knockout mice that display schizophrenia-like behavioral abnormalities also have disrupted hippocampal LTD, as well as deficits in hippocampal working and episodic memory (Zeng et al., 2001). Notably, this hippocampal LTD deficit phenotype is also seen in *Egr3*$^{-/-}$ mice (Gallitano-Mendel et al., 2007). Several additional lines of evidence suggest CN to be a crucial regulator of LTD. CN regulates dephosphorylation, and subsequent removal, of a membrane bound AMPAR subunit during LTD (Mulkey et al., 1994; Sanderson et al., 2016), which is induced by transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor activation (Chávez et al., 2010). These observations collectively highlight CN as an important regulator of behavior, memory, and LTD and as a schizophrenia candidate gene that regulates *EGR3*, making this protein an integral component of our proposed biological pathway.

Relationship to EGR3

Evidence that CN regulates expression of *Egr3* comes from the immune system. T-cell activation induces expression of *Egr3*, which, in turn, directly regulates expression of Fas-ligand (Fasl). Expression of both *Egr3* and its target gene is blocked by the CN inhibitor cyclosporin A (Mittelstadt and Ashwell, 1998). Although a similar interaction has not been investigated in the brain, the remarkable similarity in the behavioral and electrophysiologic phenotypes of CN knockout, and *Egr3*$^{-/-}$, mice strongly suggests this molecular pathway is conserved in the brain (Zeng et al., 2001; Miyakawa et al., 2003; Gallitano-Mendel et al., 2007). Both CN knockout and *Egr3*$^{-/-}$ mice show deficits in LTD, spatial learning (Zeng et al., 2001; Gallitano-Mendel et al., 2007), and heightened responsiveness to handling (Gallitano-Mendel et al., 2007; supplemental data, Miyakawa et al., 2003). These intriguing initial findings support the need to determine whether this regulatory relationship between CN and *Egr3* found in the immune system is also present in the brain.

NFATc3 (NFAT4)

The NFATs comprise a family of transcription factors that regulate activity-dependent gene expression in the immune system and the brain (Macian, 2005; Vihma et al., 2016). They reside in the cytoplasm in an inactive, phosphorylated state. Activity-triggered calcium entry into the cell stimulates CN to dephosphorylate NFAT proteins, allowing them to translocate to the nucleus where they activate expression of immediate early genes (Abdul et al., 2010). NFATs can be shuttled back into the cytoplasm via nuclear export sequences (nuclear localization signal) that, once exposed, lead to rephosphorylation of NFATs by kinases (Okamura et al., 2000) and subsequent inhibition of NFAT-mediated gene transcription (Hogan et al., 2003).

The NFAT family comprises five genes, four of which are calcium regulated and include *NFAT1-4*, and one that is osmotic tension-regulated, namely *NFAT5*, in both humans and mice (Vihma et al., 2008). NFAT proteins contain a C-terminal Rel homology domain that enables them to interact with other transcription factors (including EGRs) to co-regulate expression of downstream genes. Additional protein regions include an N-terminal domain that contains two CN binding sites (Aramburu et al., 1998; Park et al., 2000), the nuclear localization sequence, and serine residues that are sites of phosphorylation (Kiani et al., 2000).

As transcription factors, NFATs regulate expression of a wide array of genes ranging from cytokines (Klein et al., 2006), to growth factors (Hernández et al., 2001), to cell-cycle regulators (Caetano et al., 2002). In conjunction with CN, NFATs mediate a variety of physiological processes such as angiogenesis (Courtwright et al., 2009), osteogenesis (Winslow et al., 2006), and cardiovascular system development (Schulz and Yutzey, 2004). In the nervous system the NFATs have numerous roles, which include regulation of synaptic plasticity (Schwartz et al., 2009) and neurotrophin signaling (Groth et al., 2007; reviewed in Kipanyula et al., 2016).

We focus our discussion on NFATC3 (a.k.a. NFAT4), as it regulates expression of *Egr3*, and it is the only family member to show genome wide association with schizophrenia (Rengarajan et al., 2000; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Pouget et al., 2016). *Nfatc3* is expressed in the adult mouse cerebellum, hippocampus, choroid plexus and ependymal cells as shown by *in situ* hybridization studies, and in the midbrain, pons, striatum, and thalamus using reverse transcription polymerase chain reaction (RT-PCR; Vihma et al., 2008). The level of expression varies across these regions with the highest expression in the mouse cerebellum and granular cell layer of the dentate gyrus in human brain tissue (Vihma et al., 2008). *In vitro* studies demonstrate that NFATC3 is one of the most highly activated NFAT members following neuronal depolarization (Vihma et al., 2016). To date, *NFATC3* remains the only NFAT family member to show genome wide association with schizophrenia and regulate gene expression of immediate early genes, both of which are discussed below.

Evidence for a Role in Schizophrenia

NFATC3 maps to locus 85 of the 108 loci identified by the psychiatric genetics consortium and is, in fact, the index SNP for that locus (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). This initial finding has subsequently been supported by a follow-up GWAS that examined immune system-related genes from the original consortium study in an extended cohort (Pouget et al., 2016). Results of this validation study identified both *NFATC3* and *EGR1* among the six immune genes that showed genome-wide significance. Pouget et al. (2016) propose that *NFATC3*, which, like each of the 5 candidate genes they followed up, is known to have a predominantly "immune" role in peripheral organs, may be playing a non-immune role in the brain.

The vast majority of work investigating the activity and function of NFATC3 has focused on its roles in the immune system. Studies of *Nfatc3* knockout mice show reduced numbers of CD4 and CD8 cells in the thymus and spleen, and increased apoptosis of T-cells, suggesting the importance

of *Nfatc3* in T-cell development and survival. No brain or nervous system phenotypes were reported in this study (Oukka et al., 1998). However emerging data suggest that NFATC3 has neuroprotective functions. It mediates astrocyte activation in response to brain damage (Serrano-Pérez et al., 2011; Yan et al., 2014), protection against apoptosis in neuronal cells *in vitro* (Butterick et al., 2010), and growth and differentiation of neuronal precursors (Serrano-Pérez et al., 2015). NFATC3 also mediates neurodegenerative effects including methamphetamine-induced apoptosis (Jayanthi et al., 2005), and apoptosis via Fas activation in lithium-induced neurotoxicity *in vivo* (Gómez-Sintes and Lucas, 2010). The latter of these is reminiscent of *Egr3*-mediated regulation of Fasl in T-cells (Mittelstadt and Ashwell, 1998, 1999; Rengarajan et al., 2000). Although there are no published studies investigating the role of NFATC3 in LTD, its position as an activity-dependent regulator of *Egr3* in this pathway leads us to hypothesize that NFATC3 will be necessary for this form of synaptic plasticity.

Relationship to EGR3

In the immune system, CN regulates *Egr3* expression in response to T-cell activation, an interaction that is mediated via NFATs. NFATC3 regulates expression of *Egr3* in T-cells in mice. *Egr3* expression is reduced in amount and duration in T-cells of mice lacking either NFATC2 or NFATC3, and is nearly absent in NFATC2/C3 double knockout mice. This regulation is direct, as both NFATC2 and NFATC3 are able to transactivate the *Egr3* promoter *in vitro* (Rengarajan et al., 2000). We hypothesize that, in neurons, activity-dependent activation of CN dephosphorylates NFATC3, allowing it to enter the nucleus where it binds to the *Egr3* promoter and activates its expression. While NFATC2 may also regulate neuronal expression of *Egr3*, as well as of *Egr2*, and potentially *Egr1*, the strong evidence for NFATC3 interacting with EGR3 is the basis for including this family-member as a critical component of our proposed biological pathway.

EGR3

EGR3 is a member of the Egr family of immediate early gene zinc finger transcription factors, and is activated downstream of numerous schizophrenia candidate proteins, including NRG1, NMDAR and CN (Yamagata et al., 1994; Mittelstadt and Ashwell, 1998; Hippenmeyer et al., 2002). This family consists of four genes, *Egr1-4*, that are activated in response to a wide range of environmental stimuli, including stress (Senba and Ueyama, 1997).

Egr3 was identified through a screen for genes homologous to *Egr1*. Like the founding family member, *Egr3* is also highly expressed throughout the brain, including in the cortex, hippocampus, basal ganglia (Yamagata et al., 1994) and suprachiasmatic nucleus (Morris et al., 1998), as well as in the immune system and other organs (Tourtellotte and Milbrandt, 1998; Mittelstadt and Ashwell, 1999; Tourtellotte et al., 2001) *Egr3* mRNA is highly induced in response to electroconvulsive seizure in hippocampal and cortical neurons, and in hippocampal dentate gyrus granule cells, by NMDAR activation (Yamagata et al., 1994), prompting further research into its role in synaptic plasticity and behavior (Gallitano-Mendel et al., 2007, 2008).

Evidence for a Role in Schizophrenia and LTD

Investigations published by our group revealed that $Egr3^{-/-}$ mice display schizophrenia-like behavioral abnormalities, including locomotor hyperactivity that is reversed by antipsychotic treatment. In addition, these mice show immediate memory deficits, heightened reactivity to novelty, and disrupted hippocampal LTD (Gallitano-Mendel et al., 2007). Induction of LTD has recently been shown to inactivate fear-related memory *in vivo*; however, we do not know whether *Egr3* plays a role in this process (Nabavi et al., 2014). $Egr3^{-/-}$ mice also display a marked resistance to the sedating effect of clozapine, and other second-generation antipsychotics, which parallels the resistance that schizophrenia patients show to the side-effects of these medications (Cutler, 2001). This phenotype may be explained, at least in part, but the reduced level of serotonin 2A receptors found in the frontal cortex of $Egr3^{-/-}$ mice, a feature also seen in the brains of patients with schizophrenia (Williams et al., 2012; Selvaraj et al., 2014).

Our research also showed that $Egr3^{-/-}$ mice exhibit increased aggression in response to a foreign intruder (Gallitano-Mendel et al., 2008). This response was abrogated by chronic clozapine administration, despite the fact that the $Egr3^{-/-}$ mice are resistant to the sedative effects of this antipsychotic (Gallitano-Mendel et al., 2008). A similar phenomenon is seen in schizophrenia patients treated with clozapine (Hector, 1998; Chalasani et al., 2001; Chengappa et al., 2002), in whom the anti-aggressive effect of the drug can be distinguished from its sedating effect (Krakowski et al., 2006). These roles that *Egr3* plays in memory, synaptic plasticity, and behavior, and the response to antipsychotics that mimics that of patients, suggest that abnormal function of EGR3 in humans may contribute to schizophrenia pathogenesis or symptomatology.

Genetic association of *EGR3* with schizophrenia has been shown in Chinese (Ning et al., 2012; Zhang et al., 2012), Japanese (Yamada et al., 2007) and Korean (Kim et al., 2010) populations, and more recently in a population of European descent (Huentelman et al., 2015). It was also reported that decreased *EGR3* mRNA levels were observed in postmortem dorsolateral prefrontal cortex samples of schizophrenia patients compared with controls (Yamada et al., 2007). In addition, *EGR3* was identified in a screen for genes that are expressed at reduced levels in the brains of schizophrenia patients that do not smoke tobacco, and are normalized to control levels in patients that smoke. Notably, *EGR3* levels followed a pattern identical to that of *CN* in the study, consistent with its regulation by CN in the proposed biological pathway (Mexal et al., 2005).

The 8p chromosomal region, where *EGR3* resides, is a long-recognized hub for schizophrenia associations (Suarez et al., 2006; Lohoff et al., 2008). A recent review highlighted that *EGR3* was among 20 genes of interest for schizophrenia, map to the 8p locus (Tabarés-Seisdedos and Rubenstein, 2009). Association of an *EGR3* SNP with decreased prefrontal hemodynamic response was observed during a verbal fluency task in both healthy controls and schizophrenia patients (Nishimura et al., 2014).

Both human and *in vivo* animal studies suggest that overexpression of EGR3 may also negatively impact non-neural physiological processes, including immune system function. High levels of *EGR3* positively correlate with levels of proinflammatory cytokines in peripheral monocytes of schizophrenia patients (Drexhage et al., 2010). This may be mediated through the Triggering receptor expressed on myeloid cells 1 (TREM-1), a key regulator of inflammation in both brain microglia and peripheral monocytes, as EGR3 directly binds the TREM-1 promoter in monocytes (Weigelt et al., 2011).

These data support the hypothesis that EGR3 may function as a master regulator of multiple processes that are relevant to the pathophysiology of schizophrenia. In line with this theory, bioinformatic analyses have predicted EGR3 to be a central modulator of a regulatory network of microRNAs and transcription factors associated with schizophrenia (Guo et al., 2010). Despite these findings, *EGR3* was not identified as a gene residing at one of the 108 loci associated with schizophrenia. Genome-wide association studies from the PGC schizophrenia studies suggest that none of the SNPs in the region including *EGR3* showed a significant association. The SNP that showed the lowest *p* value was an intergenic SNP, rs12541654 ($p = 0.23$, OR = 0.99)[1]. However other EGR family members, such as *EGR1*, and EGR coregulatory binding factor NGFI-A Binding Protein 2 (*NAB2*), are each index SNPs at one of the 108 loci (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). EGR3 binds to NAB2 to co-regulate expression of target genes, and is involved in co-regulatory feedback relationships with EGR1, as well EGR1, EGR2 and EGR3 can regulate *NAB2* expression, demonstrating a functional interaction of both of these schizophrenia associated genes in the proposed pathway (Svaren et al., 1996; Mechta-Grigoriou et al., 2000; Kumbrink et al., 2005, 2010; Srinivasan et al., 2007).

ARC

One of the first EGR3-target genes to be elucidated was *Arc* (Li et al., 2005). *Arc* was originally identified as a novel transcript in the adult rat brain that was rapidly and strongly induced in response to electroconvulsive seizures (Link et al., 1995; Lyford et al., 1995). Induction of *Arc* was observed *in vitro* in PC12 cells following exposure to growth factors such as nerve growth factor (NGF) and epidermal growth factor (EGF; Lyford et al., 1995). *Arc* mRNA and protein are enriched in neuronal dendrites, and ARC protein colocalizes with the actin cytoskeletal matrix (F-actin; Lyford et al., 1995). These observations led to its name "ARC". Further characterization of the role of ARC protein in cytoskeletal function revealed that it also associates with microtubules and microtubule associated protein (MAP2; Fujimoto et al., 2004). ARC was also shown to maintain phosphorylation of cofilin, the actin depolymerization factor, and to promote F-actin formation (Messaoudi et al., 2007).

ARC is capable of localizing to NMDAR and postsynaptic density (PSD) 95 protein complexes (Husi et al., 2000; Donai et al., 2003) suggesting that it may be involved in synaptic neurotransmission. ARC also binds to multiple components of the clathrin-dependent endocytic machinery including endophilin and dynamin, and decreases total and surface AMPAR (GluR1) protein levels via endocytosis in hippocampal neurons *in vitro* (Chowdhury et al., 2006). Numerous proteins bind to ARC to mediate its many important roles in the nervous system. Notably 4–8 of these binding partners, termed "ARC complex proteins", were identified in schizophrenia GWAS (Kirov et al., 2012; Fromer et al., 2014; Purcell et al., 2014) and are discussed in depth in the next section.

Evidence for a Role in Schizophrenia

The behavioral phenotype of $Arc^{-/-}$ mice was recently characterized and revealed schizophrenia-like abnormalities. These include deficits in prepulse inhibition and recency discrimination (indicative of cognitive dysfunction), impairments in response to social novelty, hyperactivity in response to amphetamine administration, and region-specific alterations in dopamine (Managò et al., 2016). Though they retain the ability to form intact short-term memory, $Arc^{-/-}$ mice fail to form long lasting memories.

The necessity of *Arc* for normal memory and behavior in mice suggest that dysfunction of *ARC* in humans could result in abnormalities that increase risk to develop psychiatric illness. Indeed, *ARC* mRNA expression is decreased in the frontal cortex of schizophrenia patients (Guillozet-Bongaarts et al., 2014). In addition, genes encoding proteins that bind to ARC, "ARC-complex proteins", were identified in several large-scale, genome-wide schizophrenia genetic association studies. Kirov et al. (2012) reported that *de novo* CNVs in the genes *DLG1*, *DLG2*, *DLGAP1*, *CYFIP1*, each of which encode ARC-complex proteins, show significant enrichment in individuals with schizophrenia. A separate case-control study showed an enrichment of disruptive mutations in genes encoding ARC-complex proteins in schizophrenia cases vs. controls (Purcell et al., 2014). The nine mutations identified in this study included nonsense, essential splice site, and framseshift mutations, that occurred in nine genes, and were found *only* in schizophrenia cases, with none occurring in controls (Purcell et al., 2014). In addition, the study by Fromer et al. (2014) that identified increased *de novo* mutations in NMDAR-complex gene in schizophrenia patients also revealed that these mutations are enriched in genes encoding proteins associated with the ARC-complex. Fromer et al. (2014) showed that loss of function mutations are 17-fold enriched in genes encoding ARC-complex proteins in their cohort and 19-fold enriched in the data set from the Purcell et al. (2014) study. They concluded that "ARC disruption has particularly strong effects on disease risk" (Fromer et al., 2014).

These and other findings implicated ARC as a critical protein in a synaptic pathway involving voltage gated calcium channels, NMDARs, PSD95, FMRP, mGLuR and AMPARs, in schizophrenia risk (Hall et al., 2015). Although these studies did not report genetic association of variations in the *ARC* gene, itself, with schizophrenia, our group subsequently reported the first association of a SNP in the *ARC* gene with schizophrenia in two separate populations (discussed below; Huentelman et al., 2015). More recently, hypermethylation of eight CpG sites,

[1]http://zzz.bwh.harvard.edu/code.html

and presence of several rare variants that reduce reporter gene activity, were reported in the putative ARC promoter region of schizophrenia patients vs. controls (Chuang et al., 2016). These findings indicate that altered genetic and epigenetic regulation of *ARC* expression, specifically that reduces ARC function, may increase risk for schizophrenia.

Role in LTD

Like many of the upstream proteins in the proposed pathway, ARC is also required for hippocampal LTD. NMDA-mediated LTD deficits in Schaffer collateral-CA1 synapses were found in slices from $Arc^{-/-}$ mice compared to wildtype mice (Plath et al., 2006). In addition, mGLuR-mediated LTD was shown to depend on rapid translation of ARC, and ARC-mediated AMPAR endocytosis, in hippocampal neurons (Waung et al., 2008).

Neuronal activity stimulates expression of ARC, which then accumulates at the spines of *inactive* synapses, effectively "tagging" these synapses for remodeling. This process is dependent on Ca^{2+}/calmodulin-dependent protein kinase II (CamKIIβ), and results in ARC-mediated AMPA receptor endocytosis (Okuno et al., 2012). Minatohara et al. (2016) suggest that ARC expression and localization during LTD may increase the strength of the active synapses by causing endocytosis of AMPARs at the inactive synapses. $Arc^{-/-}$ mice also show decreased spine density and increased spine width in CA1 pyramidal neurons and DG cells (Peebles et al., 2010), suggesting that ARC plays a critical role in maintenance of spine morphology that may impact learning and behavior. These data suggest that decreased *ARC* expression results in deficits in LTD.

Relationship to EGR3

Previous research has shown that *Arc* is a transcriptional target of EGR3 (Li et al., 2005). Our proposed biological pathway (Gallitano-Mendel et al., 2007, 2008) combines environment stress-responsive proteins *EGR3*, CN, and NMDARs, each of which are associated with schizophrenia susceptibility. Since mice deficient for these proteins share phenotypes of LTD and memory deficits, similar to mice lacking *Arc*, we hypothesized that the *ARC* gene should likewise be a schizophrenia candidate gene.

To test this hypothesis, we used next generation sequencing to resequence the *ARC* gene, and flanking regions, from schizophrenia patient and control subjects from two separate ethnic groups. These studies revealed association between the *ARC* SNP and schizophrenia in both ethnic groups (Huentelman et al., 2015). In a separate study, we found that the *ARC* SNP was associated with response to cognitive remediation therapy in a cohort of new onset psychosis patients (Breitborde et al., 2017). Recently, a heritable chromosomal microdeletion that encompasses several genes, including *ARC*, was also shown to be associated with several neurodevelopmental psychiatric disorders such as attention deficit hyperactivity disorder and autism spectrum disorder (Hu et al., 2015) in addition to schizophrenia. Overall, substantial evidence suggests that ARC, an important regulator of synaptic function, memory, and LTD, influences risk for schizophrenia, and possibly other neuropsychiatric illnesses. As a direct target of EGR3, *ARC* represents an important output element of our proposed biological pathway.

ADDITIONAL GENES THAT INTERACT WITH THE PATHWAY

Neuregulin-1

NRG1 was one of the first schizophrenia candidate genes to be identified using molecular genetic methods at a genomic locus defined by linkage analysis studies in family pedigrees (Stefansson et al., 2002). It has subsequently been validated in numerous populations, and supported by preclinical studies (recently reviewed in, Mostaid et al., 2016). NRG1 regulates *EGR3* in human muscle and breast cancer cell lines (Sweeney et al., 2001; Jacobson et al., 2004). In mice NRG1 regulates *Egr3* expression in developing muscle cells (Jacobson et al., 2004), and this regulation is required to maintain development of muscle spindles. These studies demonstrate a functional link between these two schizophrenia-associated genes in two types of human cell lines, and *in vivo* in mice (Hippenmeyer et al., 2002; Jacobson et al., 2004; Herndon et al., 2013). Although this regulatory relationship has not yet been validated in the brain, we hypothesize that a similar regulatory relationship may functionally link these two schizophrenia-associated genes in the central nervous system as well.

No studies investigating LTD in NRG1 deficient mice have been reported. However, the fact that NRG1 regulation of *Egr3* in the muscle spindle is mediated by SRF (Jacobson et al., 2004; Herndon et al., 2013), and loss of either *Srf* or *Egr3* results in memory and LTD deficits (Etkin et al., 2006; Gallitano-Mendel et al., 2007), leads us to predict that NRG1 should also play a critical role in this form of hippocampal synaptic plasticity. Indeed, NRG1 impairs endocannabinoid 2-arachidonoylglycerol (2-AG)-mediated LTD in rat hippocampal slices (Du et al., 2013).

Serum Response Factor (SRF)

We have previously highlighted SRF as a protein in the proposed biological pathway for schizophrenia susceptibility based on its requirement for both novelty memory and LTD in mice (Etkin et al., 2006), as well its regulatory interactions with other proteins in the pathway (Etkin et al., 2006; Gallitano-Mendel et al., 2007). SRF is activated downstream of NRG1 in mouse muscle cells (Herndon et al., 2013, 2014). *In vitro* studies demonstrate that NRG1 is capable of stimulating *SRF* expression in human HeLa cells expressing the NRG1 receptor ErbB4, and that this is mediated by mitogen-activated protein kinase (MAPK; Eto et al., 2010). SRF is also activated by CN/NFAT, in combination with other factors, in lymphocytes (Lin et al., 2002; Hao et al., 2003). In turn, SRF regulates expression of *Egr1* and *Egr2* in the brain in response to novelty, and mediates the regulation of *Egr3* by NRG1 in muscle spindle development (Hao et al., 2003; Ramanan et al., 2005; Etkin et al., 2006; Herndon et al., 2013). SRF has also been shown to regulate *Egr3* expression in the hippocampus (personal communication from Naren Ramanan).

Together with another transcription factor Elk-1, SRF regulates expression of *Egr1* in response to chemically-induced

LTD (by 3,5-dihydroxyphenylglycine, DHPG; Lindecke et al., 2006). And *Srf*-deficient mice have deficits in LTD (Etkin et al., 2006). Interestingly two *NRG1* schizophrenia-associated SNPs from the original Icelandic haplotype occur in regions that show predicted binding sites for SRF that are abolished by presence of the SNPs (Law et al., 2006). Although we are unaware of studies demonstrating that genetic polymorphisms in *SRF* are directly associated with risk for schizophrenia at this time, we hypothesize that the positioning of SRF in this pathway, and its requirement for hippocampal LTD, will lead to identification of such roles in the future.

EGR1

Egr1 is the founding member of the Egr family of immediate early gene transcription factors. It was identified as a gene activated in response to application of NGF to PC-12 cells in culture, and thus named "NGF inducible A" NGFI-A (O'Donovan et al., 1999). *Egr1* was independently identified by other laboratories, which accounts for its numerous aliases (zif-268, Krox-24, NGFI-A, TIS8, ZIF-268, ZNF225). *Egr1* is required for long-term memory formation and late-phase LTP (Cole et al., 1989; Worley et al., 1993).

While a number of studies have identified associations between *EGR1* and schizophrenia, the most significant is that of the 2014 Schizophrenia Working Group of the Psychiatric Genomics Consortium GWAS, in which *EGR1* resides in region 69 of the 108 loci associated with the illness, and contains the Index SNP for that region (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). In a follow-up study examining the consortium dataset, Pouget et al. (2016) identified six independent regions from the 108 loci where the SNP with the lowest association *p*-value (the Index SNP) was in an immune gene. *EGR1* was one of the six.

Postmortem brain tissue studies have also identified *EGR1* as one of a small number of genes differentially expressed in schizophrenia patients compared with controls (Pérez-Santiago et al., 2012). Studies have also identified differences in mRNA levels of *EGR1* in peripheral blood cells (Cattane et al., 2015; Xu et al., 2016; Liu et al., 2017), and one study in fibroblasts (Cattane et al., 2015), of schizophrenia patients compared with controls Interestingly, the differences in *EGR1* mRNA levels identified by the two research groups were in opposite directions, which may be due to methodological differences. In particular, the two studies reporting reduced peripheral blood cell levels of *EGR1* were examining schizophrenia patients during an acute psychotic episode, while the other study did not specify the patients' current symptom status (Cattane et al., 2015; Xu et al., 2016; Liu et al., 2017).

Studies in mice deficient for *Egr1* demonstrate that both long-term memory and LTP require function of this immediate early gene (Jones et al., 2001; Bozon et al., 2003). Furthermore, over-expression of *Egr1* facilitates hippocampal LTP and enhances long-term memory of spatial location (Penke et al., 2013). Although *Egr1* is upregulated in response to LTD induction by DHPG (Lindecke et al., 2006), loss of *Egr1* has not been reported to result in LTD deficits.

As the founding member of the EGR family, EGR1 shares gene sequence homology, and DNA binding element recognition, with EGR3. EGR1 and EGR3 are activated by many of the same stimuli and are expressed in the same cells in the brain (Yamagata et al., 1994), though their proteins follow different temporal patterns of expression and perdurance (O'Donovan et al., 1998).

Together EGR1 and EGR3 bind to the promoter, and induce expression of, their coregulatory factor *NAB2* in cells of neuroectodermal origin (Kumbrink et al., 2010). NAB2 protein, via interaction with the EGRs, subsequently feedback inhibits its own expression (Kumbrink et al., 2010). These regulatory relationships that EGR1 shares with EGR3 and other proteins in the proposed pathway, as well as with *NAB2*, another GWAS-implicated gene, strengthen the likelihood that the position of *EGR1* as an Index SNP in the 108 loci indicates an actual role for EGR1 in schizophrenia susceptibility.

NGFI-A Binding Protein 2 (NAB2)

NAB2 is an activity-dependent immediate early gene that functions as a transcriptional coregulatory protein by binding to a specific recognition domain on EGR family transcription factors EGR1, EGR2 and EGR3. In cells of neuroectodermal origin EGR1 and EGR3 bind in concert to the promoter of the *NAB2* gene to induce its expression (Kumbrink et al., 2010). NAB2 protein has both co-activation and co-repression actions. Once bound to the EGRs, NAB2 feedback inhibits its own expression, as well as that of EGRs (Svaren et al., 1996; Mechta-Grigoriou et al., 2000; Kumbrink et al., 2010).

The regulatory relationships with schizophrenia-associated EGRs (EGR1 and EGR3) suggest a role for NAB2 in neuropsychiatric illness. Direct support for possible association between *NAB2* and schizophrenia comes from the 2014 Schizophrenia Working Group of the Psychiatric Genomics Consortium GWAS. *NAB2* is located at region 20 of the 108 loci, and is one of the genes at the site of the locus' Index SNP (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014).

The majority of studies involving NAB2 have investigated its role in the immune system and cancer biology (Collins et al., 2006, 2008; Hastings et al., 2017). The role of NAB2 in the nervous system is much less well investigated, and the functions of NAB2 in the brain are still largely unknown. As an immediate early gene, *Nab2* expression is induced in the brain in response to stimuli (Jouvert et al., 2002). Among the stimuli that activate *Nab2* expression in neurons is BDNF (Chandwani et al., 2013). In the peripheral nervous system loss of both *Nab2*, and its family member *Nab1*, results in severe myelination defects (Le et al., 2005).

No studies have yet been published examining the role of NAB2 in memory or hippocampal synaptic plasticity, or in behavior. However, unpublished data from our laboratory indicate significant behavioral abnormalities in mice lacking only *Nab2* (Gallitano, unpublished observation). Despite the paucity of published work on the role of NAB2 in the brain, the regulatory relationships between NAB2, EGR1 and EGR3 link this gene not only to other specific schizophrenia-associated genes, but also to the proposed pathway for illness association.

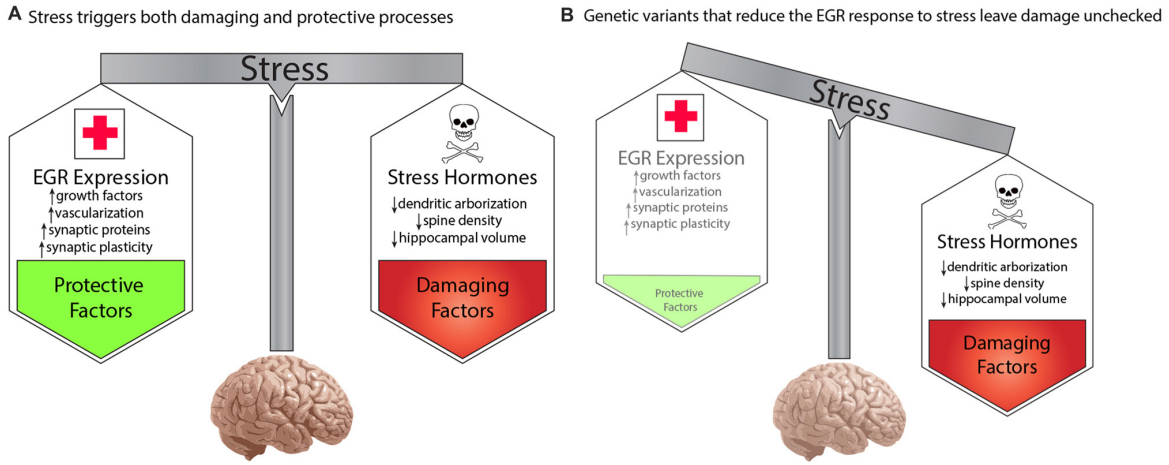

FIGURE 2 | A developmental model for genetic predisposition to stress susceptibility. **(A)** Stress hormones cause detrimental effects on the brain, such as decreased dendritic arborization, reduced density of synaptic spines, and decreased regional brain volumes, which are seen in schizophrenia. Stress also activates EGR family immediate early genes, which mediate numerous beneficial processes, such as growth factor response, vascularization, synaptic protein expression, and synaptic plasticity. The balance between these processes protects the brain from damage under stressful conditions. **(B)** Individuals carrying genetic variants that impair the activation of pathway proteins may be fine under normal conditions. However, in the context of stress, the damaging effects override the insufficient protective response, resulting in neurotoxic insults which, if sustained or repeated, may result in the neuropathology that gives rise to symptoms of schizophrenia. In this manner, genetic variations in genes of the proposed pathway create a predisposition to develop schizophrenia in a manner dependent upon the stress history of an individual.

Brain-Derived Neurotrophic Factor (BDNF)

Numerous studies have associated BDNF with mental illnesses, including schizophrenia (Islam et al., 2017). The requirement for BDNF, particularly its precursor (pro) form, for synaptic plasticity and memory, suggests a mechanism linking BDNF to neuropsychiatric disorders (reviewed in, Carlino et al., 2013). Specifically, the human *BDNF* polymorphism that converts the 66 position amino acid valine to a methionine (Val66Met) has been associated not only with mental illness risk, but also with memory (Egan et al., 2003; Hariri et al., 2003; Hashimoto et al., 2008; van Wingen et al., 2010).

BDNF is included in the proposed pathway as it has been shown to induce expression of *Egr3* in the mouse brain as an essential step in regulating expression of GABAA receptor alpha 4 subunit (Roberts et al., 2006; Kim et al., 2012). In a separate article submitted to this Research Topic issue (Meyers et al., 2017a), we demonstrate that *Egr3* is required for expression of *Bdnf* in the mouse dentate gyrus 1 h following seizure, a stimulus that induces high level *Bdnf* expression in this hippocampal region. Thus, BDNF may function both upstream, as well as downstream, of *Egr3*.

It was recently reported that BDNF-deficient mice display defects in hippocampal LTD (Novkovic et al., 2015; Bukalo et al., 2016). In addition, exogenous application of the precursor (pro) peptide of BDNF facilitates development of LTD, a process that requires function of GLUN2B containing NMDARs (Mizui et al., 2015; Kojima and Mizui, 2017). Notably, the hippocampal Schaffer collateral neurons in $Egr3^{-/-}$ mice, that fail to develop LTD, are unresponsive to the GLUN2B selective antagonist ifenprodil (Gallitano-Mendel et al., 2007), suggesting that GLUN2B-containing NMDARs are critical for both BDNF- and *Egr3*-, mediated LTD. These findings support a position for BDNF in our proposed pathway for neuropsychiatric illness risk.

DISCUSSION

In this article, we review evidence supporting an activity-dependent biological pathway that incorporates numerous schizophrenia candidate genes with critical roles in the regulation of memory and LTD, and that culminates in activation of the immediate early gene transcription factor *EGR3*. The unique position of immediate early gene transcription factors, at the nexus between environmental events and regulation of the neuronal response to activity, makes them ideally suited to account for both the genetic and environmental contributions to schizophrenia.

Additional proteins implicated in risk for schizophrenia, but that have not yet been reported to affect LTD, interact with this pathway either as upstream activators (e.g., NRG1) or as transcriptional regulators (e.g., EGR1 and NAB2). Individual genes in this pathway mediate neuroprotective functions including myelination, vascularization, growth factor regulation, and synapse formation, abnormalities in which are found in schizophrenia (Milbrandt, 1987; Mechtcheriakova et al., 1999; Jones et al., 2001; Jessen and Mirsky, 2002; Fahmy et al., 2003; Fahmy and Khachigian, 2007; Gallitano-Mendel et al., 2007). Dysfunction in any of the genes of this pathway would result in disruption of these processes, and may thereby account for the neuropathologic and clinical features of schizophrenia, including deficiencies white matter, brain volumes, and cerebral blood flow, reduced synaptic spine density, and deficits in memory and cognitive processing (Ingvar and Franzen, 1974; Saykin et al.,

1991; Glantz and Lewis, 2000; Moises et al., 2002; Davis et al., 2003; Nabavi et al., 2014).

Moreover, numerous genes in the pathway have critical functions in the immune system, particularly in T-cell activation, underscoring a longstanding recognition of a relationship between immune system and risk for schizophrenia. Finally, numerous genes that are part of the originally-defined pathway, as well as others that interact with proteins in the pathway, have subsequently been identified in the 108 loci GWAS (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Notably, each of the pathway genes that is at one of the 108 schizophrenia loci is, in fact, *the* Index SNP at that locus. Together these findings strongly support the biological relevance of our proposed pathway in schizophrenia.

In the "Introduction" section, we highlighted that there are two unanswered questions at the forefront of psychiatric genetics: (1) how can so many genes contribute susceptibility to schizophrenia? and (2) how do genes implicated in risk for schizophrenia interact with environmental factors to give rise to the disorder? The concept of a biological pathway addresses the first unanswered question by hypothesizing that numerous genes share roles in a much smaller number of critical functional processes. Disruption in these key biological processes create risk for schizophrenia.

To account for the effect of environment, we propose a model whereby genetic variations that decreased function of any protein in the pathway will result in increased risk for schizophrenia in a manner that is dependent upon the stress history of an individual. Stress is commonly thought of as detrimental. Specific negative consequences of stress demonstrated in animal and human studies include stress-hormone mediated decreases in dendritic arborization and spine density and reduced regional brain volumes (reviewed in Lupien et al., 2009). However, despite the pervasive experience of stress throughout life, most people do not develop severe mental illness. This is presumably because stress also activates molecular and cellular processes that are protective. The balance of damaging and protective responses to stress creates resilience and maintain homeostasis. This is shown in **Figure 2A**.

We propose that the activity-dependent biological pathway we have described here represents a component of the healthy biological response to stress. The normal activation of the pathway in response to stress triggers molecular and cellular processes that protects the brain from the harmful effects of stress hormones, and other detrimental elements of the stress response. While extreme, or unremitting, stress conditions may over-ride the buffering abilities of this protective arm of the stress response, it is sufficient to withstand typical episodic stress that occurs in daily life.

However, if an individual carries a genetic variation that decreases the responsiveness of a critical protein in our proposed pathway (**Figure 2B**), then exposure to stress will result in the detrimental effects of stress that are not sufficiently balanced by the protective elements. This preponderance of damaging effects over time is hypothesized to cause the neuropathologic features of schizophrenia that presumably give rise to symptoms of the illness. In the absence of sufficient stress, however, the lower level of function of the EGR pathway may be sufficient to prevent neuropathological consequences. This can explain how two individuals may carry the same genetic variation, but be discordant for schizophrenia.

In this manner, the proposed model would account for the dual genetic and environmental contributions to schizophrenia susceptibility, a crucial feature that has not been accounted for by previous gene or pathway models. We hope that this pathway and model provide the structure for investigation of additional components that may contribute to the neuropathology underlying schizophrenia and other neuropsychiatric disorders, advancing the field toward identification of more effective therapies, and perhaps one day a cure, for these illnesses.

We have focused on the role of our proposed pathway in schizophrenia due to the large body of supporting studies from human genetic, post-mortem, and basic science animal studies. However, we hypothesize that this pathway is relevant for other neuropsychiatric and neurodegenerative disorders in which stress plays a role and memory is affected. Indeed, *EGR* genes, and *EGR3* in particular, has been implicated in bipolar disorder, depression and Alzheimer's disease (Hokama et al., 2014; Pfaffenseller et al., 2016; Francis et al., 2017). Although in-depth discussion of these disorders is beyond the scope of this review, we look forward to application of our proposed pathway to these illnesses by others in the future.

AUTHOR CONTRIBUTIONS

ALG conceived the hypothesis that is the focus of the manuscript. KKM and ALG were both responsible for collecting literature, review and writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00023/full#supplementary-material

TABLE S1 | Supplemental table contains details regarding human variant studies discussed in the review. For each study discussed the associated gene, protein, variant, *p* value, effect size (if reported), and major findings have been summarized.

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REVIEW
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Immediate Early Genes, Memory and Psychiatric Disorders: Focus on c-Fos, Egr1 and Arc

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Many psychiatric disorders, despite their specific characteristics, share deficits in the cognitive domain including executive functions, emotional control and memory. However, memory deficits have been in many cases undervalued compared with other characteristics. The expression of Immediate Early Genes (IEGs) such as, *c-fos*, *Egr1* and *arc* are selectively and promptly upregulated in learning and memory among neuronal subpopulations in regions associated with these processes. Changes in expression in these genes have been observed in recognition, working and fear related memories across the brain. Despite the enormous amount of data supporting changes in their expression during learning and memory and the importance of those cognitive processes in psychiatric conditions, there are very few studies analyzing the direct implication of the IEGs in mental illnesses. In this review, we discuss the role of some of the most relevant IEGs in relation with memory processes affected in psychiatric conditions.

Keywords: Egr1, c-Fos, Arc, fear memory, episodic memory, rodents, psychiatric disorders

INTRODUCTION

The diagnoses of psychiatric disorders are based on a cluster of specific symptoms. The genetic, clinical and neuroimaging evidence suggests that they share important features including common affected functions (Bearden and Freimer, 2006). Dysregulation of some mnemonic processes and the mechanisms of neuroplasticity could contribute to prevalent neuropsychiatric diseases. Many psychiatric disorders, despite their specific characteristics, share deficits in the cognitive domain including executive functions, emotional control and memory (Pittenger, 2013). Even though memory deficits have been in many cases undervalued compared with other characteristics, they can be considered as an endophenotype across many psychiatric disorders (Kéri and Janka, 2004; Gur et al., 2007; Henry et al., 2012). Of the many types, episodic and fear memories are two main categories commonly affected in different psychiatric disorders (Dickerson and Eichenbaum, 2010). Episodic memory deficits have been reported in schizophrenia, autism, bipolar disorder, obsessive-compulsive disorder, panic disorder and major depression (Muller and Roberts, 2005; Exner et al., 2009; Brezis, 2015; Czepielewski et al., 2015; Herold et al., 2015; Oertel-Knöchel et al., 2015; Ragland et al., 2015; Vrabie et al., 2015; Meconi et al., 2016; Solomon et al., 2016; Whitton et al., 2016; Green et al., 2017). Fear memories can be linked to anxiety and particularly to post-traumatic stress disorder (PTSD; Parsons and Ressler, 2013; Briscione et al., 2014).

For decades, Immediate Early Genes (IEGs) were used as an indirect marker to measure neuronal activity and, although many of them are routinely measured in thousands of labs around the world, their role in many biological processes is still unknown. The IEGs most commonly used to map neuronal activity, *c-fos* and *Egr1*, have had their expression time-course systematically studied (for mRNA and protein expression dynamics; Mello and Ribeiro, 1998; Bisler et al., 2002; Zangenehpour and Chaudhuri, 2002). They encode transcription factors that influence neuronal physiology by regulating the expression of downstream target genes, normally referred to as late-response genes (Curran and Morgan, 1987; Curran and Franza, 1988; Herdegen and Leah, 1998; O'Donovan et al., 1999; Tischmeyer and Grimm, 1999; Pinaud, 2004; Pinaud et al., 2005). Both c-Fos and Egr1 interact with an array of other transcription factors (Herdegen and Leah, 1998; Knapska and Kaczmarek, 2004). Besides, other IEGs encode proteins that directly influence cellular function, as in the case of *arc* (reviewed in Lanahan and Worley, 1998).

The expression of, *c-fos*, *Egr1* and *arc* are related in literature to learning and memory. Changes in their expression observed in a limited number of cells in regions associated with learning and memory lead to widespread use as readout of plastic changes subserving long-term memory formation and maintenance in specific neuronal populations (Rosen et al., 1998; Guzowski et al., 1999; Ramírez-Amaya et al., 2005; Minatohara et al., 2016). In this review, we focus on the role of, c-Fos, Egr1 and Arc since they have been associated to different memory processes, becoming good candidates as markers or effectors of these processes usually affected in psychiatric conditions.

MEMORY AND PSYCHIATRIC DISORDERS

Memory is a complex process through which the information acquired during learning is stored. Memory processes can be studied by their stages (acquisition, consolidation, and retrieval), duration (short-term and long-term) and to the type of information that it is stored (explicit and implicit), which are reviewed somewhere else (Eysenck, 1988; Abel and Lattal, 2001; Kandel et al., 2014; Squire and Dede, 2015). We now have significant knowledge and consensus regarding the phases of the memory process as well as some of the key structures, signaling pathways and genes involved in many different types of memories. Though it is still a matter of debate "where" and "how" memories are stored, memories are "stored" as spatiotemporal *representations* within a given neuronal network (Davis et al., 2006). Then, a particular memory might produce an identifiable pattern due to expression of a specific set of activity dependent genes.

Memory consolidation is a process that is proposed to occur at the same synapses involved in the encoding of the information (Dudai, 2002). It includes the activation of transcription factors, protein synthesis, and post-translational modification that lead to plastic changes that make the memory trace stable (Lamprecht and LeDoux, 2004; Morris, 2006; Alberini, 2009; Ruediger et al., 2011). During retrieval, memory reactivation can trigger two quite different processes, *extinction* or *reconsolidation*. The reactivated memory trace becomes labile and is susceptible to being modified or disrupted in the process called reconsolidation. If there is a reinforcement of the original trace during retrieval, the reconsolidation—a protein synthesis-dependent process—re-stabilizes the original memory trace (Lee, 2008). On the contrary, after repeated reactivations in the absence of the appropriate reinforcement, memory extinction is triggered, leading to the loss in memory expression. This process is also protein-synthesis dependent and evidence shows that a new memory trace is formed, replacing the original one without erasing it (Quirk and Mueller, 2008). Then, it emerges that from the clinical point of view, it is important to understand the underlying mechanisms of these phenomena in order to develop new tools as novel treatment in Psychiatry (Monfils et al., 2009; Schiller et al., 2010).

In psychiatric disorders, episodic, working and fear memories are the most commonly affected types of memories, making them relevant to be studied in animal models (Dickerson and Eichenbaum, 2010; Parsons and Ressler, 2013; Briscione et al., 2014; Oertel-Knöchel et al., 2015; Meconi et al., 2016; Whitton et al., 2016; Martinussen et al., 2005; Rasetti and Weinberger, 2011). Episodic memories encode series of events that occur at a particular place and a specific time. Despite that episodic memories bear a strong anthropocentric character, it is now accepted that some characteristics of episodic memory, like recognition, are found in different species, allowing the study of their mechanisms and substrates in different models (Binder et al., 2015; Morici et al., 2015).

Working memory is the ability to maintain current representations of goal relevant knowledge. It is an executive component that is distributed across the frontal lobe together with sensory cortices of various modalities which interact through attentional processes (Postle, 2006; Carruthers, 2013). Over the years, working memory tasks were developed to allowed the study of working memory, its processes and neural substrates in different animal models (Carruthers, 2013; Dudchenko et al., 2013).

Although the expression of fear in animals might not have its exact correlate in humans, fear memories have been extensively studied in animal models to identify the neurobiological basis underlying memory processes (LeDoux, 2014; Izquierdo et al., 2016). Historically, the most common tasks used to study fear memories have been fear conditioning and inhibitory avoidance tasks. Both behavioral approaches are associative learning tasks, were an "unconditioned stimulus" (context or cue) is linked to an aversive "conditioned stimulus" (shock) resulting in a "conditioned response" (freezing or place avoidance). These kinds of tasks allowed the study of the molecular mechanisms and brain structures involved in the processing of fear memories.

IMMEDIATE EARLY GENES IN LEARNING AND MEMORY

IEGs represent a class of genes that respond rapidly and transiently to a variety of cellular stimuli

(Hughes and Dragunow, 1995; Kaczmarek and Chaudhuri, 1997; Herdegen and Leah, 1998; Pinaud et al., 2005; Terleph and Tremere, 2006; Bahrami and Drabløs, 2016). There are more than 100 genes classified as IEGs (Sheng and Greenberg, 1990; Minatohara et al., 2016), although only a small subgroup was found in neurons (Sheng et al., 1995). Since the discovery of the regulation of IEGs in the brain by neural activity, there has been an extensive research using IEGs as neural activity markers in studies of behavior and cognition. However, there is some controversy regarding the physiological role that these genes might have. Over the years, different hypotheses were put forward. For example, some groups assigned IEGs a role in homeostatic maintenance or replenishment, while others found in IEG's expression a role for the maintenance of activity dependent plastic changes (for review Dragunow, 1996). Another hypothesis suggested that they might be involved in mechanisms of information integration (Kaczmarek, 2000). Independently of the role that these genes have at a cellular level, it is clear that their expression is affected by neuronal activity and they are used as neuronal activity markers. In particular, some studies have reported changes in IEG expression associated with learning and memory processes and in connection with psychiatric disorders. In the next subsections we will discuss three of the most common IEGs studied in relation with memory processes and psychiatric disorders.

MOLECULAR MECHANISMS OF c-Fos

c-Fos was one of the first transcription factors whose induction was shown to be activity-dependent (Morgan and Curran, 1988; Sagar et al., 1988). In neurons, *c-fos* expression appears to be stimulated by cAMP and Ca^{2+} through the activation of the CREB/CRE complex. The *c-fos* gene codes for the Fos protein that dimerizes with transcription factors of the Jun family to build up the transcription factor AP-1 (Chiu et al., 1988; Pennypacker, 1995).

c-Fos AND MEMORY

c-fos is a clear example of an IEG whose increased expression is routinely used as an indicator for neuronal activation. For instance, it has been shown that *c-fos* expression following behavioral training specifically correlates with learning (Maleeva et al., 1989, 1990; Kaczmarek and Nikołajew, 1990; Tischmeyer et al., 1990), performance (Sakai and Yamamoto, 1997; Radulovic et al., 1998; Bertaina-Anglade et al., 2000; Vann et al., 2000a,b) and with cellular ensembles that are activated following memory retrieval (Maviel et al., 2004; Kubik et al., 2007; Lopez et al., 2012; Bravo-Rivera et al., 2015), supporting its role as activity marker.

Interestingly, changes in c-Fos expression were observed mainly during the first sessions of multiple-session training protocols, indicating an adaptive response (Maleeva et al., 1989, 1990; Kaczmarek and Nikołajew, 1990; Nikolaev et al., 1992a,b; Hess et al., 1995a,b, 1997; Gall et al., 1998; Lukasiuk et al., 1999; Anokhin et al., 2000; Bertaina-Anglade et al., 2000).

Genetic manipulations of the *c-fos* gene were performed during the first genetic engineering Era. One of the first approaches aimed to analyze the function of c-Fos in memory was done using whole-body knockout mice. With this model they were found deficits in complex but not in simple behavioral tasks, indicating that *c-fos* is not necessary for all types of learning tasks and memories. It is important to point out that these animals showed many developmental malformations which might affect the interpretation of the behavioral studies (Paylor et al., 1994).

Using the cre/loxP system Fleischmann et al. (2003) developed a central-nervous-system-selective knock out mouse. This model had normal locomotion and emotional related responses but was impaired in hippocampus-dependent spatial and associative learning tasks, such as Morris water maze and contextual fear memory. The mutant mice displayed a reduction of synaptic plasticity mechanisms in hippocampal CA3-CA1 synapses. These specific deficit were consistent with cued and spatial tasks in which an increase in hippocampal c-Fos expression was observed (Guzowski et al., 2001a) and also with experiments done with *c-fos* antisense oligonucleotide (ASO) in rats in which the infusion of the ASO produce deficits in spatial long-term memory (Guzowski, 2002; Kemp et al., 2013).

Fear memory paradigms were also able to produce changes in c-Fos expression in different structures: using an inhibitory avoidance task (Bekinschtein et al., 2010; Katche et al., 2010; Katche and Medina, 2017). It was shown that fear memory persistence required c-Fos expression and that blockade of c-Fos expression by infusion of *c-fos* ASO into the dorsal CA1 region of the hippocampus or retrosplenial cortex produces deficits in the consolidation and persistence of this type of memory (Katche et al., 2010, 2013; Katche and Medina, 2017). Similarly, the infusion of c-*fos* ASO in the prefrontal cortex affected long-term cued fear memory (Morris and Frey, 1999).

The results described here concerning c-Fos expression and function in learning and memory still do not answer the question of the biological role of c-Fos within the brain. Then, is c-Fos expression a response to the activity of neurons within a memory circuit? Or, does it play a role in maintenance of cellular homeostasis? An important obstacle in solving these questions is that the role of c-Fos in synaptic plasticity is still unclear. c-Fos is part of a complex, the AP-1, whose target genes downstream have yet to be fully characterized. Independently of the mechanism operating after c-Fos activation, it is clear that understanding its role at the cellular level is more complex than for other IEGs, such as *narp*, *homer1a* and *arc*, that are known to encode proteins that have a direct effect on the synapses (Chowdhury et al., 2006; Chang et al., 2010; Roloff et al., 2010; Lu et al., 2013). Still, it is important to increase our knowledge of the role of c-Fos at the cellular level to be able to address if it is really a marker of specific neuronal engrams associated with specific memory episodes.

More recently, the *c-fos* promoter was combined with optical sensitive proteins to mark and manipulate a particular subset of cells involved in contextual learning. With this technique, Cowansage et al. (2014) found that c-Fos-expressing neurons in

retrosplenial cortex are involved in the acquisition of contextual memories and that the reactivation of this particular set of cells can control behavioral response. Using a similar approach Liu et al. (2012) attempted to test the existence of memory engrams with a strategy of optogenetic and temporary control of cellular activation. Their results lead to two important conclusions. First, they provide evidence that IEGs, combined with optogenetics, can tag neurons that are activated during memory encoding for later manipulation. Second that artificial light-reactivation of these memory engrams is sufficient for behavioral memory recall for contextual fear conditioning. These results support the role of c-Fos in learning and memory while, at the same time demonstrate the power that this type of approach have to understand memory processing in more detail. If it is combined with other genetic models, it can become an extraordinary tool for better understanding the functional link between IEG, memory (Semon, 1904; Morris, 1999; Martin and Morris, 2002; Gerber et al., 2004; Josselyn, 2010) and psychiatric disorders (Chowdhury et al., 2006; Chang et al., 2010; Roloff et al., 2010; Lu et al., 2013).

MOLECULAR MECHANISMS OF EGR1

Egr1, also known as Zif-268, NGFI-A, Krox 24 or ZENK (Milbrandt, 1987; Lemaire et al., 1988), is a member of the zinc finger family of transcription factors. Its expression can be induced by a variety of signals that includes injury, stress, differentiation factors, as well as extracellular signals like peptides, neurotransmitters and growth factors (Herdegen and Leah, 1998; O'Donovan et al., 1999; Davis et al., 2003; Clements and Wainwright, 2010). Compared to *c-fos*, *Egr1* has a distinct pattern of expression in the brain (Milbrandt, 1987; Herdegen et al., 1990; Mack et al., 1990; Waters et al., 1990) and mediates the expression of a number of late-response genes involved in different neuronal processes from growth control to plastic changes (Sukhatme et al., 1988; Williams et al., 2000; Bozon et al., 2003; Maddox et al., 2011). *Egr1* has a relatively high expression maintained by normal ongoing neuronal activity (Worley et al., 1991; Beckmann et al., 1997; Herdegen and Leah, 1998) in the hippocampus (Hughes et al., 1992; Cullinan et al., 1995; Okuno et al., 1995; Desjardins et al., 1997), including the dentate gyrus (Cole et al., 1989; Wisden et al., 1990), as well as other brain regions (Herdegen et al., 1990, 1995; Cullinan et al., 1995; Okuno et al., 1995).

EGR1 AND MEMORY

The first approach to study Egr1 expression and its relation with behavior employed two-way avoidance training (Nikolaev et al., 1992b). In this study it was shown, for the first time, an increased level of *Egr1* mRNA in the hippocampus after one training session (Nikolaev et al., 1992a). In general, most studies use fear conditioning paradigms. With fear conditioning, *Egr1* is rapidly induced by behavioral training in the amygdala (Rosen et al., 1998; Kwon et al., 2012), the hippocampus (Nikolaev et al., 1992a; Miyashita et al., 1998; Guzowski et al., 2001b) and the retrosplenial cortex (Pothuizen et al., 2009). Nonetheless, inconsistent results were also found: Hall et al. (2000) reported non-specific expression of this IEG. Similarly Weitemier and Ryabinin (2004) showed a lack of *Egr1* expression in the septum, amygdala, hippocampus and the anterior cingulate cortex when studying fear conditioning in C57BL/6J mice.

Despite these discrepancies, it is accepted that Egr1 has an important role in learning and memory. Deletion of *Egr1* produces impairment across a broad number of behavioral tasks related to different brain regions. Typically, *Egr1* mutant mice displayed intact short-term memory in several types of tasks; however, long-term memory was drastically impaired in tasks such as social transmission of food preference, taste aversion memory, spatial memory, object recognition memory and object-place recognition memory (Jones et al., 2001; Bozon et al., 2003; Davis et al., 2010). Interestingly, studies using ASOs to partially knock down Egr1 in specific structures showed that the knockdown of Egr1 in the hippocampus does not impair contextual fear conditioning (Lee et al., 2004) but impairs inhibitory avoidance memory persistence (Katche et al., 2012) as well as recognition memory (Zalcman et al., 2015). Egr1 knockdown in the amygdala impairs both contextual (Malkani et al., 2004) and cued-fear memory formation (Maddox et al., 2011) suggesting that the deficits observed in the knockout might be attributed to its role in specific structures or circuits. *Egr1* knockout mice were also shown to be impaired in the consolidation and reconsolidation of contextual fear memory, while heterozygous mice showed impairment only in the reconsolidation (Besnard et al., 2013). This difference supported the hypothesis that memory reconsolidation is not mechanistically a repetition of consolidation. Consistent with the phenotype observed in knockout mice, overexpression of *Egr1* improved spatial memory, but not memory for the objects, during a recognition memory task (Penke et al., 2014) and enhanced aversive memories' resistance to extinction (Baumgärtel et al., 2008). On the other side, failure to induce *Egr1* allowed spontaneous recovery of fear memory after extinction (Herry and Mons, 2004).

Historically, a number of studies supported the view that proposed that *Egr1* expression is sensitive to information gained after the exposure to novelty or learning associated environments (Tischmeyer and Grimm, 1999; Bozon et al., 2002; Guzowski, 2002; Davis et al., 2003, 2006; Knapska and Kaczmarek, 2004). This idea came from the increments in the expression of *Egr1* mRNA or protein after different learning paradigms. Some examples are cited above, others are: brightness discrimination (Grimm and Tischmeyer, 1997), visual paired associate learning in monkeys (Okuno and Miyashita, 1996; Tokuyama et al., 2002), birds song learning (Mello et al., 1992; Jarvis et al., 1995; Bolhuis et al., 2000), learning and retrieval of contextual and cue fear memory (Frankland et al., 2004; Weitemier and Ryabinin, 2004) and spatial learning (Guzowski et al., 2001b). Mechanistically, a role of Egr1 in learning and memory is supported partially by its modulation of plastic changes including spine and synapse remodeling as well as growth of new synaptic connections

(Lamprecht and LeDoux, 2004; Miniaci et al., 2008; Lai et al., 2012).

These studies support a role of Egr1 in processes of learning and memory formation and they do determine a *necessity* of Egr1 for these neural and cognitive functions. But what is the specific role of Egr1 in plasticity or memory processes? It is a highly regulated transcription factor with many identified target genes and probably many more still unknown. Unlike c-Fos, there is a multitude of genes related to vesicular transport and neurotransmitter release, clathrin-dependent, or actin, which are commonly observed as direct Egr1 targets (Koldamova et al., 2014; Duclot and Kabbaj, 2015, 2017b), supporting its role in synaptic plasticity and, through this mechanism, in learning and memory. Then, alterations in the normal expression of *Egr1*, or in its protein function, could affect the encoding of information in the engram and, therefore, affect higher orders of organization.

MOLECULAR MECHANISMS OF ARC

The activity-regulated cytoskeletal (Arc) protein also known as Arg3.1, is one of the most characterized molecules involved in the consolidation of different types of memories. In contrast to *c-fos* or *egr1*, this gene is known to code for a synaptic protein. Arc is one of the effectors of the BDNF, glutamatergic, dopaminergic and serotonin signaling (Chowdhury et al., 2006; Granado et al., 2008; Karabeg et al., 2013; Leal et al., 2014; Panja and Bramham, 2014; Pastuzyn and Keefe, 2014; Managò et al., 2016; Mastwal et al., 2016). *arc* expression is under regulation of Egr1 (Li et al., 2005). It is well characterized that the *arc* mRNA is transported to the dendrites (Fujimoto et al., 2004; Steward et al., 2015) and is usually used as marker for neural activity (Chowdhury et al., 2006; Shepherd et al., 2006; Li et al., 2015; Ivashkina et al., 2016). In this sense, the post-synaptic dendrites are enriched with *arc* mRNA in contrast to the absence at the pre-synaptic axons (Moga et al., 2004; Dynes and Steward, 2012; #6010; Steward et al., 2015). Arc is involved in the generation of new synapses and the maintenance of old ones required for some plasticity mechanisms such as long-term potentiation (LTP) and long-term depression (LTD; Korb and Finkbeiner, 2011; Minatohara et al., 2016). Besides, *arc* encodes a growth factor that associates with F-actin (Lyford et al., 1995). These features led to postulate that Arc is involved in experience-dependent dendritic reconfiguration (Pinaud et al., 2001; Steward and Worley, 2001; Pinaud, 2004).

Arc gene has specific sequences normally found in retroviruses such as HIV (Campillos et al., 2006). Recent evidence was found of a plausible novel molecular mechanism by which genetic information could be transferred between neurons. It was shown that Arc protein forms a virus-like particle that can enclose RNA and be transferred through the synapse (Pastuzyn et al., 2018). Interestingly it was also proven in *Drosophila* (Ashley et al., 2018). Though the mechanism is not entirely new, considering there have been studies suggesting these pathways and their potential role in synaptic plasticity before Budnik et al. (2016); Zappulli et al. (2016) this was the first time that Arc was proven to form these particles and enclose RNA. Further research is needed on this mechanism to understand how it can be linked with synaptic plasticity.

ARC AND MEMORY

Up-regulation of *arc* in the Morris water maze was observed in cortical and para-hippocampal regions during memory spatial retention and after fear conditioning training, triggered by context exploration (Lonergan et al., 2010; Barry et al., 2016). Gusev and Gubin (2010) found differences in *arc* expression with memory durability; select segments in the prefrontal, retrosplenial, somatosensory and motor cortex showed similar robust increases in *arc* expression in recent and remote spatial memories. In another work, the study on the expression of *arc* yielded its requirement for the consolidation of long-term memory, but not for learning or short-term memory (Plath et al., 2006). Kubik et al. (2012) showed that the inactivation of dorsal CA1 was sufficient to impair the spatial performance in the Morris water maze task and this was also followed by a reduction of the expression of *arc* in the retrosplenial cortex. The requirement of Arc expression for long-term plasticity and memory consolidation was shown by infusing *arc* ASO into the dorsal hippocampus, the lateral amygdala or the anterior cingulate cortex (Guzowski et al., 2000; Ploski et al., 2008; Holloway and McIntyre, 2011; Nakayama et al., 2015). Arc also facilitates the consolidation of weak memories, and has been reported to play a role in behavioral tagging in the hippocampus (Moncada and Viola, 2007; Ballarini et al., 2009; Wang et al., 2010; Moncada et al., 2011; Martínez et al., 2012).

A genetic KO for *arc* expression generates deficits in the consolidation of different types of long-term memories (spatial, fear and episodic-like) together with changes in long-term plasticity (Plath et al., 2006; Peebles et al., 2010; Yamada et al., 2011). In addition, a significant correlation between *arc* mRNA expression and behavioral performance was found during spatial reversal task suggesting a role in cognitive flexibility (Guzowski et al., 2001b). The same group, Guzowski et al. (2006) showed that both the activity and the *arc* dynamic expression of CA1 neurons depend on the recent behavioral history of the animal: when the animals were exposed repeatedly to the same context, *arc* expression in the CA1 region was decreased. A recent study showed that the infusion of *arc* ASO into the perirhinal cortex in an object-pattern-separation task affects the consolidation of overlapping object memories (Miranda and Bekinschtein, 2018).

In summary, of the three genes reviewed here, a large and robust body of evidence suggests that *arc* and *Egr1*, although to a different extent, influence the dynamics of large networks associated with learning and memory, indicating that they have a more specific role than *c-fos*.

PSYCHIATRIC DISORDERS: MEMORY DEFICITS AND IEGs

Going from genes to neuropsychiatric disorders has proven to be a hard task, partly because psychiatric disorders have polygenic

origin with increasing levels of complexity. Each gene will have an effect at a cellular level but it will also interact with other genes with its own regulation.

Another relevant issue is the environmental factor. It is well established that complex interactions between genes and the environment are involved in multiple aspects of neuropsychiatric disorders. It can determine the vulnerability to a particular disorder and even the response to therapeutic intervention. Then, it seems crucial to achieve a better comprehension of the reaction of individuals to environmental stimuli in a particular genetic context. This level of analysis exceeds the scope of this review. Here we will approach how IEGs can intervene with fundamental neurobiological mechanisms of behavior, bearing in mind that in the central nervous system neuronal plasticity and neurotransmission are among the main processes of interactions between genes and the environment. In particular, IEGs are critical components of these interactions for they provide the molecular framework for a rapid and dynamic response to neuronal activity, opening the possibility of a lasting and sustained adaptation by regulating the expression of a wide range of genes.

The defining characteristics of the IEGs, i.e., activation within the range of minutes and independent expression of protein synthesis, give us a very strong guideline that they are a necessary mechanism of action that will, in turn, allow encoding and storing of memories. Then, deciphering the functions of IEG can provide relevant information on how these mechanisms fail in pathological conditions and thus provide new insights into the molecular mechanisms that are responsible for symptoms associated with neuropsychiatric disorders.

IEGs IN MAJOR DEPRESSION DISORDER

Major depressive disorder (MDD) is a mood disorder with prominent disturbances in cognitive functions such as certain types of memory, executive function, and attention (Jaeger et al., 2006; Mcintyre et al., 2013). MDD patients have reduced hippocampal and prefrontal cortex volumes. These alterations are mainly due to a clear-cut reduction in the neuropil and neurons in both regions have less complex dendritic trees (Saylam et al., 2006; Arnone et al., 2012). Animal models of depression have also shown a reduction in the volume of some regions of the prefrontal cortex and hippocampal formation (Hains et al., 2009; Czeh et al., 2016) with decreases in the length and complexity of dendrites and reduced number of dendritic spines.

One IEG that participates in synaptic transmission and dendritic plasticity is *arc*. Given that it has been shown that the expression of *arc* in the hippocampus and prefrontal cortex in animal models of depression change in a similar way to the alterations found in post-mortem tissues of MDD patients (Lee et al., 2012), it was postulated as a candidate target for intervening to ameliorate cognitive deficits in MDD. Interestingly, chronic stress *reduces arc* expression in medial prefrontal cortex and increases it in the amygdala (Ons et al., 2010), mirroring what is observed in MDD patients (Lee et al., 2012). Interestingly, chronic—but not acute—treatments with antidepressants restore *arc* expression in the hippocampus and prefrontal cortex (Pei et al., 2003; Molteni et al., 2008, 2010) supporting its involvement in psychiatric disorders.

Another IEG associated to MDD is *Egr1*. Covington et al. (2010) found a decreased expression of *Egr1* in the medial prefrontal cortex in depressed patients refractory to treatment and it was also observed in non-medicated subject. It should be noticed that this region has consistently been reported to be affected in depressed patients and in animal models of depression (Krishnan and Nestler, 2008; Koenigs and Grafman, 2009; Duclot and Kabbaj, 2017a,b; Lefaucheur et al., 2017). Based on these and other studies, a direct link between *Egr1* in the mPFC and the depressive phenotype had begun to be analyzed as a possible marker to predict the effectiveness of antidepressants (Morinobu et al., 1995, 1997; Bjartmar et al., 2000; Duclot and Kabbaj, 2017a,b).

Repeated exposure to stressful experiences is one of the main risk factors for the development of stress-related mood disorders like anxiety and depression (Kessler and Wang, 2008). Depending on the nature, duration, and intensity of the stress, changes in the expression of *Egr1* may vary across the entire central nervous system (Knapska and Kaczmarek, 2004). In addition, Egr1 is a critical factor in encoding the behavioral enduring effects of stress in the hippocampus. Moreover, stress-related fear memory is associated with an increased expression of Egr1 and the fear related response is blocked by knocking down Egr1 expression (Revest et al., 2005, 2010; Saunderson et al., 2016).

In line with this clinical observations, it was found that chronic stress provoked in rodents a decreased expression of *Egr1* in the medial prefrontal cortex (Covington et al., 2010) and hippocampus (Xu et al., 2015), and an increase of *Egr1* expression in the lateral amygdala (Monsey et al., 2014). In addition, using social isolation in rodents, another animal model of depression, it was observed a marked reduction in *Egr1* mRNA levels in the hypothalamus, the hippocampus, and the medial prefrontal cortex (Matsumoto et al., 2012; Hodges et al., 2014; Okada et al., 2014). This can be paralleled to the general idea that stress-related depressive disorders are associated with a down-regulated activity in the prefrontal cortex and the hippocampus, and an up-regulation of neuronal activity in the amygdala.

A new player in the neurobiology of depression is another member of the FOS family, ΔFosB. In marked contrast to what happens with the other members of the family, this transcriptional regulator is not rapidly activated by environmental stimuli. Instead, its activation is delayed and accumulates to repeated stimulation (chronic stress) mainly in nucleus accumbens neurons (Perrotti et al., 2004; Nestler, 2008). In addition, chronic administration of antidepressants, like fluoxetine and ketamine, induce ΔFosB in nucleus accumbens (Vialou et al., 2010; Donahue et al., 2014), a brain region associated to reward and motivation. Nestlers group has established that increased expression of ΔFosB within accumbal D1-type medium spiny neurons promotes

stress resilience, and mediates antidepressant-like responses (Nestler, 2015). In addition, ΔFosB overexpression in nucleus accumbens promotes several rewarding behaviors, including sucrose drinking, consumption of high-fat food, and sexual activity. Despite its role in nucleus accumbens associated behaviors, since depression is a complex disorder affecting many brain regions, it will be important to study ΔFosB role in other brain structures in which its expression is induced by chronic antidepressant administration (Vialou et al., 2014).

FEAR-RELATED DISORDERS AND THE INVOLVEMENT OF IEGs

Most anxiety disorders are associated with a strong environmental component (fear) and a physiological defect in response to that component. Anxiety disorders include simple phobia, social phobia—which involves fear and avoidance of social situations—, and panic disorder; they all share abnormal fear responses associated with different environmental stimuli but also differ in important and specific symptoms. For example, PTSD is a chronic neuropsychiatric disorder that results from a very strong traumatic event and it is characterized by intrusive and persistence fear related memories. The etiology of PTSD remains largely unknown but different neurobiological systems have been identified as participants in the disorder (Parsons and Ressler, 2013).

The first opportunity to apply treatments designed to modify the formation and persistence of fear memories is around the aversive experience, mainly through pharmacological manipulations (Monfils et al., 2009; Quirk and Milad, 2010; Quirk et al., 2010). Modulation of opioid systems has been proven to be effective in PTSD (Holbrook et al., 2010). However, other pharmacological manipulation yielded mixed results (Maren and Chang, 2006; Myers et al., 2006; Parsons and Ressler, 2013). A new strategy was developed to attenuate fear-related memories by manipulating memory reconsolidation and extinction processes. Immediate or delayed extinction procedures induce a reduction in those aversive memories that are context-dependent and short-lived (Hermans et al., 2006; Woods and Bouton, 2008) suggesting that its efficacy as a treatment depends on how old the memory is (Milekic and Alberini, 2002; Suzuki et al., 2004). A novel behavioral design that involves a mixed reconsolidation-extinction procedure has proved to be a better strategy (Monfils et al., 2009; Chan et al., 2010; Schiller et al., 2010).

Few studies have analyzed the role of IEG in PTSD related animal models, though they reported interesting results. Changes in *arc* expression was observed along the septo-temporal axis of the hippocampus of PTSD susceptible rats (Nalloor et al., 2014), suggesting that it might exist a basal difference among susceptible population. *arc* expression in the hippocampus is also involved in the perpetuation of fear related memories (Nakayama et al., 2015) while changes in the amygdala appeared to be related to the response to uncontrollable stress (Machida et al., 2018). Then, the IEGs analyzed here directly or indirectly play a role in these processes, so that a better understanding of the molecular mechanisms underlying memory processing, including the role of specific IEGs, may be essential to obtain new targets and strategies as treatments for fear-related disorders.

IEG IN SCHIZOPHRENIA

Schizophrenia is characterized by profound cognitive deficits that are not alleviated by currently available medications. Many of these cognitive deficits involve dysfunction of the newly evolved, dorsolateral prefrontal cortex. The brains of patients with schizophrenia show atrophy in the dendrites of the pyramidal cells, particularly from dorsolateral prefrontal cortex (Weinberger et al., 1986; Bonilha et al., 2008; Konopaske et al., 2014).

It has been shown that *Egr1* expression is decreased in the dorsolateral prefrontal cortex of schizophrenic patients. This down-regulation correlates with the levels of *gad1* mRNA, which is also down-regulated in schizophrenia (Yamada et al., 2007; Pérez-Santiago et al., 2012; Kimoto et al., 2014). Antipsychotics administration, in contrast, up-regulates *Egr1* and related IEGs in frontal and striatal regions (de Bartolomeis et al., 2015a).

Searching for biological markers of schizophrenia in peripheral tissues, it was found that whole blood samples of patients suffering this disease have an increased expression of *Egr1* (Kurian et al., 2011; Cattane et al., 2015). Besides, *Egr1* and other IEGs are also associated with response to antipsychotic drugs (MacGibbon et al., 1994; Robbins et al., 2008; Bruins Slot et al., 2009; Wheeler et al., 2014; de Bartolomeis et al., 2015b; Duclot and Kabbaj, 2017b).

Changes in *c-fos* expression have been reported in schizophrenia related animal models. Acute treatment with the NMDA antagonist MK-801 produced deficits in novel object recognition, conditioned test aversion and a moderate increase in locomotion. Interestingly, animals treated with this drug showed increased levels of c-Fos expression in cortical regions associated with cognitive processing (Vishnoi et al., 2015). Subchronic, but not acute, treatment with PCP, another NMDA antagonist, produces deficits in working memory tasks and evokes a particular pattern of *c-fos* expression (Castañé et al., 2015) suggesting that there are plastic changes related to the behavioral output. Interestingly, *c-fos* expression in the mPFC does not correlate with glutamic acid decarboxylase 67 expression as does *Egr1* (Kimoto et al., 2014). CamKII is a Serine/Threonine protein that plays a key role in neural plasticity. α-CamkII heterozygous and knock out mice have working memory deficits. This behavioral deficits correlate with decrease c-Fos expression in key areas associated with working memory like mPFC and hippocampus (Matsuo et al., 2009). This changes in c-Fos expression appears to be task specific since the same α-CamkII +/- mice showed normal c-Fos expression pattern in the mPFC after fear conditioning training (Frankland et al., 2004). An interesting and new target is matrix metalloproteinase 9 (MMP-9), an enzyme activated outside the cell by proteolytic cleavage that degrades extracellular matrix. It has been implicated in synaptic plasticity and is under

c-fos regulation (Michaluk and Kaczmarek, 2007; Michaluk et al., 2007; Huntley, 2012). Aberrant levels of MMP-9 has been observed in different psychiatric disorders, including schizophrenia and importantly, blood levels of MMP-9 or gene responsiveness to antipsychotics has been related to this disorder (Lepeta and Kaczmarek, 2015; Vafadari et al., 2016).

The interaction between Arc and dopamine appears to be bidirectional. Using a genetic model in which *arc* was deleted in the whole brain, Managò et al. (2016) found a dysregulation in the dopaminergic system with reduced dopamine levels in the cortex and increased levels in the striatum. Arc deletion induced these changes that are characteristic of a schizophrenia-related model. Consistent with this finding, the authors also found deficits in multiple domains correlated with cognitive, positive and negative associated symptoms of schizophrenia and identified a complex interaction between Arc, dopaminergic system and schizophrenia (Managò et al., 2016; Managò and Papaleo, 2017).

PSYCHIATRIC RELATED DRUGS AND IEGs

IEGs have not only been associated directly with particular psychiatric disorders but they have also been used as markers of the specific action of different psychiatric drugs that are related. Haloperidol, a typical antipsychotic drug induces changes in *c-fos* and *Egr1* expression in the striatum and nucleus accumbens (MacGibbon et al., 1994). Olanzapine, in acute doses show increased *c-fos* expression in the medial prefrontal cortex (Robertson and Fibiger, 1996; Ohashi et al., 2000), and the locus coeruleus (Ohashi et al., 2000; Dawe et al., 2001), while a chronic treatment showed a downregulation of *Egr1* and an upregulation of FOS-like expression in the same structures (Verma et al., 2006). These results, together with other studies (Nguyen et al., 1992), suggest those antipsychotics do affect in some way IEG expression, however, they do not do it in a concerted fashion. It would be interesting to address if the IEGs' expression pattern correlates with antipsychotics' activity.

Antidepressants' activity has also been analyzed in relation to their effect on IEG expression. Acute administration of fluoxetine, imipramine, LiCl, or mirtazapine produced changes in IEG expression in different regions. There were changes found for *c-fos* in the anterior insular cortex, the septum and the amygdala using different antidepressants. In the case of *Egr1* a consistent change was found only in the amygdala (Slattery et al., 2005). Chronic antidepressants can restore the up-regulation of *c-fos* in the frontal cortex following an acute stress experience (Beck and Fibiger, 1995a,b). Furthermore, chronic administration of fluoxetine and vortioxetine decreased Arc and c-Fos, but not Egr1 in the frontal cortex, hippocampus and amygdala of rodents (Waller et al., 2017).

Although these studies show changes in the expression of IEGs in apparent response to different drugs, they are correlational studies and do not address any role of these proteins in their mechanism of action. Despite this caveat, even these studies provide important information regarding circuits involved in learning and memory that can be affected in psychiatric disorders.

CONCLUSION

Although the prolific use of IEGs in neuroscience, our understanding of their role in neurobiological processes remains insufficient. This is due at least to the large number of IEGs that are currently under study, but also because of the difficulty in fully comprehending their roles and their downstream effectors. Independently of this, IEGs are used as markers of synaptic activity and as that, are useful. Even more, when combined with modern techniques, like optogenetic or chemogenetics (Reijmers et al., 2007; Garner et al., 2012; Liu et al., 2012; Cowansage et al., 2014) IEGs can become powerful tools to identify activity-dependent cell populations. However, important gaps in the study of IEGs exist. As we mentioned above we still do not know much regarding the cellular effectors of these genes specifically in the Central Nervous System. Likewise, their role in memory processes requires further investigation. The lack of information regarding this particular point is surprising, especially if we consider that tools to address it have been around for decades (e.g., use of antisense or inducible genetic manipulations). A better understanding of these points becomes particularly relevant considering their role in psychiatric conditions. Understanding neuropsychiatric disorders is principally challenging since their etiology and pathophysiology are mostly unknown. Nonetheless, by focusing on the study of endophenotypes (Bearden and Freimer, 2006) together with the use of animal models (Krishnan et al., 2008; Nestler and Hyman, 2010; Anderzhanova et al., 2017), our understanding of these disorders has considerably improved in the last decade. Analyzing the role of IEGs in specific endophenotypes or animal models of psychiatric disorders could shed light on both the genetic and molecular basis of these diseases. Together with a better understanding of their biological roles, the manipulation of these genes in animal models could provide a robust model for the discovery of new pharmacological targets. In this review, we aimed to resume the literature looking at the intersection between psychiatric disorders, IEG and memory. Interestingly, we found that most of these studies were correlational; with surprisingly few studies analyzing a causal role of IEGs in psychiatric disorders. It is clear now that IEGs are much more than simple activation markers and they deserve to be analyzed as possible key participants in psychiatric conditions. Genetic manipulations that allow the temporal and spatial control of expression of these genes are powerful tools to analyze the role that IEGs have in memory and other cognitive domains in psychiatric animal models. We consider that, until now, the intersection between psychiatric models, cognition and IEG function did not receive the attention that it deserves. The manipulation of IEG expression as a means of intervention on the onset and progression of psychiatric conditions could generate new targets in the development of their treatments, targets that nowadays are completely unnoticed.

AUTHOR CONTRIBUTIONS

FTG, CK, JFM, JHM and NVW conceived the content, wrote and organized the manuscript.

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The Immediate Early Gene *Egr3* Is Required for Hippocampal Induction of *Bdnf* by Electroconvulsive Stimulation

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Early growth response 3 (*Egr3*) is an immediate early gene (IEG) that is regulated downstream of a cascade of genes associated with risk for psychiatric disorders, and dysfunction of *Egr3* itself has been implicated in schizophrenia, bipolar disorder, and depression. As an activity-dependent transcription factor, EGR3 is poised to regulate the neuronal expression of target genes in response to environmental events. In the current study, we sought to identify a downstream target of EGR3 with the goal of further elucidating genes in this biological pathway relevant for psychiatric illness risk. We used electroconvulsive stimulation (ECS) to induce high-level expression of IEGs in the brain, and conducted expression microarray to identify genes differentially regulated in the hippocampus of Egr3-deficient (−/−) mice compared to their wildtype (WT) littermates. Our results replicated previous work showing that ECS induces high-level expression of the brain-derived neurotrophic factor (*Bdnf*) in the hippocampus of WT mice. However, we found that this induction is absent in *Egr3*−/− mice. Quantitative real-time PCR (qRT-PCR) validated the microarray results (performed in males) and replicated the findings in two separate cohorts of female mice. Follow-up studies of activity-dependent *Bdnf* exons demonstrated that ECS-induced expression of both exons IV and VI requires *Egr3*. *In situ* hybridization demonstrated high-level cellular expression of *Bdnf* in the hippocampal dentate gyrus following ECS in WT, but not *Egr3*−/−, mice. *Bdnf* promoter analysis revealed eight putative EGR3 binding sites in the *Bdnf* promoter, suggesting a mechanism through which EGR3 may directly regulate *Bdnf* gene expression. These findings do not appear to result from a defect in the development of hippocampal neurons in *Egr3*−/− mice, as cell counts in tissue sections stained with anti-NeuN antibodies, a neuron-specific marker, did not differ between *Egr3*−/− and WT mice. In addition, Sholl analysis and counts of dendritic spines in golgi-stained hippocampal sections revealed no difference in dendritic morphology or

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synaptic spine density in *Egr3*−/−, compared to WT, mice. These findings indicate that *Egr3* is required for ECS-induced expression of *Bdnf* in the hippocampus and suggest that *Bdnf* may be a downstream gene in our previously identified biologically pathway for psychiatric illness susceptibility.

Keywords: electroconvulsive therapy, immediate early genes, early growth response 3, brain-derived neurotrophic factor, schizophrenia, psychosis treatment

INTRODUCTION

The risk to develop neuropsychiatric illnesses is determined by both genetic and environmental factors. We have hypothesized that immediate early gene (IEG) transcription factors are poised to modulate this dual contribution, as they are rapidly activated in the brain in response to environmental stimuli and, in turn, influence numerous neurobiological processes that are dysfunctional in the brains of patients with mental illness (Gallitano-Mendel et al., 2007, 2008; Huentelman et al., 2015). These include memory formation and synaptic plasticity (Gallitano-Mendel et al., 2007; Forbes et al., 2009), myelination (Jessen and Mirsky, 2002; Davis et al., 2003; Dwork et al., 2007), vascularization (Mechtcheriakova et al., 1999; Fahmy et al., 2003; Hanson and Gottesman, 2005), and growth factor response (Moises et al., 2002; Fahmy and Khachigian, 2007; Zakharyan et al., 2014). Our prior work has focused on investigating the functions of the IEG transcription factor early growth response 3 (*Egr3*), as it is regulated downstream of numerous proteins that are associated with risk for neuropsychiatric disorders (Gallitano-Mendel et al., 2007, 2008; Huentelman et al., 2015). We have previously reported that *Egr3*-deficient (−/−) mice have behavioral abnormalities consistent with animal models of mental illness, including schizophrenia and bipolar disorder, that are rescued by the antipsychotic medications used to treat these disorders (Gallitano-Mendel et al., 2007, 2008). *EGR3* has been associated with risk for schizophrenia in Japanese, Korean, Han Chinese, and US populations of European descent (Yamada et al., 2007; Kim et al., 2010; Zhang et al., 2012; Huentelman et al., 2015) and levels of *EGR3* are reduced in the brains of schizophrenia patients (Mexal et al., 2005; Yamada et al., 2007). Recently, *EGR3* was identified as a master regulator in a network of genes differentially expressed in the postmortem brains of bipolar disorder patients, compared with controls, in two independent cohorts (Pfaffenseller et al., 2016). In addition, although *EGR3* was not identified within one of the 108 loci found to be associated with schizophrenia risk in the Psychiatric Genomics Consortium genome-wide association study (GWAS), numerous genes encoding proteins that regulate or are regulated by *EGR3* do map to these schizophrenia-associated loci (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Marballi and Gallitano, 2018).

The few confirmed downstream target genes of EGR3 have also been implicated in neuropsychiatric disorders. These include glutamic acid decarboxylase A4 (*GABRA4*; Roberts et al., 2005, 2006), and activity-regulated cytoskeleton associated protein (*Arc-Arg3.1*; Li et al., 2005). In addition, our prior studies identified a specific deficit in GluN2B-containing *N*-methyl D-aspartate receptors (NMDARs) in the hippocampus of *Egr3*-deficient (−/−) mice, indicating a requirement of *Egr3* for the normal function of this receptor (Gallitano-Mendel et al., 2007). Hypofunction of NMDARs is one of leading hypotheses for the etiology of schizophrenia (Javitt and Zukin, 1991; Olney et al., 1999). Indeed, GRIN2A, the gene encoding the NMDAR subunit GluN2A, is also located at one of the 108 schizophrenia-associated loci (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014).

Based on these prior studies, we hypothesized that other EGR3 target genes in the brain may be critical contributors to neuropsychiatric disorders. Since neuronal expression of IEGs is activity dependent, we used electroconvulsive seizure (ECS) to maximally activate IEGs, and compared hippocampal gene expression between mice lacking *Egr3* and their wildtype (WT) littermates. This experimental approach is particularly relevant as ECS is an experimental model of electroconvulsive therapy (ECT), which remains one of the most effective treatments for severe mood and psychotic disorders. However, the mechanisms underlying the efficacy of ECT remain elusive.

Here we show that induction of brain-derived neurotrophic factor (*Bdnf*), a critical neurotrophin involved in a wide range of neurobiological functions, is significantly diminished or absent in the hippocampus of mice lacking *Egr3*. Dysfunction of BDNF has been implicated in numerous neuropsychiatric disorders (Autry and Monteggia, 2012), including bipolar disorder and schizophrenia (Durany et al., 2001). Like *Egr3*, *Bdnf* is also induced in the hippocampus following ECS, and this expression is associated with the effectiveness of antidepressant treatments in reversing mood disorder-like phenotypes in rodents (Altar et al., 2003; Adachi et al., 2008; Inta et al., 2013).

MATERIALS AND METHODS

Mice

Previously generated *Egr3*−/− mice (Tourtellotte and Milbrandt, 1998) were backcrossed to C57BL/6 mice for greater than 20 generations. Studies were conducted on homozygous adult littermate progeny of heterozygote matings, and assigned as "matched pairs" at the time of weaning. Matched pairs were exposed to identical conditions for all studies. The term WT refers to +/+ littermates of *Egr3*−/− mice generated from these crossings. Male mice ages 6–12 months ($n = 4$) were utilized for the microarray and follow-up quantitative real-time PCR (qRT-PCR) study. Results were validated in two separate cohorts of female *Egr3* mice. The first cohort included older adult females, ages 12–15 months, from the same C57BL/6 background ($n = 4$–5 per group). A second cohort of females, age 3.5–6 months ($n = 4$–5 per group), had a mixed background resulting from

crossing the above *Egr3* C57BL/6 background mice to a GENSAT reporter line in a mixed FVB/N and Swiss Webster background. Since these animals contain a bacterial artificial chromosome expressing EGFP, in addition to a mixed background, results were analyzed separately from other groups.

Animals were housed on a 12 h light/dark schedule with *ad libitum* access to food and water. All studies were performed in accordance with the University of Arizona, Institutional Animal Care and Use Committee (IACUC). This study was carried out in accordance with the recommendations of IACUC guidelines, IACUC. The protocol was approved by IACUC.

Electroconvulsive Stimulation and Tissue Collection

To anesthetize the corneas of all animals, 0.5% proparacaine hydrochloride ophthalmic solution (Akorn, Inc., Lake Forest, IL, United States) was applied 5 min prior to electroconvulsive stimulation (ECS). Male mice utilized in the microarray study underwent ECS without general anesthesia. Female mice used in the replication studies were anesthetized with isoflurane (VetOne, Boise, ID, United States) administered in an enclosed chamber at a flow rate of 0.5 mL/min in oxygen. Animals were removed from the chamber after 2 min of full anesthetization, transferred to room air to recover to a level of light anesthesia, and then administered electrical stimulation of 260 A for 1 ms duration and a pulsewidth of 0.3 mm, 1 ms. (Ugo Basile, Varese, Italy) via orbital electrodes. Mice were observed to undergo seizure, and were placed in their home cage to recover for one hour prior to sacrifice. Control animals remained in their home cages undisturbed.

Tissue Collection and RNA Isolation

Animals were sacrificed via isoflurane overdose, followed by decapitation. The brains were removed, rinsed in sterile ice-cold phosphate buffered saline (PBS), and hemisected along the central sulcus into right and left hemispheres for further studies to quantify both the expression of mRNA with qRT-PCR and *in situ* hybridization, respectively.

Hippocampal tissue was dissected and immediately placed in RNAlater (Ambion, Waltham, MA, United States). Tissue was transferred to 1.5-mL Eppendorf tubes, immediately placed on dry ice, and transferred to −80°C until further use. For male cohort microarray and follow-up qRT-PCR, RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, United States) followed by phenol/chloroform extraction, chloroform extraction, and isopropanol precipitation, per the manufacturer's protocol. RNA was resuspended in RNAse-free water and quantitated by spectrophotometry. RNA quality and concentration was validated by Agilent Bioanalyzer 2100 prior to microarray analysis and reverse transcription for qRT-PCR. An aliquot of the RNA samples was sent to the Microarray Resource Center, Yale/NIH Neuroscience Microarray Center (New Haven, CT, United States) for analysis using an Illumina Mouse WG6 v3.0 expression beadchip microarray. For female cohorts, RNA isolation was performed using TRI reagent (Sigma-Aldrich, St. Louis, MO, United States) and MagMax^{TM} Total RNA isolation kit (Ambion, Waltham, MA, United States) according to the manufacturer's protocol, and quantified using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, United States).

Microarray Procedure and Analysis

Data analysis and quality control was initially performed using Gene Pattern (Reich et al., 2006), with normalization using the cubic spline method using the following settings (FDR < 0.05) to determine significantly differentially expressed genes between the WT and *Egr3*−/− groups 1 h following ECS. This was the timepoint at which we expected the most enrichment for EGR3 transcription factor targets, as *Egr3* is maximally induced by ECS (O'Donovan et al., 1998). A parallel analysis using the Illumina Genome studio 2010 software was used to determine differentially expressed genes by comparing the same two groups using the following settings: background subtraction, quantile normalization, $p < 0.05$ (T-test). Finally, a list of common differentially expressed genes using both programs was generated. Complete microarray results will be published separately.

qRT-PCR

For qRT-PCR studies, mRNA was reverse transcribed into cDNA using a standard protocol (Maple et al., 2015), and used as a template for qRT-PCR using FastStart SYBR Green Master mix (Roche Applied Science, Indianapolis, IN, United States) on a 7500 Fast Real-Time PCR machine (ThermoFisher Scientific, Waltham, MA, United States). Each sample was amplified in triplicate for both *Bdnf* and the housekeeping gene phosphoglycerate kinase 1 (*Pgk1*). *Pgk1* was selected for use as a housekeeping gene as one of the least changed genes across experimental groups in the microarray data and validated by qRT-PCR in all cohorts.

General *Bdnf* primers were used to assess overall levels of total *Bdnf* mRNA (*Bdnf* F, R) (Tallaksen-Greene et al., 2014). These primers target a region common to all 12 transcripts of *Bdnf* as revealed by NCBI primer blast analysis. For follow-up exon-specific studies, we focused on *Bdnf* exons IV and VI as removal of these exons *in vivo* significantly reduces BDNF protein levels in the mouse hippocampus (Maynard et al., 2016). In addition, *Bdnf* transcripts containing exon IV and VI are highly induced *in vivo* following ECS in the mouse frontal cortex (Martinowich et al., 2011). Exon specific primers for *Bdnf* exons IV and VI (Zheng and Wang, 2009) were used in our study. Fold changes in gene expression and data were plotted using the $2^{-\Delta CT}$ method (Le et al., 2013). Primer sequences used were as follows, *Bdnf* F: TGG CCC TGC GGA GGC TAA GT; *Bdnf* R: AGG GTG CTT CCG AGC CTT CCT; *Pgk1* F: TGT TAG CGC AAG ATT CAG CTA GTG; *Pgk1* R: CAG ACA AAT CCT GAT GCA GTA AAG AC; *Bdnf* exon IV F: CTC CGC CAT GCA ATT TCC AC; *Bdnf* exon VI F: GTG ACA ACA ATG TGA CTC CAC; and *Bdnf* exon IV/VI R: GCC TTC ATG CAA CCG AAG TA.

Prediction of EGR3 Binding Sites in *Bdnf* Gene Regulatory Regions

Bioinformatic identification of putative EGR binding sites in the promoter of the mouse *Bdnf* gene was carried out using

the TFBIND[1] (Tsunoda and Takagi, 1999) website that utilizes the transcription factor database TRANSFAC R. 3.3. Briefly, the nucleotide sequence 4000 bp upstream of the start ATG in the mouse *Bdnf* gene was exported from the UCSC genome browser[2] using genome assembly GRCm38/mm10 and used to query the TFBIND site. This generated an output that showed all the putative binding sites for the EGR3 TF.

Golgi-Cox Preparation

Tissue was treated using the Golgi-Cox method, as previously described (Gibb and Kolb, 1998; Gallitano et al., 2016). Briefly, 10 animals (5 *Egr3*−/− and 5 WT) were decapitated under isoflurane and the brains were rapidly extracted. The brains were rinsed in 0.9% saline and immersed in Golgi-Cox solution for at least 14 days followed by 30% sucrose for at least 3 days. The solution was made in the laboratory from ingredients purchased from Sigma-Aldrich (St. Louis, MO, United States). Brains were then sectioned on a Vibratome at 200 μm and mounted on gelatin-coated slides.

Golgi-Cox Imaging and Analysis

Ten fully impregnated cells were selected in each region, from the hippocampal Cornu Ammonis (CA) regions CA1 and CA3, and the dentate gyrus (DG) of each animal, and were imaged in a z-stack throughout the thickness of the section using a brightfield microscope equipped with a digital camera (AmScope, Irvine, CA, United States). Using ImageJ (NIH) or Metamorph (Molecular Devices) software, several measures of dendritic arborization were obtained (**Figure 3**). The Sholl technique (Sholl, 1953) involves overlaying the neuron with a series of concentric circles (20 μm apart) and recording the number of dendritic processes intersecting each circle to a maximal distance of 380 μm. In addition, branching order was calculated for all cells. The primary dendrites originating from the soma are assigned a branch order of one, while dendritic processes originating from that dendrite are second-order branches, and each subsequent bifurcation is assigned a progressively higher branch order (van Pelt et al., 1986). Dendritic spine density and morphology were analyzed at 100x under oil immersion. Dendritic spines were counted in a sample of two random 20-μm-long segments in several distinct regions of each cell according to the classification of Lorente de No (1934). For CA1 and CA3, these regions were: the basal tuft (stratum oriens), the mid-point of the apical tuft (near the distal edge of the stratum radiatum), and the distal tip of the apical tuft (stratum lacunosum moleculare). In the DG, dendritic spines were counted in the inner molecular layer (IML), middle molecular layer (MML), and outer molecular layer (OML) as defined by dividing the length of the molecular layer into three equal parts.

Immunohistochemistry

Animals were sacrificed by isoflurane euthanasia and were perfused with PBS followed by fixation with 4% paraformaldehyde (PFA). Brains were harvested and were post-fixed in 4% PFA for 24 h, rinsed in Tris-buffered saline (TBS, pH 7.4), transferred into 30% sucrose, and stored at 4°C until saturated, rinsed, and stored in TBS at 4°C. Tissue was sectioned coronally at a thickness of 20 μm on a sliding microtome and stored until further processing in a cryopreservative solution.

Immunohistochemistry

Tissue was rinsed in TBS, 3x × 5 min. Endogenous peroxidase was quenched with 1% H_2O_2 × 10 min in a buffer/detergent solution (0.4% Triton-X in TBS). The reaction was stopped by rinsing the tissue 3x × 5 min in TBS. Tissue was blocked with 4% normal goat serum (Sigma-Aldrich, St. Louis, MO, United States) prepared in 0.4% Triton-X in TBS at room temperature (RT) for 2 h. Polyclonal rabbit primary antibody raised against neuronal nuclei, NeuN (1:1000, Millipore, Cat #ABN78) was prepared in 4% normal goat serum in 0.4% Triton-X in TBS and was incubated at RT for 1 h with a 24-h incubation at 4°C. A secondary biotinylated antibody raised against rabbit (1:1000, Vector Labs, #BA-1000) was prepared in 4% normal goat serum in 0.4% TBS-TX and was incubated with the tissue for 1 h at RT. Avidin–biotin complex (ABC; ThermoFisher Scientific, Waltham, MA, United States) was prepared at a concentration of 1:1000 in TBS. Tissue was incubated in ABC for 1 h at RT. Detection of the antigen was performed with 3,3′-diaminobenzidine (DAB) kit (ImmPACT DAB Peroxidase Substrate, Cat No. SK-4105, Vector Laboratories, Burlingame, CA, United States). Tissue sections were imaged with a Zeiss Imager M2 microscope using a 40x objective, photographed using an Axiocam 506 camera, and tiled together using Zen 2012 software (Zeiss Microscopy, Oberkochen, Germany).

Neurons were counted from defined anatomical regions of CA1 and CA3 pyramidal neurons, as well as both the suprapyramidal (DG_{sp}) and infrapyramidal (DG_{ip}) blades of the DG. For each brain, neurons were counted from four sections spanning a total rostral–caudal distance of 200 μm including, and caudal to, coordinates: interaural, 1.74 mm; bregma, 2.06 mm (Paxinos and Franklin, 2001). The number of neurons within each analyzed region of the hippocampus was estimated using Abercrombie's (1946) correction.

In Situ Hybridization

Tissue Preparation

Tissue was flash frozen in −40°C methylbutane and was sectioned coronally at 10 μm thickness with a cryostat (Leika, Buffalo Grove, IL, United States), mounted on super-charged slides and stored at −80°C. Tissue sections from matched experimental pairs of animals were mounted on a single slide to control for possible staining differences (*Egr3*−/− control, *Egr3*−/− ECS, WT control, and WT ECS).

Probe Generation

Mouse BDNF DNA plasmid (accession #X55573) was kindly donated by Dr. Stanley Watson, University of Michigan, Ann Arbor. The DNA sequence was validated by comparison to *Bdnf* reference sequence using FinchTV software (Geospiza, Inc., Seattle, WA, United States). Sense and antisense strands were

[1]http://tfbind.hgc.jp

[2]http://genome.ucsc.edu/

generated by *in vitro* transcription with the AMBION Maxiscript T7 kit (Ambion, Waltham, MA, United States), labeled with digoxigenin RNA labeling mix (Roche, Basel, Switzerland), and purified with mini columns according to the manufacturer's protocol. (Roche, Basel, Switzerland).

In Situ Hybridization

Tissue was fixed in 4% PFA at 4°C for 5 min., followed by a wash in 2x SSC for 2 min at RT. Tissue was acetylated in acetic anhydride (0.625%) for 10 min at RT, rinsed in sterilized water, and delipidized with a 1:1 mixture of acetone and methanol for 5 min. Tissue was rinsed in 2x SSC for 5 min, before application of the probe (300 ng) prepared in hybridization buffer (Sigma, St. Louis, MO, United States). The probe was applied directly to the mounted tissue (250 μL/slide) and was cover-slipped (FisherBrand, Fisher Scientific). Tissue was hybridized in a humid, sealed chamber with a mixture of 2x SSC and formamide overnight at 56°C. Stringency washes were performed on Day 2 to remove any non-specific binding (protocol available on request). Endogenous peroxidase was quenched with 2% hydrogen peroxide, and tissue was permeabilized with 1x SSC-Tween followed by rinsing in 0.1 M TBS (pH 7.4). The tissue was blocked with a mixture of 0.5% TSA blocking buffer (PerkinElmer, Waltham, MA, United States) and 5% normal sheep serum prepared in 0.1 M TBS. Anti-digoxigenin (Roche, Basel, Switzerland; 1:400 dilution in blocking buffer) was applied to the slides at RT for 2 h. Slides were washed in 0.1 M TBS-Tween 4x × 15 min at RT and the antibody was detected through alkaline phosphatase development, SIGMA*FAST* BCIP/NBT tablets (Sigma, St. Louis, MO, United States). Slides were imaged with bright-field microscopy (Axiovision, Zeiss, Oberkochen, Germany) at a magnification of 40x.

Statistical Analysis

All statistical analyses for qRT-PCR and *in situ* data were carried out using graphpad prism. In all cases, two-way analysis of variance (ANOVA) was performed followed by Tukey's *post hoc* test, corrected for multiple comparisons, and significance was set at $p < 0.05$. Golgi-Cox impregnated granule cells and pyramidal cells were analyzed in independent ANOVAs. Sholl data were analyzed by repeated-measures ANOVA, while branching order and spine density measures used general factorial ANOVA, with genotype and hippocampal region (i.e., either CA1, CA3a/b, and CA3c or DGsp and DGip) as factors. The neuron density (number of neurons per unit area) was compared between WT and *Egr3*-/- brains using a 2 × 5 ANOVA with genotype and hippocampal region (i.e., CA1, CA3a/b, CA3c, DG_{sp}, and DG_{ip}) as factors.

RESULTS

Activity-Dependent Hippocampal *Bdnf* Expression Depends on *Egr3*

To identify genes regulated by *Egr3* in the mouse hippocampus, we compared the complement of genes expressed in *Egr3*−/− mice to that of WT mice. Since *Egr3* is an activity-dependent IEG, and thus expressed at low levels in the absence of a stimulus, we used ECS to maximally activate IEG expression in both *Egr3*−/− and WT mice, and compared levels of induced genes to those at baseline. We conducted an expression microarray to screen for genes that were differentially expressed in response to seizure in the hippocampus of male WT, compared to *Egr3*−/−, mice.

Results of the microarray showed 65 genes to be differentially expressed between WT and *Egr3*−/− mice following ECS. Of these, 40 genes were increased (greater than 1.5-fold), while 13 were decreased, in WT mice following ECS compared to *Egr3*−/− mice following ECS. Twelve genes were minimally changed across both groups (1–1.4-fold). *Bdnf* was the only growth factor among these 65 putative EGR3-dependent, ECS-induced genes. Complete results of the microarray will be published separately.

Bdnf was of particular interest to us, as it has been shown to contribute to the therapeutic effects of antidepressant treatments, including ECS, in rodent models (Altar et al., 2003; Adachi et al., 2008; Inta et al., 2013). Results of the expression microarray demonstrated that, in WT mice, ECS produced a 2.5-fold increase in *Bdnf* mRNA levels, measured 1 h following seizure, compared to baseline unstimulated controls. This induction is dependent upon *Egr3*, as *Egr3*−/− mice did not show a change in *Bdnf* mRNA expression following seizure (**Figure 1A**). A two-way ANOVA revealed a significant interaction of genotype and treatment [$F_{(1,12)} = 12.65$, $p = 0.004$]. *Post hoc* analysis showed that WT mice had significantly higher levels of *Bdnf* induction after ECS compared to the untreated WT ($p < 0.001$) mice, while *Egr3*−/− mice did not show a significant increase in *Bdnf* expression following ECS compared to baseline. In addition, there was a significant difference in *Bdnf* levels between WT and *Egr3*−/− mice following ECS ($p < 0.01$).

To validate these findings, we performed qRT-PCR from the RNA samples that were used for the expression microarray (**Figure 1B**). ECS induced a greater than six-fold increase in *Bdnf* expression in WT mice (compared to non-stimulated control), while it failed to induce a significant increase in *Bdnf* expression in the *Egr3*−/− mice. These results validate the microarray data. A main effect of treatment was observed using two-way ANOVA [$F_{(1,12)} = 9.393$, $p = 0.0098$] and *post hoc* analysis demonstrated that ECS-treated WT mice had significantly higher levels of *Bdnf* after ECS compared to the untreated WT ($p < 0.05$). ECS did not result in a significant change in *Bdnf* expression in *Egr3*−/− mice ($p = 0.8133$).

To determine whether the requirement of *Egr3* for activity-dependent induction of *Bdnf* is sex specific, we conducted ECS in female *Egr3*−/− and WT mice. **Figure 1C** shows that, as in male mice, ECS induced a significant increase (3.8-fold) in *Bdnf* mRNA in WT female mice, but did not result in a statistically significant increase in *Egr3*−/− mice. Cohort 1 (**Figure 1C**) showed a significant interaction of genotype and treatment [$F_{(1,14)} = 20.52$, $p = 0.0005$] and *post hoc* analysis revealed significant elevations in *Bdnf* levels post ECS in the WT group compared to WT controls ($p < 0.0001$) and *Egr3*−/− ECS-treated mice ($p < 0.0001$), respectively.

To determine whether the effect was also seen in younger animals, we used a second cohort of female mice. **Figure 1D**

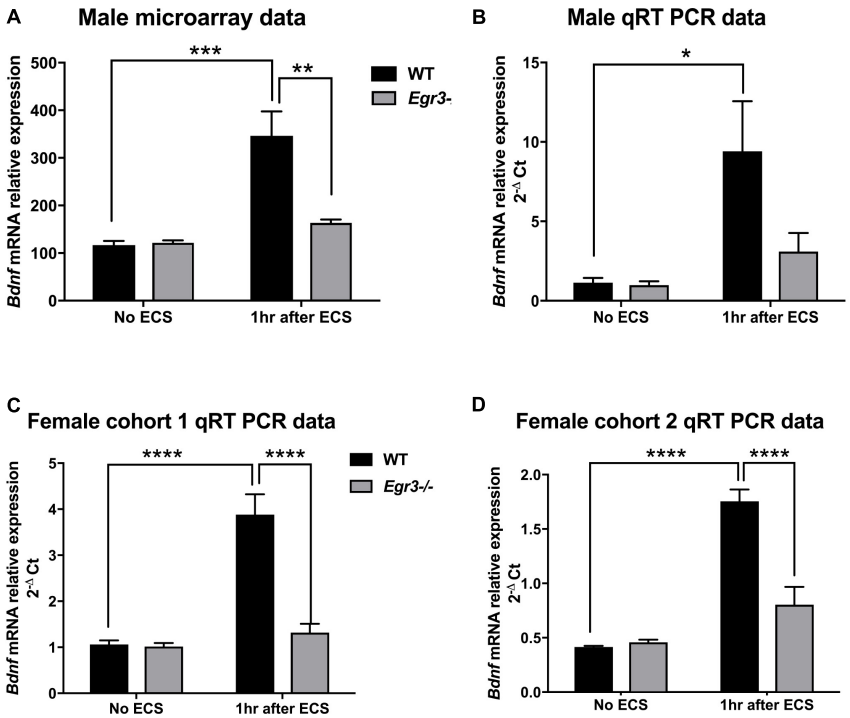

FIGURE 1 | Electroconvulsive seizure (ECS)-induced hippocampal *Bdnf* expression is *Egr3*-dependent. Expression microarray analysis **(A)**, and validation using qRT-PCR **(B–D)**, of hippocampal *Bdnf* expression from WT, and *Egr3*–/–, mice at baseline (no ECS) and 1 h after ECS. Two-way ANOVAs showed significant interaction of genotype and ECS treatment on *Bdnf* expression in **(A)** male microarray data ($p = 0.004$) and qRT-PCR data in **(C)** female cohort 1 ($p = 0.0005$) and **(D)** female cohort 2 ($p = 0.0003$), and **(B)** significant effect of treatment in original male cohort ($p = 0.0098$; $n = 4$–5 animals/group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ controlled for multiple comparisons).

shows a similar result to that seen in adult male, and older female, C57BL/6 mice, that ECS produces a fourfold increase in *Bdnf* expression in WT mice that is dependent upon *Egr3*. Two-way ANOVA demonstrated a significant interaction of genotype and treatment [$F_{(1,13)} = 23.85$, $p = 0.0003$]. *Post hoc* analysis revealed significant elevations in *Bdnf* post ECS in the WT group compared to WT controls ($p < 0.0001$) and to *Egr3*−/− ECS-treated mice ($p < 0.0001$). No significant effect of ECS on *Bdnf* induction was observed in *Egr3*−/− mice in either group. These data suggest that *Bdnf* induction by ECS is dependent, at least in part, on *Egr3*.

ECS-Induced Exon IV and Exon VI *Bdnf* Expression Is *Egr3* Dependent

Given that the *Bdnf* gene has multiple splice variants, we next examined whether *Egr3* is required for ECS-induced expression of specific splice variants. This was of particular interest as ECS induces specific exon variants in the mouse brain *in vivo* (Martinowich et al., 2011). Martinowich et al. (2011) showed that mRNAs containing exon IV and exon VI are highly upregulated following ECS compared to untreated mice, and mice that lack exon IV fail to show induction of six splice variants following ECS *in vivo* (Martinowich et al., 2011). In addition, both exon IV and exon VI containing mRNAs maximally contribute to total BDNF protein levels in the mouse hippocampus (Maynard et al., 2016). Since exons IV and VI are required for expression of the remaining BDNF exons, we decided to evaluate levels of transcripts containing these two critical exons in WT and *Egr3*−/− mice following ECS compared with untreated controls.

Quantitative real-time PCR using exon specific primers showed that WT mice demonstrated a 10-fold increase in *Bdnf* exon IV expression, 1 h post-ECS that was absent in *Egr3*−/− mice (**Figure 2A**). Two-way ANOVA revealed a significant interaction of genotype and treatment [$F_{(1,12)} = 12.52$, $p = 0.0041$]. *Post hoc* analysis indicated that WT mice had significantly higher levels of *Bdnf* exon IV mRNA induction after ECS compared to the untreated WT ($p < 0.001$) mice. *Egr3*−/− mice did not show a significant increase in *Bdnf* exon IV expression following ECS compared to baseline, but showed decreased *Bdnf* exon IV expression compared to WT mice following ECS ($p < 0.01$). Similar results were seen in both female cohorts. Female cohort 1 (**Figure 2B**) WT mice exhibited a sevenfold induction in *Bdnf* exon IV post ECS compared to baseline. Two-way ANOVA showed a significant interaction of genotype and treatment [$F_{(1,14)} = 14.46$, $p = 0.0019$]. *Post hoc* analysis demonstrated that WT mice had a significantly greater induction of *Bdnf* exon IV following ECS compared to baseline ($p < 0.0001$) and compared to *Egr3*−/− mice post ECS ($p < 0.001$). WT mice from the second female cohort demonstrated a ninefold induction in *Bdnf* exon IV post ECS compared to non-stimulated controls (**Figure 2C**). Two-way

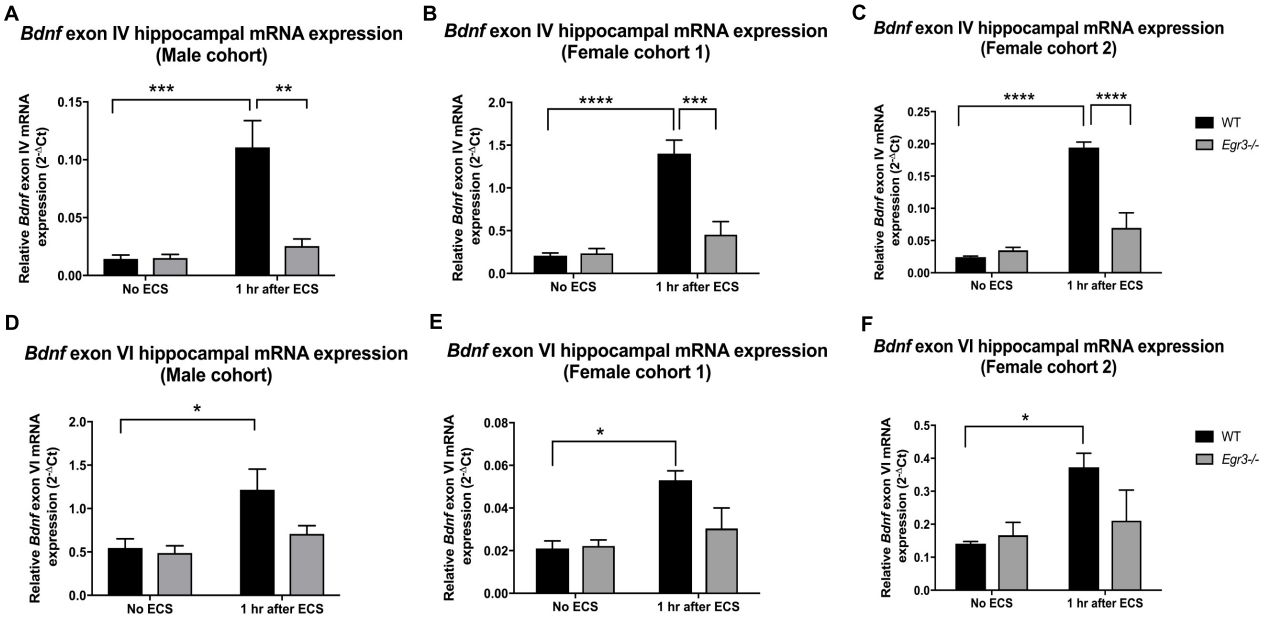

FIGURE 2 | Electroconvulsive seizure (ECS)-mediated hippocampal induction of *Bdnf* exon IV and VI requires *Egr3*. Exon IV **(A–C)** and VI **(D–F)** hippocampal *Bdnf* expression determined using qRT PCR from WT, and *Egr3–/–*, mice at baseline (no ECS) and 1 h after ECS. Two-way ANOVAs showed significant interactions of genotype and ECS treatment on *Bdnf* exon IV expression in **(A)** original male cohort data ($p = 0.0041$), **(B)** female cohort 1 ($p = 0.0019$), and **(C)** female cohort 2 ($p = 0.0001$). Two-way ANOVAs showed significant effect of treatment on *Bdnf* exon VI expression in original **(D)** male cohort ($p = 0.0099$), **(E)** female cohort 1 ($p = 0.0064$), and **(F)** female cohort 2 ($p = 0.0237$; $n = 4$–5 animals/group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ controlled for multiple comparisons).

ANOVA revealed a significant interaction of genotype and treatment [$F_{(1,13)} = 30.24$, $p = 0.0001$]. *Post hoc* analysis revealed WT mice showed a significant induction of *Bdnf* exon IV post ECS compared to baseline ($p < 0.0001$) and *Egr3−/−* mice that underwent ECS ($p < 0.0001$).

In comparison, *Bdnf* exon VI mRNA was induced 2.2-fold in male WT mice following ECS compared to baseline that was absent in *Egr3−/−* mice (**Figure 2D**). Two-way ANOVA showed a significant effect of treatment [$F_{(1,12)} = 9.361$, $p = 0.0099$] and *post hoc* analysis revealed a significant induction of *Bdnf* exon VI in WT mice that underwent ECS vs. non-stimulated WT mice ($p < 0.05$). These data were replicated in both female cohorts. WT mice showed a 2.24- (**Figure 2E**) and a 2.64-fold (**Figure 2F**) induction post ECS compared to baseline, in the first and second female cohorts, respectively, that was absent in *Egr3−/−* mice. Two-way ANOVA revealed a significant effect of treatment in both female cohorts; female cohort 1 [$F_{(1,14)} = 10.26$, $p = 0.0064$] and female cohort 2 [$F_{(1,13)} = 6.557$, $p = 0.0237$]. *Post hoc* analysis revealed that significant induction of *Bdnf* exon VI in WT mice that underwent ECS compared to baseline ($p < 0.05$) in both female cohorts, while this effect was not observed in *Egr3−/−* mice.

ECS-Induced *Bdnf* Expression in Dentate Gyrus Requires *Egr3*

To identify the location of *Bdnf* expression in the hippocampus following ECS, and to determine in which regions it is dependent on EGR3, we conducted *in situ* hybridization to detect expression of *Bdnf* mRNA in hippocampal tissue sections. *In situ* hybridization histochemistry was performed on tissue sections from female *Egr3−/−* and WT mice at baseline, and 1-h post ECS. In WT mice, ECS resulted in a strong induction of *Bdnf* mRNA in the DG of the hippocampus 1 h after ECS (**Figure 3C**) compared with baseline, unstimulated controls (**Figure 3A**). Expression of *Bdnf* in the hippocampus of WT mice at baseline is found in sparsely distributed individual cells in the DG as well as CA regions 2 and 3 (**Figure 3A**). One hour following ECS, high level *Bdnf* expression is evident uniformly throughout the dorsal and ventral blades of the DG (**Figure 3C**). There is no evident increase in the individual cellular pattern of labeling in CA2 and CA3 (**Figure 3C**). In *Egr3−/−* mice ECS did not result in increased expression of *Bdnf* (**Figure 3D**) compared to baseline (**Figure 3B**), and neither condition demonstrated strong labeling of *Bdnf* in the DG, or in CA1, CA2, or CA3 regions (**Figures 3B,D**).

EGR3 Binding Sites Are Present in *Bdnf* Regulatory Regions

If *Egr3* is required to directly regulate expression of *Bdnf*, then EGR3 binding sites should be present in the promoter of the *Bdnf* gene. The genomic sequence 4000 bp upstream of the *Bdnf* transcription start site contains eight high-probability putative binding sites for EGR3 (**Figure 4** and **Table 1**), with a cutoff value of 0.74 designated by TFBIND that calculates transcription factor-specific

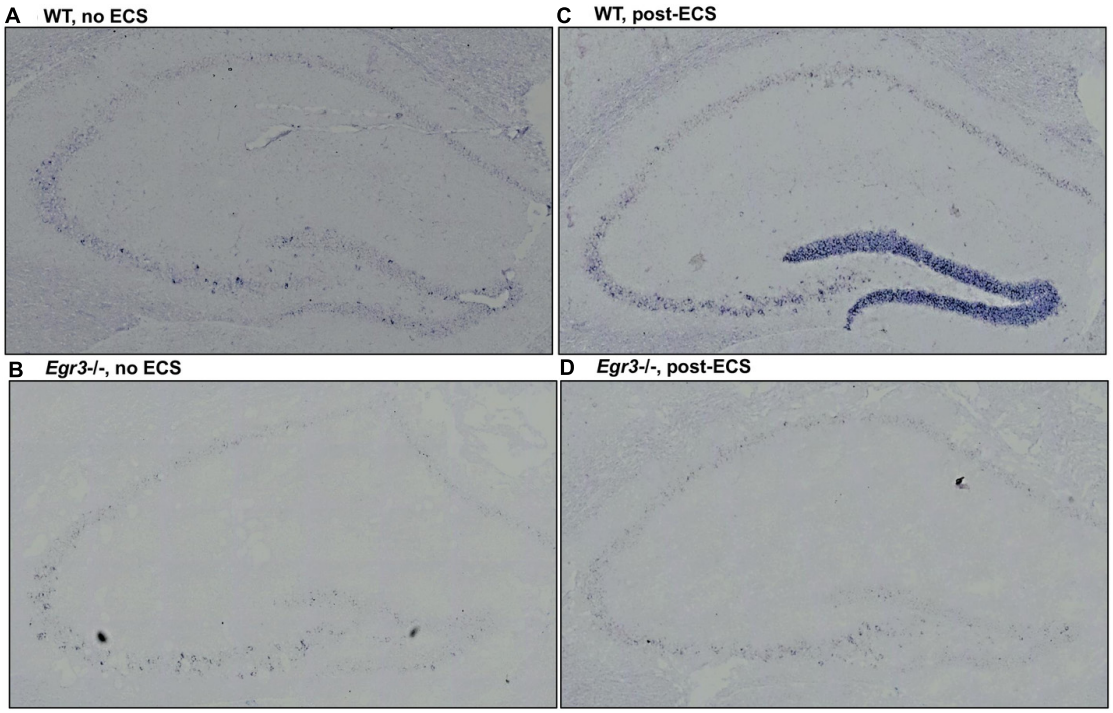

FIGURE 3 | *Bdnf* induction in the hippocampal dentate gyrus requires *Egr3*. Representative tissue sections demonstrating *in situ* hybridization labeling of *Bdnf* mRNA expressing cells in the hippocampus of WT and *Egr3*–/– mice at baseline **(A,B)** and 1 h following ECS **(C,D)**. **(A)** *Bdnf* is expressed sparsely in CA3, CA2, and the DG of WT mice at baseline. **(C)** ECS induces high-level *Bdnf* expression in the DG, and increased expression in CA3, CA2, and CA1 of WT mice. **(B)** *Bdnf* expression in *Egr3*–/– mice is limited to rare cells in the CA3 region of the hippocampus, with no clear expression above background in the DG, CA2, or CA1, at baseline. **(D)** ECS produces little to no increase in *Bdnf* expression in the DG, CA3, CA2, and CA1 regions of *Egr3*–/– mice.

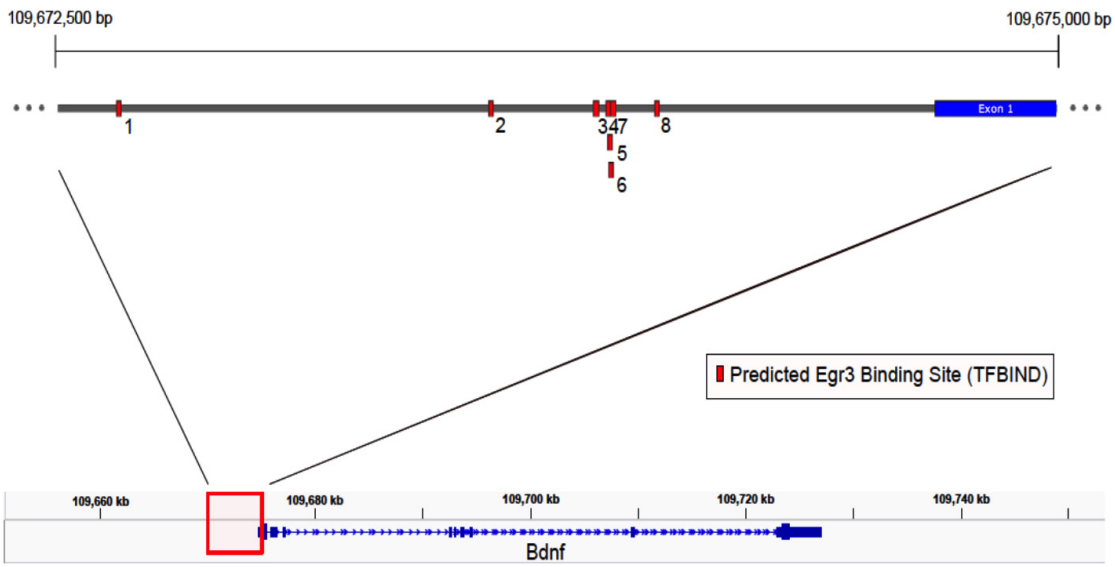

FIGURE 4 | Map of predicted EGR3 binding sites in *Bdnf* upstream region. EGR3 binding sites identified in **Table 1** are indicated in red. Positions are shown relative to exon 1 of the mouse *Bdnf* gene as identified in the NCBI Refseq database. Numbers under each binding site correspond to numbered rows in **Table 1**.

cutoffs using an algorithm (Tsunoda and Takagi, 1999). These sites represent a potential mechanism by which EGR3 could regulate expression of the *Bdnf* gene. Further studies will be necessary to determine whether EGR3 binds to these sites *in vivo*, and whether this binding may be functional.

TABLE 1 | EGR3 binding sites identified in the *Bdnf* promoter using TFBIND.

ID*[1]	Score*[2]	Position*[3]	Strand*[4]	Consensus sequence*[5]	Identified sequence*[6]
V$EGR3_01	0.807500	1947	(–)	NTGCGTGGGCGK	CCTCCCACGTCA
V$EGR3_01	0.753542	2881	(–)	NTGCGTGGGCGK	ACTCGCACTCAT
V$EGR3_01	0.741042	3144	(–)	NTGCGTGGGCGK	CCGCATCCGCCT
V$EGR3_01	0.901667	3175	(–)	NTGCGTGGGCGK	ACGCCCGCGCAC
V$EGR3_01	0.748958	3179	(–)	NTGCGTGGGCGK	CCGCGCACACGC
V$EGR3_01	0.771042	3181	(–)	NTGCGTGGGCGK	GCGCACACGCGC
V$EGR3_01	0.805417	3187	(–)	NTGCGTGGGCGK	ACGCGCACACAC
V$EGR3_01	0.777083	3296	(–)	NTGCGTGGGCGK	CAGCCTGCGCAG

*[1]*ID: transcription factor matrix ID (from TRANSFAC R.3.4). V: vertebrate.* *[2]*Score: degree of similarity between input sequence and registered sequence for the transcription factor binding sites at the position shown in the position column (ranging from 0 to 1, representing low–high degree of similarity).* *[3]*Position: location of putative EGR3 binding site in the 4000 nucleotides upstream of Bdnf transcription start site.* *[4]*Strand: location of EGR3 consensus sequences on + vs. − strand of DNA.* *[5]*Consensus sequence (fixed) of the transcription factor binding sites. K = G or T; N = any base pair.* *[6]*Putative EGR3 binding sequence identified by TFBIND.*

Hippocampal Neuronal Numbers Are Not Affected by Developmental Absence of *Egr3*

Egr3−/− mice lack expression of the functional gene throughout development and life. It is therefore possible that the absence of induction of *Bdnf* in the hippocampus following ECS in these animals may be a consequence of abnormal development, or survival, of hippocampal neurons in the absence of *Egr3*. To determine whether this may be the case, we conducted detailed regional cell counts of anti-NeuN antibody immunolabeled neurons in the hippocampus of matched pairs of adult *Egr3*−/− and WT littermate mice. WT and *Egr3*-/- brains display a comparable distribution and density of neurons (**Figure 5**). As expected (van Strien et al., 2009), a significant difference was observed on the basis of hippocampal region [$F_{(4,50)} = 9.13$, $p < 0.001$], with the DG showing the tightest cell packing density, while the CA3 regions were the least densely packed. However, there was no difference in the number of neurons in any hippocampal regions between *Egr3*−/− and WT control mice. No significant main effect of genotype [$F_{(1,50)} = 1.19$, $p = 0.28$] or interaction between genotype and hippocampal region [$F_{(4,50)} = 0.10$, $p = 0.98$] was observed.

Dendritic Complexity Remains Normal in *Egr3*−/− Mice

To further characterize how the absence of functional *Egr3* may alter the structure of hippocampal neurons, we conducted detailed analyses of cells impregnated with Golgi-Cox solution in adult WT and *Egr3*−/− littermate mice (**Figures 6A,B**). In pyramidal cells (**Table 2**), no significant differences were observed in the Sholl analysis [$F_{(1,24)} = 2.71$, $p = 0.11$] or branching order [$F_{(1,24)} = 0.81$, $p = 0.38$] on the basis of genotype. Moreover, no significant main effect of region was observed on Sholl analysis [$F_{(2,24)} = 1.32$, $p = 0.29$], or branching order [$F_{(2,24)} = 0.68$, $p = 0.51$], and no interaction between genotype and region was observed ($p > 0.05$ in all cases). Similarly, granule cells (**Table 3**) showed no significant differences in the Sholl analysis [$F_{(1,16)} = 0.11$, $p = 0.75$) or branching order [$F_{(1,16)} = 1.678$, $p = 0.21$] on the basis of genotype. A significant main effect of region was observed on Sholl analysis [$F_{(1,16)} = 20.52$, $p < 0.001$], indicating that granule cells in the DG_{sp} are significantly larger and more complex than granule cells from the DG_{ip}, consistent with previous observations (Gallitano et al., 2016), although branching order was not significantly different [$F_{(1,16)} = 3.15$, $p = 0.09$]. No interaction between genotype and region was observed ($p > 0.05$ in all cases).

Spine Density Is Not Affected by Loss of *Egr3*

Consistent with the dendritic analysis, ANOVAs showed no significant differences in spine density in any brain region (**Figure 6**). In pyramidal cells, no significant difference on the basis of genotype in the density of basal spines in stratum oriens [$F_{(1,24)} = 0.35$, $p = 0.5588$], in apical spines in stratum radiatum proximal [$F_{(1,24)} = 0.64$, $p = 0.43$] or distal [$F_{(1,24)} = 0.35$, $p = 0.56$] to the soma, nor in stratum lacunosum/moleculare [$F_{(1,24)} = 1.31$, $p = 0.26$]. Similarly, no significant differences were observed on the basis of hippocampal region (i.e., CA1, CA3a/b, and CA3c) basal spines in stratum oriens [$F_{(2,24)} = 0.92$, $p = 0.41$], in stratum radiatum proximal [$F_{(2,24)} = 0.42$, $p = 0.66$] or distal [$F_{(2,24)} = 0.27$, $p = 0.77$] to the soma, nor in stratum lacunosum/moleculare [$F_{(2,24)} = 0.5624$, $p = 0.5772$]. Similarly, no significant interactions were observed between region and genotype ($p > 0.05$ in all cases).

In granule cells, there was no significant effect of genotype on the density of spines in the IML [$F_{(1,16)} = 0.31$, $p = 0.58$], MML [$F_{(1,16)} = 0.14$, $p = 0.71$], or OML [$F_{(1,16)} = 0.33$, $p = 0.58$]. Similarly, no significant differences in spine density were observed on the basis of blade of the DG (i.e., DG_{sp} vs. DG_{ip}) in IML [$F_{(1,16)} = 0.28$, $p = 0.64$], MML [$F_{(1,16)} = 0.33$, $p = 0.57$], or OML [$F_{(1,16)} = 0.76$, $p = 0.30$]. Similarly, no significant interactions were observed between region and genotype ($p > 0.05$ in all cases).

DISCUSSION

Egr3 is an IEG transcription factor that is induced in response to environmental stimuli (Thompson et al., 2010; Maple et al., 2015). We have hypothesized that dysfunction of *Egr3* could disrupt the brain's normal neurobiological response to stress,

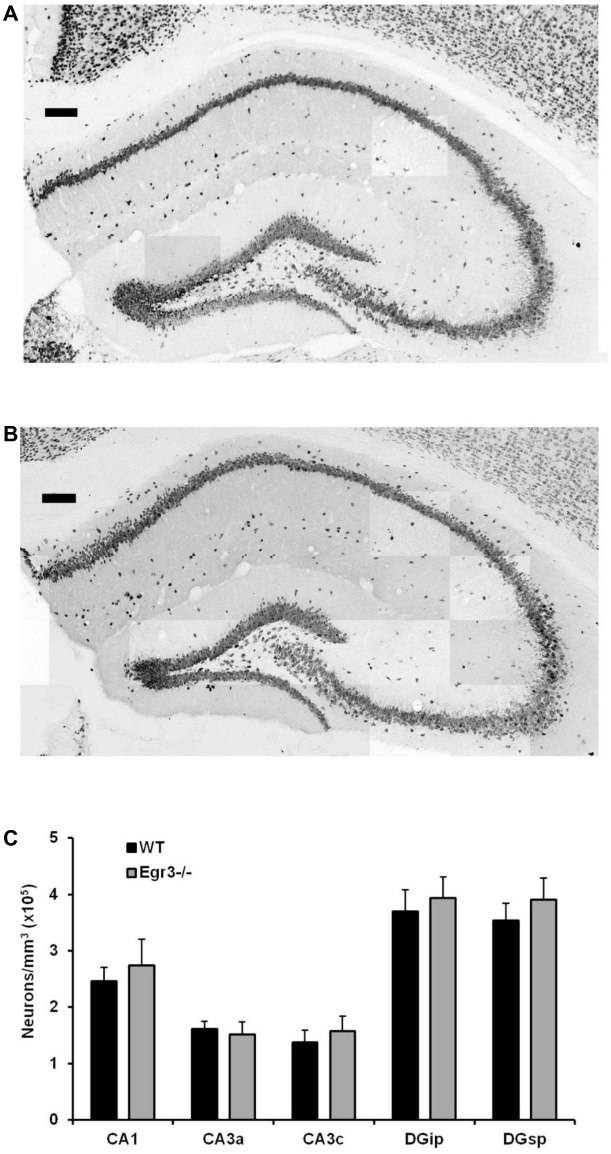

FIGURE 5 | *Egr3* is not required for development of normal numbers of hippocampal neurons. Sample montaged photomicrographs from the hippocampus of anti-NeuN stained **(A)** WT and **(B)** *Egr3*–/– mice show no obvious signs of changes in gross morphology (scale bar = 100 μm). Consistent with this observation, **(C)** quantification of NeuN+ cells (n = 6 animals/genotype) demonstrated no significant differences in neuron density in any hippocampal region between WT (black) and *Egr3*–/– (gray) mice.

resulting in increased risk to develop mental illnesses such as schizophrenia and bipolar disorder (Gallitano-Mendel et al., 2007, 2008; Williams et al., 2012; Huentelman et al., 2015; Pfaffenseller et al., 2016; Marballi and Gallitano, 2018). In support of this hypothesis, our prior work has shown that *Egr3*−/− mice display behavioral abnormalities consistent with animal models of psychotic disorders that can be reversed by administration of medications used to treat these illnesses in humans (Gallitano-Mendel et al., 2007, 2008). In addition, *Egr3* has been associated with risk for schizophrenia in Japanese, Korean, Han Chinese populations, and populations of European Descent (Yamada et al., 2007; Kim et al., 2010; Zhang et al., 2012; Huentelman et al., 2015), and *Egr3* expression is decreased in the brains of patients with schizophrenia, compared with controls (Mexal et al., 2005; Yamada et al., 2007). Bioinformatics approaches have identified *Egr3* as a central gene in a network of transcription factors and microRNAs implicated in schizophrenia risk (Guo et al., 2010), as well as a master regulator of genes differentially expressed in the brains of bipolar disorder patients in two independent cohorts (Pfaffenseller et al., 2016). Together, these findings suggest that disruption of *Egr3* activity, or function of other proteins that act either upstream or downstream in the EGR3 pathway, may mediate both the genetic and environmental factors that contribute to risk for severe mental illnesses (Marballi and Gallitano, 2018).

Since EGR3 functions as a transcription factor, we hypothesized that the downstream target genes regulated by EGR3 may also influence risk for psychotic disorders. IEGs, including *Egr3*, are expressed at low levels at baseline. We therefore conducted ECS to induce rapid, high-level, expression of *Egr3* (O'Donovan et al., 1998). We used a microarray approach to compare genes expressed 1 h following ECS in the hippocampus of WT, compared to *Egr3*−/−, mice to identify EGR3-dependent target genes. This study revealed increased expression of *Bdnf*, in the WT mice, but not in the *Egr3*−/− mice, indicating that ECS-induced *Bdnf* expression requires *Egr3*.

BDNF is a neurotrophic factor that promotes growth and differentiation of neurons and synapses (Barde et al., 1987; Binder and Scharfman, 2004; Bennett and Lagopoulos, 2014). In addition, BDNF has been found to play a role in the therapeutic effectiveness of treatments for psychiatric illnesses. Animals treated with antidepressant medications demonstrate increased expression of *Bdnf* in the hippocampus (Bjorkholm and Monteggia, 2016). Disruption of BDNF function is implicated in the pathology underlying numerous psychiatric disorders, ranging from schizophrenia and bipolar disorder to depression (Grande et al., 2010; Lee and Kim, 2010; Autry and Monteggia, 2012). Reduction of hippocampal BDNF attenuates the effect of antidepressants (Monteggia et al., 2004; Adachi et al., 2008), and infusion of BDNF into the hippocampus reverses depression-like behavior in rodents (Shirayama et al., 2002).

The mouse *Bdnf* gene consists of nine exons; eight of which are in the 5′ untranslated region of the gene (exons I–VIII) and only one of which encodes protein (exon IX; Aid et al., 2007). Different *Bdnf* splice variants are formed from a combination of one of the 5′ untranslated exons (I–VIII) with the common protein-coding exon (exon IX; Liu et al., 2005; McDowell et al., 2010). Two alternative polyadenylation signals result in transcription termination and result in either the short (0.3 kB) or long (2.9 kB) 3′ UTR of *Bdnf* (Timmusk et al., 1993).

Prior studies have shown that activity-dependent transcription of *Bdnf* can be mediated by calcium influx (Shieh and Ghosh, 1999; West et al., 2001; Aid et al., 2007; Hong et al., 2008; West, 2008). Calcium responsive elements, including cAMP-responsive element-binding (CREB) protein (West et al., 2001), Carf (Tao et al., 2002), calcium-dependent phosphorylation of the methyl

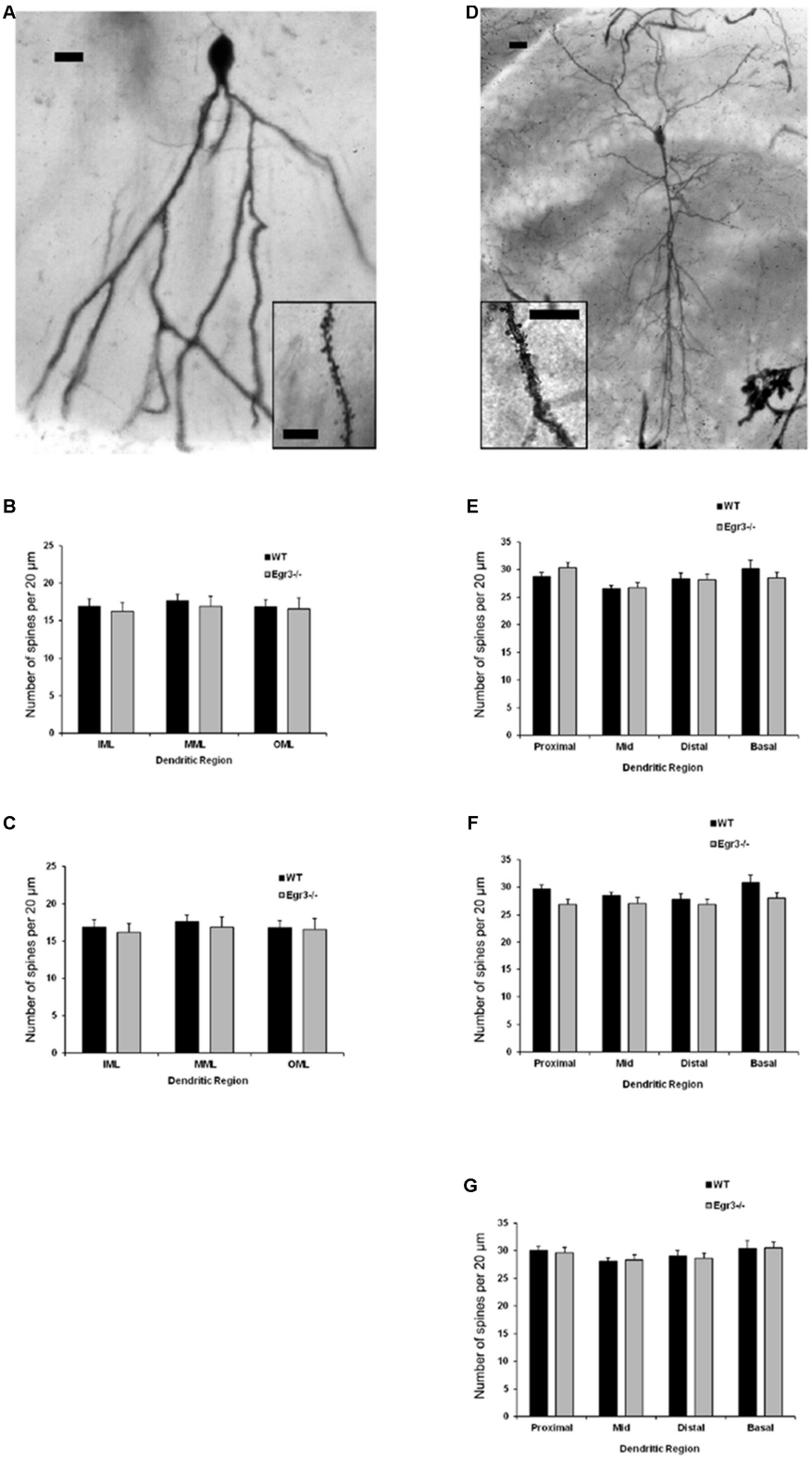

FIGURE 6 | *Egr3* is not required for normal development of neuronal morphology. Samples of Golgi-Cox staining in **(A)** granule cells and **(D)** pyramidal cells shows impregnation of both gross dendritic structure (scale bar = 20 μm) as well as spines (inset, scale bar = 10 μm). Quantitative data show no statistically significant differences in either dendritic morphology or spine number between WT (black) and *Egr3*–/– (gray) mice in the **(B)** DG_{sp}, **(C)** DG_{ip}, **(E)** CA1, **(F)** CA3a/b, or **(G)** CA3c regions.

TABLE 2 | Dendritic arborization in hippocampal cells of WT and *Egr3−/−* mice.

		WT						Egr3−/−					
		CA1		CA3a/b		CA3c		CA1		CA3a/b		CA3c	
		Apical	Basal	Apical	Basal	Apical	Basal	Apical	Basal	Apical	Basal	Apical	Basal
Branching	1	1.30 ± 0.21[1]	2.50 ± 0.47	2.53 ± 0.42	3.35 ± 1.07	1.77 ± 0.38	4.73 ± 0.85	1.13 ± 0.07	4.00 ± 0.10	2.80 ± 0.23	2.13 ± 0.22	2.01 ± 0.92	3.04 ± 0.17
Order	2	2.19 ± 0.55	4.75 ± 0.47	3.28 ± 0.27	3.50 ± 0.87	2.50 ± 0.84	4.38 ± 0.94	3.66 ± 0.63	6.10 ± 0.64	3.10 ± 0.17	2.50 ± 0.14	3.80 ± 1.04	3.46 ± 0.41
	3	5.89 ± 0.32	7.00 ± 0.74	3.05 ± 0.84	5.90 ± 0.87	2.53 ± 0.85	5.12 ± 0.78	6.08 ± 0.46	7.28 ± 0.88	4.00 ± 0.32	3.96 ± 0.17	3.20 ± 0.42	4.79 ± 0.22
	4	4.57 ± 0.39	7.00 ± 0.65	2.23 ± 0.31	5.50 ± 1.03	1.30 ± 0.78	5.30 ± 0.12	5.71 ± 0.34	5.23 ± 0.01	2.50 ± 0.27	3.38 ± 0.65	1.30 ± 0.16	2.83 ± 0.20
	5	6.02 ± 0.90	5.75 ± 1.08	3.03 ± 0.45	4.40 ± 1.03	2.37 ± 1.18	4.90 ± 0.10	4.30 ± 0.32	2.90 ± 0.06	1.80 ± 0.10	1.13 ± 0.07	2.00 ± 0.32	0.67 ± 0.19
	6	2.00 ± 0.49	2.50 ± 0.95	1.70 ± 0.37	2.10 ± 0.07	0.83 ± 0.59	1.80 ± 0.09	2.17 ± 0.31	1.15 ± 0.38	1.40 ± 0.23	0.50 ± 0.14	0.60 ± 0.12	0.29 ± 0.02
	7	1.57 ± 0.31	1.00 ± 0.47	1.60 ± 0.92		0.27 ± 0.19		0.89 ± 0.23	0.01 ± 0.01	4.00 ± 0.32		0.51 ± 0.43	
	8+	0.84 ± 0.27	0.25 ± 0.12	0.50 ± 0.29		–		0.57 ± 0.33	–	–		–	
	Total	24.51 ± 2.48	30.75 ± 4.90	19.90 ± 3.68	16.48 ± 2.73	16.17 ± 3.65	16.80 ± 2.77	22.94 ± 1.54	25.19 ± 4.98	21.60 ± 3.12	15.93 ± 3.34	14.95 ± 1.99	13.10 ± 0.64
Scholl (μm)	20	2.03 ± 0.14	1.50 ± 0.70	1.70 ± 0.17	6.78 ± 0.56	1.73 ± 0.30	6.93 ± 0.19	1.91 ± 0.20	7.48 ± 1.00	1.70 ± 0.17	5.80 ± 0.46	1.38 ± 0.33	5.78 ± 0.01
	40	3.50 ± 0.12	3.75 ± 0.60	2.58 ± 0.68	8.78 ± 1.14	3.68 ± 0.62	9.73 ± 0.30	3.58 ± 0.17	10.88 ± 0.65	2.58 ± 0.68	6.80 ± 0.46	2.75 ± 0.72	6.95 ± 0.84
	60	5.19 ± 0.22	5.00 ± 2.14	3.63 ± 0.36	7.15 ± 1.36	5.28 ± 0.27	10.43 ± 0.48	5.04 ± 0.31	10.03 ± 0.45	3.63 ± 0.36	5.58 ± 0.48	3.25 ± 0.82	5.25 ± 1.01
	80	4.51 ± 0.26	4.75 ± 1.72	4.85 ± 0.38	4.63 ± 2.09	6.60 ± 0.35	6.68 ± 1.20	5.61 ± 0.06	8.53 ± 0.74	4.85 ± 0.38	3.78 ± 0.59	5.25 ± 1.43	3.83 ± 0.91
	100	4.22 ± 0.57	6.00 ± 3.51	4.98 ± 0.45	1.85 ± 0.95	6.45 ± 1.13	5.45 ± 1.18	5.79 ± 0.53	4.00 ± 1.15	4.98 ± 0.45	1.73 ± 0.27	5.34 ± 1.24	2.75 ± 0.72
	120	5.18 ± 0.32	7.00 ± 3.79	4.65 ± 0.66	0.85 ± 0.38	4.35 ± 0.49	3.15 ± 0.78	5.78 ± 0.38	2.00 ± 0.58	4.65 ± 0.66	0.95 ± 0.26	6.13 ± 1.47	0.65 ± 0.09
	140	4.55 ± 0.45	4.25 ± 2.04	5.08 ± 0.76	0.50 ± 0.29	3.48 ± 0.16	1.35 ± 0.66	5.27 ± 0.59	0.85 ± 0.20	5.08 ± 0.76	0.20 ± 0.12	6.75 ± 1.67	0.19 ± 0.10
	160	5.63 ± 0.60	4.75 ± 1.98	4.48 ± 1.00	–	3.38 ± 0.36	–	5.08 ± 0.70	0.50 ± 0.15	4.48 ± 1.00	–	6.50 ± 1.90	–
	180	6.00 ± 0.53	4.50 ± 0.68	3.88 ± 1.23	–	2.50 ± 0.29	–	4.88 ± 0.65	0.19 ± 0.06	3.88 ± 1.23	–	4.13 ± 1.30	–
	200	5.09 ± 0.41	3.50 ± 0.24	3.00 ± 1.15	–	1.68 ± 0.53	–	4.73 ± 0.57	0.10 ± 0.06	3.00 ± 1.15	–	3.00 ± 0.94	–
	220	3.50 ± 0.41	2.75 ± 0.24	2.08 ± 0.76	–	1.35 ± 0.49	–	4.46 ± 0.48	0.03 ± 0.09	2.08 ± 0.76	–	1.50 ± 0.47	–
	240	1.99 ± 0.21	1.75 ± 0.98	1.90 ± 0.52	–	1.15 ± 0.38	–	2.32 ± 0.10	–	1.90 ± 0.52	–	1.00 ± 0.26	–
	260	1.11 ± 0.10	0.50 ± 0.28	1.28 ± 0.30	–	0.40 ± 0.23	–	1.40 ± 0.02	–	1.28 ± 0.30	–	0.50 ± 0.16	–
	280	0.69 ± 0.18	–[1]	–	–	0.20 ± 0.12	–	0.58 ± 0.17	–	–	–	0.13 ± 0.06	–
	300	0.30 ± 0.06	–	–	–	–	–	0.13 ± 0.07	–	–	–	–	–
	320	0.11 ± 0.09	–	–	–	–	–	–	–	–	–	–	–
	Total	52.00 ± 9.93	39.10 ± 19.02	44.95 ± 4.88	26.30 ± 4.33	42.60 ± 4.23	33.08 ± 4.93	52.63 ± 8.30	35.25 ± 6.33	44.95 ± 4.88	24.83 ± 3.64	42.15 ± 3.94	35.20 ± 4.58

[1]*All data are reported as mean ± SE.* [2]*Denotes that no observations could be made in this category.*

TABLE 3 | Dendritic arborization in granule cells of WT and *Egr3−/−* mice.

		WT		Egr3−/−	
		DG_{sp}	DG_{ip}	DG_{sp}	DG_{ip}
Branching	1	2.05 ± 0.17[1]	2.04 ± 0.10	2.23 ± 0.12	2.08 ± 0.50
Order	2	4.13 ± 0.36	3.89 ± 0.28	4.65 ± 0.22	3.93 ± 0.47
	3	5.08 ± 0.39	4.91 ± 0.21	5.55 ± 0.27	5.03 ± 0.68
	4	3.63 ± 0.16	3.58 ± 0.77	4.38 ± 0.35	4.28 ± 1.02
	5	1.225 ± 0.25	1.70 ± 0.39	1.50 ± 0.36	1.55 ± 1.15
	6	0.45 ± 0.17	0.40 ± 0.21	0.63 ± 0.31	0.35 ± 0.37
	7	0.45 ± 0.16	0.40 ± 0.21	0.53 ± 0.26	0.30 ± 0.37
	8+	0.09 ± 0.04	0.01 ± 0.01	0.10 ± 0.06	0.05 ± 0.01
	Total	17.00 ± 0.61	16.91 ± 1.20	19.55 ± 1.36	17.55 ± 2.74
Scholl (μm)	20	2.85 ± 0.28	3.13 ± 0.85	3.08 ± 0.26	3.27 ± 0.28
	40	4.425 ± 0.20	4.48 ± 1.20	4.55 ± 0.38	4.39 ± 0.43
	60	4.7 ± 0.16	5.83 ± 0.73	5.58 ± 0.49	5.53 ± 0.45
	80	5.73 ± 0.20	6.05 ± 0.71	6.32 ± 0.38	5.94 ± 0.35
	100	6.10 ± 0.16	6.33 ± 0.84	6.80 ± 0.39	6.02 ± 0.58
	120	6.85 ± 0.19	6.63 ± 0.62	7.20 ± 0.31	6.52 ± 0.48
	140	6.975 ± 0.21	6.50 ± 0.76	7.75 ± 0.49	6.55 ± 0.38
	160	7.05 ± 0.26	6.30 ± 0.85	7.90 ± 0.40	6.50 ± 0.41
	180	6.98 ± 0.28	5.65 ± 1.20	7.85 ± 0.46	5.95 ± 0.38
	200	6.58 ± 0.27	5.08 ± 0.93	7.48 ± 0.50	5.45 ± 0.49
	220	6.53 ± 0.23	4.48 ± 0.96	6.83 ± 0.50	5.40 ± 0.49
	240	6.10 ± 0.18	3.48 ± 1.34	6.43 ± 0.44	4.36 ± 0.45
	260	5.28 ± 0.25	2.50 ± 1.45	5.95 ± 0.31	3.55 ± 0.50
	280	4.65 ± 0.33	1.70 ± 1.80	5.15 ± 0.28	2.29 ± 0.37
	300	3.5 ± 0.27	0.95 ± 1.83	3.80 ± 0.23	1.37 ± 0.38
	320	2.3 ± 0.20	0.40 ± 0.97	2.48 ± 0.29	0.64 ± 0.29
	340	1.5 ± 0.44	0.25 ± 0.96	1.88 ± 0.55	0.48 ± 0.19
	Total	88.08 ± 2.15	69.68 ± 10.45	97.01 ± 4.26	74.17 ± 5.41

[1]*All data are reported as mean ± SE.*

CpG binding protein 2 (MeCP2), and the basic helix-loop-helix upstream signaling factors (USFs; Chen et al., 2003), positively regulate expression of *Bdnf*. In contrast, stressful environmental stimuli, ischemia (Lindvall et al., 1992), and prenatal stress (Boersma et al., 2014) lead to decreased expression levels of *Bdnf* in the hippocampus.

One of the stimuli known to activate *Bdnf* is ECS. ECS is the experimental equivalent of ECT, a treatment that has been in use for more than 80 years, and that remains the most effective treatment for severe mood disorders and psychotic disorders with a mood component (Fink, 2001, 2014; Pagnin et al., 2004; Rosenquist et al., 2014). ECT results in increased levels of BDNF in peripheral blood in humans (Bocchio-Chiavetto et al., 2006). Studies in rodents demonstrate that ECS induces high levels of BDNF expression in the hippocampus (Altar et al., 2004). ECS also induces expression of IEGs, including *Egr3*, as well as other growth factors in addition to BDNF, and stimulates neurogenesis and dendritic sprouting in the hippocampus (Duman and Vaidya, 1998; Malberg et al., 2000; Altar et al., 2004; Perera et al., 2007; Kato, 2009; Youssef and McCall, 2014; Thomann et al., 2017).

Our findings show that hippocampal induction of *Bdnf* in response to ECS requires *Egr3*. The presence of EGR3 binding sites in the *Bdnf* promoter suggests the possibility that EGR3, a transcription factor, may directly regulate expression of *Bdnf*. However, since the current study did not confirm binding of EGR3 to these sites *in vivo*, we are not able to determine this at this time.

Exon IV in particular seems to be an important component of activity-dependent *Bdnf* expression, as deletion of exon IV impedes both ECS-induced, and sleep deprivation-mediated, activation of several *Bdnf* transcripts *in vivo* (Martinowich et al., 2011). Notably, both ECS and sleep deprivation activate expression of *Egr3* as well (Thompson et al., 2010; Maple et al., 2015). Deletion of exon IV also causes impairments in spatial memory reversal and fear memory extinction (Sakata et al., 2013), deficits in GABAergic transmission in the cortex (Sakata et al., 2009), and impairment of hippocampal synaptic plasticity (Sakata et al., 2013) *in vivo*.

Another important activity-induced exon in the hippocampus is exon VI (Chiaruttini et al., 2008). Both *Bdnf* exon IV and VI contribute significantly to BDNF protein levels in the hippocampus (Maynard et al., 2016). We found that both exon IV and VI expression failed to show induction in *Egr3−/−* mice following ECS compared to WT mice. Follow-up bioinformatic

analysis of the 4 kb proximal upstream region of *Bdnf* using TFBind revealed eight high-probability EGR3 binding sites (**Table 1**). Further studies will be important to confirm the binding of EGR3 to these sites and confirm the specific conditions and cell types in which they occur.

Psychotic disorders are characterized by cognitive impairments (Goff et al., 2011). In animals, disrupting the expression of BDNF results in deficits in learning, memory, attention, executive function, and cognition (Sharma and Antonova, 2003). Both *Egr3* and BDNF play critical roles in hippocampal synaptic plasticity, a process associated with memory formation. Specifically, long-term depression, a form of hippocampal plasticity that is facilitated by novelty and stress exposure, requires *Egr3*, and is stimulated by the immature form of BDNF, proBDNF (Kim et al., 1996; Woo et al., 2005; Gallitano-Mendel et al., 2007; Yang et al., 2014; Bukalo et al., 2016). The results of these, and many other studies, have led to the hypothesis that BDNF may be a critical molecule in mediating the therapeutic effects of psychiatric therapies, including ECT (Bocchio-Chiavetto et al., 2006; Li et al., 2016).

One limitation of our study is that it employed mice that lack *Egr3* function throughout development and life. It is therefore possible that the failure of ECS to induce *Bdnf* expression in *Egr3*−/− mice could be explained by a developmental deficit of the cells that normally express *Bdnf*. However, our neuroanatomical studies demonstrated no regional differences in neuronal counts in the DG or CA regions of the hippocampus in *Egr3*−/− mice. Furthermore, no differences were found in the spine density or branch order of pyramidal cells, or in the density DG granule cells.

The absence of neuroanatomical deficits in *Egr3*−/− mice is supported by our prior findings of normal activation of *Arc* expression in DG granule cells following exposure to a novel environment (Maple et al., 2017) and prior studies demonstrating seizure-induced *Arc* expression in the hippocampus of *Egr3*−/− mice (Li et al., 2005). However, this *Arc* expression dissipates over several hours in *Egr3*−/− DG cells, compared to WT mice in which *Arc* expression perdures for up to 8 h (Maple et al., 2017). Thus, the hippocampal DG granule cells of *Egr3*−/− mice are capable of expressing *Arc* in response to activity, but maintenance of this expression requires *Egr3*. Our results indicate that the numbers of DG cells remain consistent in the WT and *Egr3*−/− mice, which suggests that the loss of *Bdnf* in these cells is due to failed activation in the absence of *Egr3*.

Another limitation is that, although we show that *Bdnf* fails to be induced following ECS in the *Egr3*−/− mice, and the *Bdnf* promoter contains putative EGR3 binding sites, we cannot rule out the possibility that an intermediary factor regulates expression of *Bdnf*. Future studies will employ chromatin immunoprecipitation (ChIP) to assess binding of EGR3 to the *Bdnf* promoter in the hippocampus, as well as luciferase reporter assays to demonstrate functionality of this binding *in vitro*.

The expression of IEGs is, by definition, independent of protein synthesis. Since exons III and IV of the mouse BDNF have been reported to have IEG activity (Lauterborn et al., 1996; Binder and Scharfman, 2004; Sakata et al., 2009), this indicates that *Bdnf* transcription should be dependent upon transcription factor proteins that are already present and poised for activation. However, in our study, we demonstrate that *Egr3*, an IEG transcription factor, mediates *Bdnf* mRNA expression in response to ECS. Specifically, we found that *Egr3* is required for ECS-induced expression of *Bdnf* exons IV and VI, with a larger effect on exon IV, in the hippocampus. Since our study examined gene expression 1 h following ECS, it is possible that *Bdnf* is initially activated in the brains of *Egr3*−/− mice, but fails to be maintained in the absence of *Egr3*, as seen with *Arc* expression in prior studies by our group and others (Li et al., 2005; Maple et al., 2017). If this is the case, then the induced IEG *Bdnf* transcripts would have to be degraded within 1 h following ECS, a timeframe more rapid than that of *Arc*.

Our studies identify the novel requirement of EGR3 for hippocampal *Bdnf* expression in response to ECS. We further show that EGR3 is required for seizure-induced expression of *Bdnf* transcripts containing exons IV and VI. Notably, these two isoforms are the greatest contributors to total BDNF protein in the hippocampus (Maynard et al., 2016). These data suggest the possibility that EGR3 may play a role in other forms of activity-dependent *Bdnf* induction in the hippocampus, such as that induced by high-frequency stimulation or exposure to novelty. However, this will have to be tested in future studies. This finding is of interest as both *Egr3* and *Bdnf* share several common features. These include essential roles in hippocampal long-term depression (Gallitano-Mendel et al., 2007; Novkovic et al., 2015; Bukalo et al., 2016) calcium-signaling-dependent activation (Mittelstadt and Ashwell, 1998; Shieh et al., 1998), and genetic association with schizophrenia (Gratacos et al., 2007; Yamada et al., 2007; Kim et al., 2010; Ning et al., 2012; Zhang et al., 2012; Huentelman et al., 2015). We have hypothesized that both *Bdnf* and *Egr3* are crucial components of a biological pathway implicated in mental illness risk (Marballi and Gallitano, 2018). It will be interesting to determine whether activity-dependent, EGR3-mediated *BDNF* expression contributes to the therapeutic effects of ECT on the mood and psychotic symptoms of patients with severe psychiatric illnesses.

AUTHOR CONTRIBUTIONS

MC accumulated images and data for the manuscript and assisted in analyzing NeuN counts. BR contributed to data analysis and image collection for the hippocampal neuroanatomical studies. SB analyzed the original microarray study, identified downstream targets of the immediate early gene, *Egr3*, and developed figures and tables for the binding regions of EGR3 within the Bdnf promoter. KKM validated the work of SB, performed validation studies in the female cohorts of mice, analyzed the qRT-PCR results, formulated graphs, and assisted in the conceptualization of the experiments and the development of the manuscript. AG funded the research with her NIMH funds, conceptualized the project, and developed the hypothesis of immediate early genes synergistically acting with genetic factors to contribute toward the pathogenesis of schizophrenia. DM utilized his funds

obtained from the Natural Sciences and Engineering Research Council of Canada, collaborated with AG to establish the theory of regulation of *Bdnf* by *Egr3*, and contributed directly to the manuscript with his writing, editing, and neuroanatomical studies. KTM performed ECS, collected tissue, performed *in situ* hybridization, analyzed the results, and assisted in writing the manuscript.

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