Converging Biochemical Pathways in Psychiatric Disorders

by

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Abstract:

According to the World Health Organization, neuropsychiatric diseases account for approximately one third of years lost to disability. Yet, despite this huge disease burden, there is a lack of new treatments under development: approved treatments all essentially target the same target(s), if the target itself is known. There is now considerable evidence for a common set of heritable risk for psychiatric disorders including schizophrenia, bipolar disorder, as well as autism. Many of these risk alleles affect genes implicated in neuronal development with known roles at an early stage; these genes would have an effect on the individual before the onset of overt symptoms or diagnosis. Furthermore, many of the genes identified are known to participate in established pathways that are relevant for neuronal development and function. It is important then to address the causality between these signaling pathways that are important for neurodevelopment, and the risk of developing neuropsychiatric disorder.

The work presented in this thesis represents two projects that aim to work toward this goal. The first project pertains to the mechanisms of transcriptional repression by DISC1 on ATF4-mediated gene transcription. The second project presents some initial steps towards uncovering the role of BCL9 in neuronal development.

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CHAPTER 1: INTRODUCTION

Psychiatric diseases: cost, burden and need for basic research

According to the World Health Organization, neuropsychiatric diseases account for approximately one third of years lost to disability (YLD) (Table 1). The major depressive disorders (unipolar and bipolar depression) as well as schizophrenia all separately rank within the top ten causes of YLD and account for roughly half of the YLD due to neuropsychiatric causes (Table 2) (source: http://apps.who.int/ghodata/?vid=110001 accessed 04/09/2012).

Cause of YLD by Category	**% total YLD**
Neuropsychiatric disorders	31.0
Sense organ disorders	14.5
Unintentional injuries	8.1
Infectious and parasitic diseases	8.0
Musculoskeletal diseases	5.0
Respiratory diseases	4.7
Nutritional deficiencies	4.5
Maternal conditions	3.9
Cardiovascular diseases	3.8
Perinatal conditions (e)	3.4
Digestive diseases	2.5
Congenital abnormalities	1.8
Intentional injuries	1.7
Diabetes mellitus	1.6
Oral diseases	1.3
Respiratory infections	1.0
Nutritional/endocrine disorders	1.0

Diseases of the genitourinary system	0.8
Malignant neoplasms	0.7
Skin diseases	0.5
Other neoplasms	0.0

Table 1: Cause of years lost to disability (YLD) as recorded by the World Health Organization.

Cause, Specific Condition	%YLD
Unipolar depressive disorders	10.9
Refractive errors	4.6
Hearing loss, adult onset	4.6
Other unintentional injuries	4.0
Alcohol use disorders	3.7
Cataracts	3.0
Schizophrenia	2.7
Osteoarthritis	2.6
Bipolar affective disorder	2.4
Iron-deficiency anemia	2.2

Table 2: Top ten causes of years lost to disability as recorded by the World Health Organization.

These statistics highlight the immense burden these disorders place on the human species as a whole and also the need to better treat the patients that have been identified as having these debilitating disorders. Yet, despite this huge disease burden, there is a lack of new treatments under development: approved treatments all essentially target the same target(s), if

the target itself is known, investment by the private sector into novel therapeutics for these disorders have stalled (GlaxoSmithKline PLC, Sanofi-Aventis, AstraZeneca, and Merck, WSJ http://online.wsj.com/article/SB10001424052748704474804576222463927753954.html accessed 4/9/2012). The great majority of pharmaceutical companies have halted or refocused their efforts to other disorders, citing high costs, high probability of failure, and the lack of promising novel therapeutic targets in the treatment of psychiatric disorders. This places an even heavier responsibility on basic scientists in academia to identify the molecular mechanisms underlying these disorders, as the causes for these conditions remain unknown, and only with a better understanding of the etiology could more drug targets be identified.

The work presented in this thesis pertains to genes with associations to major depression, bipolar disorder, and schizophrenia. I will briefly introduce the key aspects of each disorder before going into what is currently known about these disorders, based on the genetic studies conducted on affected patients, as well as insights gained from research on the mechanism of action of currently used therapies.

Major depression, bipolar disorder, and schizophrenia

There are many overlapping features between major depression, bipolar disorder, and schizophrenia, however they can be clinically distinguished by the application of various criteria. Major recurrent depression is diagnosed based on the presence of a major depressive episode including the following symptoms nearly every day for a period of at least two weeks, causing significant distress or dysfunction: depressed mood, loss of interest in enjoying pleasure, loss of energy, thoughts of worthlessness or guilt, poor concentration, sleep disturbances, change in appetite/weight, psychomotor retardation/agitation, and thoughts of suicide, with at least one of the five being either depressed mood or loss of interest or pleasure (American Psychiatric Association., 2000) (Table 3). It is estimated that 8% of Americans have had major depressive

episodes, with upwards estimates of prevalence at 11.9% of the population in the United States. Suicide plays a major role in the risk for suicide, which is the leading cause for death in adolescents and young adults under the age of 30 (Richards, 2011; Kupfer et al., 2012).

A. The presence of at least one major depressive episode, defined as the presence of five or more of the following symptoms, present most of the day nearly every day for a minimum of two consecutive weeks. •**Depressed mood*** •**Loss of interest or pleasure in most or all activities*** •Insomnia or hypersomnia •Change in appetite or weight •Psychomotor retardation or agitation •Low energy •Poor concentration •Thoughts of worthlessness or guilt * At least one of these must be present B. The symptoms do not meet criteria for a mixed episode. C. The mood disturbance is sufficiently severe to cause marked impairment in occupational functioning, usual social activities, or relationships with others. D. The symptoms are not due to the direct physiological effects of a substance (eg, a drug of abuse, a medication, or other treatment) or a general medical condition (eg, hyperthyroidism). E. The symptoms are not due to bereavement
Table 3. Diagnostic Criteria for Major Depressive Disorder

Bipolar disorder, though less common, with an estimated 1-3% of the population affected (Kieseppa et al., 2004; Miller, 2006; Edvardsen et al., 2008; Sachs et al., 2011), nonetheless has a significant impact on society. The diagnosis of bipolar disorder I is made based on the presence of a manic or hypomanic episode, which is defined as a period of abnormally and persistently elevated mood such as distractibility, inflated self-esteem, racing thoughts, a decreased need for sleep, and risk-taking behavior (Table 4). The presence of a major depressive episode, while not necessary for diagnosis, is very common: in fact, patients with bipolar I experience depressive episodes approximately 3 times as often as manic episodes. Bipolar II is the diagnosis given to patients that experience hypomania and also have at least one episode of major depression (Benazzi, 2007a). Patients with bipolar II experience depressive symptoms as many as 37 times more frequently than symptoms of hypomania (Judd et al., 2003). A major cause of death in patients with bipolar disorder is suicide, with 15% of

patients diagnosed successfully committing suicide (Miller, 2006; Benazzi, 2007b, a). It should be noted that during episodes of mania, affected individuals could experience delusions, as well as hallucinations, both of which are characteristics of psychosis. Mood stabilizers such as lithium and valproic acid are commonly used to treat bipolar disorder, though several recent reviews of the literature have found that the antipsychotics, which will be mentioned below, are more effective in the treatment of mania than mood stabilizers (Cruz et al., 2010; Chwieduk and Scott, 2011; Sachs et al., 2011; Zupancic, 2011).

Diagnostic criteria common to Mania and Hypomania: A. A distinct period of abnormally and persistently elevated, expansive, or irritable mood, lasting at least 1 week (or any duration if hospitalization is necessary) for mania, and at least 4 days for hypomania. B. During the period of mood disturbance, three (or more) of the following symptoms have persisted (four if the mood is only irritable) and have been present to a significant degree: • Inflated self-esteem or grandiosity •Decreased need for sleep (eg, feels rested after only 3 hours of sleep) •More talkative than usual or pressure to keep talking •Flight of ideas or subjective experience that thoughts are racing •Distractibility •Increase in goal-directed activity or psychomotor agitation •Excessive involvement in pleasurable activities that have a high potential for painful consequences E/F. The symptoms are not due to the direct physiological effects of a substance (eg, a drug of abuse, a medication, or other treatment) or a general medical condition (eg, hyperthyroidism).
Diagnostic criteria specific to mania C. The symptoms do not meet criteria for a mixed episode. D. The mood disturbance 1) is sufficiently severe to cause marked impairment in occupational functioning, usual social activities, or relationships with others, 2) necessitates hospitalization to prevent harm to self or others, or 3) has psychotic features.
Diagnostic criteria specific for hypomania C. The episode is associated with an unequivocal change in functioning that is uncharacteristic of the person when not symptomatic. D. The disturbance in mood and the change in functioning are observable by others. E. The episode 1) is not severe enough to cause marked impairment in social or occupational functioning, 2) does not necessitate hospitalization, and 3) does not have psychotic features.
Table 4. Diagnostic Criteria for Bipolar Disorder.

Schizophrenia is a chronic debilitating disorder that affects 0.7-3% of the population (2008). The symptoms of schizophrenia can be broadly put into three categories: positive, negative, and cognitive. The positive symptoms, most widely associated with schizophrenia, are

synonymous with psychosis and include hallucinations, delusions, and disorganized thought. The negative symptoms are the decrease in, or absence of characteristics such as motivation, affective response, verbal speech, attention, and enjoyment. People with schizophrenia also suffer from cognitive impairment including attention, language, memory, and executive function. Relative cognitive impairment, along with other deficits such as low social function, is often noted before the onset of positive symptoms, and is seen more often seen in people with affected siblings. Schizophrenics also exhibit a moderate and appreciable decline in cognitive function throughout their lifetime. Suicide risk is increased in schizophrenia, particularly amongst those early in the diagnosis and with high cognitive function, with estimates between 5-10% successfully committing suicide (Laursen et al., 2012). Even after taking into account the increased rate of suicide, the diagnosis of schizophrenia is associated with higher mortality; this can only be partially explained to be due to decreased rate of adherence to medication (Saha et al., 2007).

A. Two (or more) of the following, each present for a significant portion of time during a 1-month period (or less if successfully treated)*:
- •Delusions
- •Hallucinations
- •Disorganized speech (eg, frequent derailment or incoherence)
- •Grossly disorganized or catatonic behavior
- •Negative symptoms, ie, affective flattening, alogia, or avolition

B. For a significant portion of the time since the onset of the disturbance, one or more major areas of functioning such as work, interpersonal relations, or self-care are markedly below the level achieved prior to onset (or when the onset is in childhood or adolescence, failure to achieve expected level of interpersonal, academic, or occupational achievement).

C. Continuous signs of the disturbance persist for at least 6 months.
- 1). This 6-month period must include at least 1 month of symptoms (or less if successfully treated) that meet Criterion A, above (ie, active-phase symptoms) and
- 2). May include periods of prodromal or residual symptoms during which signs of the disturbance may be manifested by
  - a). Only negative symptoms or
  - b). Two or more symptoms listed in Criterion A present in an attenuated form (eg, odd beliefs, unusual perceptual experiences).

D. Schizoaffective disorder and mood disorder with psychotic features have been ruled out

E. The disturbance is not due to the direct physiological effects of a substance (eg, a drug of abuse, a medication) or a general medical condition.

F. Relationship to a pervasive developmental disorder

If there is a history of autistic disorder or another pervasive developmental disorder, the additional diagnosis of schizophrenia is made only if prominent delusions or hallucinations are also present for at least a month (or less if successfully treated).

Table 5. Diagnostic Criteria for Schizophrenia

Therapeutic interventions in place for the treatment of major depression, bipolar disorder, and schizophrenia

Recent literature reviews have shown a modest, at best, effect of current therapies for the treatment of severe depression, and no observable effect – beyond placebo- of current pharmacologic therapies on mild to moderate depression, as defined by a Hamilton Depression Rating Scale score of less than 25 (Hamilton, 1980). Most major depressive episodes go undiagnosed (Cepoiu et al., 2008), and most resolve within a matter of months, without treatment. Current pharmacological therapies used in the treatment of major depressive disorder (MDD) include the use of monoamine oxidase inhibitors (MAOi's) which act in the monoamine releasing neurons to inhibit the breakdown of monoamines; tricyclic antidepressants, which block monoaminergic neurotransmitter reuptake; and more selective reuptake inhibitors, such as selective serotonin reuptake inhibitors (SSRI's), and serotonin-norepinephrine reuptake inhibitors (SNRI's) (Crupi et al., 2011). The first line therapies for treatment have become the selective reuptake inhibitors, because of their relatively modest side effect profile compared to the MAOi's and tricyclics: in fact, MAOi's are now rarely used, because its use often necessitates dietary restriction and the risk for serotonin syndrome (Gillman, 2006; Sun-Edelstein et al., 2008). However, even the first line therapies come with a number of side effects, including drowsiness, sexual dysfunction, weight gain, insomnia, and anxiety, which are seen in over 10% of patients taking these medications. That all these medications are effective suggests that all of these neurotransmitter systems play a role in affect and mechanisms downstream of each of these signaling cascades may play a role in the manifestation and treatment of MDD, which will be discussed later.

The reuptake inhibitors all take about a week before improvements in mood can be discerned from placebo (Racagni and Popoli, 2010), and the current theory of this delay in efficacy is due to the requirement of a secondary effect that takes place after the drugs have reached its site of action. Two potential secondary effects have been presented, which are neither mutually exclusive nor comprehensive in their explanations. Several animal model studies (Overstreet et al., 2003; Richardson-Jones et al., 2010) and numerous human brain imaging studies (Hirvonen et al., 2008; Saijo et al., 2010) have implicated the role of presynaptic autoreceptors, 5-HT1A in particular for serotonergic neurons of the dorsal raphe (Serretti et al., 2004) and both 5HT1A and alpha-2-adrenergic receptors in adrenergic neurons of the locus coeruleus (Berrocoso and Mico, 2007; Ortega et al., 2010) , in the delay of onset of antidepressants. The initial blockade of reuptake triggers an increase in extrasynaptic levels of the monoamine neurotransmitters, resulting in an activation of autoreceptors at the presynaptic cell soma of the monoamine-releasing neurons. The activation of autoreceptors at the soma results in a reduced rate of action potentials in these neurons, which leads to a compensatory reduction in the level of released neurotransmitter at their postsynaptic sites of action: it is only with downregulation of these autoreceptors that a functional increase in monoamine neurotransmitter can be observed (Richardson-Jones et al., 2010). This observation has led to an interest in using a 5HT1A antagonist in conjunction with SSRI's to improve clinical efficacy or abridging the time of antidepressant effect. Some trials have shown promising results (Whale et al., 2010; Portella et al., 2011), while others have shown mixed results (Scorza et al., 2011).

A second reason that has been proposed for the delay in action of reuptake inhibitors, is that the increase in monoamine levels leads to a cascade of effects postsynaptically that take weeks to manifest. Some of the effects seen after chronic antidepressant treatment that are hard to appreciate immediately include things such as increase in dendritic arborization in numerous brain regions (Alves et al., 2002; Jones et al., 2009), changes in gene transcription of

numerous factors that have been implicated in cell survival (Mannari et al., 2008), chronic stress response(Aubry et al., 1999; Anacker et al., 2011), metabolism (Duman et al., 2009), and proliferation (Malberg et al., 2000; Santarelli et al., 2003)). I will group these effects and talk about them as effects on neurons that are already present and established in the neural circuitry underlying depressive disorders, and effects on newly formed cells that become incorporated into the neural circuitry underlying depressive disorders.

Effects on the generation of neurons that become incorporated into circuitry

The subgranular zone of the dentate gyrus and subventricular zone have been established as two areas in which neurogenesis continues into adulthood (Ma et al., 2009). In the rodent brain, 6% of the neurons in the dentate gyrus are estimated to be new neurons generated within a month (Cameron and McKay, 1998). While a significantly smaller percentage is estimated to generated in humans (Amrein et al., 2011), the same mechanisms are presumed to be important across species. Neurogenesis in the dentate gyrus, in particular, plays a role in both the development of depression (Snyder et al., 2011) and antidepressant effect (Sahay and Hen, 2007). Therapeutic interventions that induce a depressive phenotype, such as corticosterone/glucocorticoid treatment (Brummelte and Galea, 2010), chronic stress (Pham et al., 2003), interferon treatment (Kaneko et al., 2006) lead to a significant reduction in neurogenesis. On the other hand, treatments that have an antidepressant effect increase neurogenesis, whether it be pharmacologic, in the case of MAOi's (Malberg et al., 2000; Nakagawa et al., 2002a), Tricyclics, SSRI's (Malberg et al., 2000; Santarelli et al., 2003), SNRI's (Shankaran et al., 2006), Lithium (Chen et al., 2000), antipsychotics (Kippin et al., 2005), or electroconvulsive treatment (Madsen et al., 2000).

Antidepressants' effects on already-born neurons

Antidepressants have also been shown to have effects on neurons independent of neurogenesis. Newly born neurons have a higher rate of survival in the presence of antidepressants, independent of the effect of SSRI's on progenitor proliferation (Bath et al., 2012). SSRI administration results in structural changes in non-neurogenic brain regions (McCabe and Mishor, 2011). IV Ketamine, currently under investigation in clinical trials for antidepressant treatment, is also known to facilitate the massive reorganization of the dendritic architecture of neurons from numerous cortical brain regions (Surget et al., 2011).

Schizophrenia: Antipsychotics

The treatments for bipolar disorder and schizophrenia fare better than placebo (Leucht et al., 2012) but their efficacy is limited to only a subset of symptoms, and also come with a high-side effect profile that often results in treatment discontinuation, either by the choice of the patient or due to the development of dangerous side effects (Daumit et al., 2008; Caroff et al., 2011).

The first antipsychotic, chlorpromazine, was discovered in 1952, rather accidentally: it was initially developed for use as a surgical anesthetic, but was used in psychiatric patients for its sedative effects. The discovery of chlorpromazine led to a revolution in psychiatric care, as patients that responded to treatment were discharged from confinement in psychiatric inpatient hospitals: previous to this, the diagnosis meant a lifetime of respite and confinement in these wards. The current pharmacological treatments for schizophrenia, referred to as the antipsychotics have some antagonist activity at the dopamine D2 receptor. The pharmacologic agents available to date for the treatment of the disorder only provide symptomatic relief, and are effective for only a portion of the symptoms (mainly positive). The treatments themselves have significant adverse effects, including tardive dyskinesia, sedation, metabolic changes including diabetes, and because of this, discontinuation rate is high (Salimi et al., 2009). The

current pharmacologic treatments for bipolar disorder include a combination of mood stabilizers plus an antipsychotic (Correll et al., 2010).

Current hypothesis pertaining to risk for major depressive disorder (MDD)

There are many hypotheses as to what results in the manifestation of these psychiatric conditions, based on neurochemical, neuroanatomical, and epidemiological findings, the pharmacology or effect of treatments, and, most recently, genome-wide association studies (GWAS). The leading hypothesis is that there is dysfunction of numerous brain areas related to the generation and processing of emotions, such as the prefrontal cortex, amygdala, hypothalamus, medial septal areas and the hippocampus. Significant efforts have been made to examine the role of neurogenesis on the etiology of depression. This is based on the findings that antidepressants, as well as other interventions that have antidepressant like effects such as deep brain stimulation and environmental enrichment in animal models, all increase the number of newly-born neurons in the dentate gyrus, and because the time course for the maturation of adult-born neurons is consistent with the onset of antidepressant effectiveness. However, a change in adult neurogenesis is not sufficient to explain all the activity of antidepressants, as there are neurogenesis-independent actions of antidepressants (Sahay and Hen, 2007). Furthermore, research to date suggests that adult neurogenesis in the hippocampus is a substrate for the behavioral effects of antidepressants, but is not an essential contributor to the etiology of depression. Two large-scale genome-wide association studies have been conducted to date with no significant correlation identified between disease and a genomic region (Muglia et al., 2010). Thus, the etiology of unipolar depression remains a significant challenge to address.

Current hypothesis pertaining to risk for bipolar disorder

The concordance rate of bipolar I disorder in monozygotic twins is around 40% compared to ~10% in dizygotic twins and in non-twin siblings (Kieseppa et al., 2004). The

overall heritability of bipolar disorder is 0.71 (Edvardsen et al., 2008). There have been several genome-wide association studies to date that have identified novel genetic loci associated with the disease (2007; Ferreira et al., 2008; Sklar et al., 2008; Purcell et al., 2009). These studies point to the heterogeneity of underlying genetic contributions to the risk of developing bipolar disorder. Recent studies have identified linkage disequilibrium for polymorphisms within the CACNA1C L-type voltage gated calcium channel subunit and ANK3 (Ferreira et al., 2008), as well as other genetic regions close to miR137 (Ripke et al., 2011), CDH7 (Soronen et al., 2010), and c10orf26 open reading frames (Ferreira et al., 2008; Sklar et al., 2008; Moskvina et al., 2009). As ANK3 plays an important role in clustering Na+ and K+ channels at the axon initial segment (Pan et al., 2006), it is tempting to speculate that changes in channel property or synaptic transmission contribute to the etiology of bipolar disorder. There is, however, a significant proportion of the heritability of the disorder that has yet to be taken into account, illustrating the need for novel and, as yet unexplored mechanisms to probe for disease heritability that go beyond the sequence of the genes themselves.

Current Hypotheses on Disease Etiology: schizophrenia

Numerous hypotheses to the etiology of schizophrenia have been postulated to date such as the dopamine, glutamate, and GABA hypotheses, based on medications, pharmacological mimicry and postmortem neuropathological findings, but none are sufficient to explain the onset of the syndrome. The neurodevelopmental hypothesis for schizophrenia has also been gaining traction in the field. This hypothesis predicts that alterations during the development of the brain result in cognitive deficits and renders the individual more susceptible to developing psychiatric disorders at a later age. Indeed, the cognitive deficits seen in patients with schizophrenia are often detected long before the diagnosis is made, and these same deficits are often seen in family members (Gold and Weinberger, 1995). Though there are no pathomnemonic features or tests to date for the disorder, and there is no identified organic

cause, several lines of evidence suggest that such a cause may exist. Endophenotypes, including those from neurological testing and functional brain imaging have been identified in some people with schizophrenia and their family members. These endophenotypes may also point to a brain with more susceptibility for developing schizophrenia. Genetic abnormalities that result in aberration of neural development have been associated with schizophrenia, as will be discussed below.

There is a 50% concordance rate for schizophrenia between monozygotic twins, and the offspring of affected individuals have a ten-fold increase in risk for developing the disorder relative to the general population, highlighting a genetic basis for the disorder. Recent genetic evidence indicates that microdeletions or duplications of certain chromosomal loci, known as copy number variations (CNVs), increase the risk for schizophrenia. Chromosome 22q11.2 microdeletions occur in 1 of every 4,000 live births. These deletions produce a wide range of clinical presentations including velo-cardio-facial syndrome, DiGeorge syndrome, and mental retardation (Debbane et al., 2005). About 30% of carriers develop psychosis (Debbane et al., 2006a). Reduced brain volume, or microcephaly, is a common pathological feature of these carriers (Debbane et al., 2006b). Recently, additional CNVs have been identified that increase the risk for schizophrenia including microdeletions of chromosome 1q21.1, 15q13.3, 15q11.2, 3q29, 16p13.1, and 17p12 and microduplications at 16p11.2 (International Schizophrenia Consortium2008; Stefansson et al., 2008a; Vacic et al., 2011). Interestingly, 1q21.1 microdeletions and duplications are also involved in other neurodevelopmental disorders such as microcephaly/macrocephaly with behavioral abnormalities (Brunetti-Pierri et al., 2008) and autism (Mefford et al., 2008), whereas 15q13.3 microdeletions and duplications are associated with idiopathic epilepsy, mental retardation, autism, ADHD, and anxiety disorder (Ben-Shachar et al., 2009; Helbig et al., 2009). Another study identified rare CNVs disrupting multiple genes in neurodevelopmental pathways in schizophrenia (Walsh et al., 2008). Collectively, genetic

evidence strongly supports the notion that impaired brain development increases the risk for the manifestation of mental illnesses. The genetic findings are also consistent with the general description of the neuropathology of schizophrenia, namely enlarged lateral ventricles and reduced cortical/hippocampal size (Tamminga and Holcomb, 2005). It remains to be determined why a given genetic lesion gives rise to clinically heterogeneous phenotypes.

A Common set of heritable risk for psychiatric disorders

There is now considerable evidence for a common set of heritable risk for psychiatric disorders including schizophrenia, bipolar disorder, as well as autism. For starters, the concordance between monozygotic twins, and the increased incidence amongst family members, for bipolar disorder and schizophrenia support the existence of a such a common genetic factor (Lichtenstein et al., 2009). There are a number of recurrent CNV's that are common to both schizophrenic and autistic patients, albeit some go in opposite directions, suggesting a possible gene-dosage effect (Mefford et al., 2008; Stefansson et al., 2008a; Moreno-De-Luca et al., 2010; Malhotra and Sebat, 2012; Rodriguez-Murillo et al., 2012) Further evidence for the shared genetic risk for these psychiatric disorders comes from genome-wide association studies (GWAS) that have identified numerous genetic risk SNPs that are common between disorders(Sklar et al., 2008; Ripke et al., 2011). Current evidence suggests that schizophrenia, bipolar disorder, and autism, either treated separately or treated as a unitary set, would have an inheritance pattern similar to that of several non-neuropsychiatric disorders, including adult-onset diabetes and inflammatory bowel disease, all of which follow a complex inheritance pattern. It is interesting that schizophrenia has common risk variations for both autism and bipolar disorder, both in SNP alleles and recurrent CNV's, almost as if it is an intermediary phenotype.

Putting everything together: biochemical pathways.

Many of these risk alleles affect genes implicated in neuronal development with known roles at an early stage; these genes would have an effect on the individual before the onset of overt symptoms or diagnosis. Furthermore, many of the genes identified through GWAS are known to participate in established pathways that are relevant for neuronal development and function.

One could hypothesize that the risk for developing psychiatric disorders can be accounted for by disruptions to specific biochemical pathways. This type of approach has been successfully implemented for cancer treatment, where the identification of disease targets based on alterations in pathways has led to the development of targeted therapeutics (de Bono and Ashworth, 2010). This would enable the classification of patients based on the presence of risk, grouped by biochemical pathways, as well as provide a more tractable predictor of patient response to medications. Some of the recently identified risk factors affect neural development at an early stage; before the onset of overt symptoms leading to diagnosis with the disorder. Importantly, many of the genes that have been identified through these genetic studies encode proteins that are known to be pivotal for signaling pathways associated with neuronal development and function. It is important then to address the causality between these signaling pathways that are important for neurodevelopment, and the risk of developing neuropsychiatric disorder.

Several pathways have been clearly implicated in neuronal development: these include the Wnt, Akt, Lis1/NudeL, PKA, and EGR/Map Kinase pathways. These signaling pathways are not straightforward; there is substantial parallel signaling, as well as feed-forward and feedback signaling within one biochemical pathway, as well as substantial crosstalk between these pathways.

In the following paragraphs I will briefly identify some pathways important for neuronal development. I will elaborate on the Wnt pathway moreso than the others because of the intriguing, and notably undocumented, number of genes that are in or near affected gene regions that converge onto the pathway: it is not meant to diminish the importance of the other pathways in neuronal development.

cAMP-PKA-CREB pathway

The cyclic adenosine monophosphate (cAMP)-Protein Kinase A (PKA)-cyclic-AMP response element binding protein (CREB) pathway is a pathway that is activated upon the binding of ligands to G-protein coupled receptors (GPCRs) that are coupled to the $G\alpha_s G\beta\gamma$ heterotrimeric G-protein complex. In the baseline state, the G-protein complex is bound to GDP and is inactive. Upon ligand binding to these GPCR, there is an exchange of GDP to GTP, leading to the dissociation and activation of $G\alpha_s$. $G\alpha_s$ –GTP binds to stimulate the activity of adenylyl cyclase, which converts adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). cAMP acts as a second messenger, and the increase in the levels of cAMP lead to the activation of several key downstream events. cAMP can bind to and open cyclic-nucleotide gated ion channels, influencing neuronal conductance. It can also activate exchange proteins activated by cAMP (EPACs). It's most well characterized function is the activation of protein kinase A (PKA). PKA normally exists as a holoenzyme consisting of two regulatory and two catalytic subunits. cAMP binds to the regulatory subunits, releasing the catalytic subunits for downstream activity. PKA phosphorylates a plethora of substrates: one of them is CREB, a transcription factor that binds to the cAMP response element (CRE). Phosphorylated CREB (pCREB) induces the transcription of many genes involved in neuronal development. pCREB is found in almost all newborn neurons and correlates with the expression of doublecortin (Nakagawa et al., 2002a; Nakagawa et al., 2002b), and is important for newborn neuron survival(Herold et al., 2011). However, the role of CREB in the proliferation

of neural precursors is yet unknown (Merz et al., 2011). However, it is well established that increasing cAMP levels by inhibiting the phosphodiesterase 4 family of enzymes with rolipram, does increase the rate of neuronal progenitor proliferation, raising the possibility that there are other factors that contribute downstream of PKA (Li et al., 2009).

The PI3K-AKT-mTOR pathway

The phosphatidylinositol (3,4,5)-triphosphate kinase (PI3K)-AKT/PKB-mammalian Target of Rapamycin (mTOR) pathway is activated by another class of membrane-bound receptors, the receptor tyrosine kinases. In the baseline state, AKT is bound to phosphatidylinositol(3,4)-bisphosphate (PIP2). Upon activation of a receptor tyrosine kinase, or, in some cases, a G-protein coupled receptor, PI3K phosphorylates PIP2 to form PIP3. AKT bound to PIP3 is then phosphorylated by mammalian target of rapamycin complex 2 (mTORC2), then phosphorylated by phosphinositide dependent kinase 1 (PDPK1). The phosphorylation by both kinases activates AKT to then phosphorylate its targets, including mTOR, implicated in autism, GSK3β, involved in the Wnt signaling cascade, BAD, a pro-apoptotic mitochondrial protein, and IκB kinase (IKK), which regulates NFκB mediated transcription.

The intersection of the AKT pathway with the pharmacology of psychiatric disorder treatment is notable. Dopamine d2 receptors, which are targets of all antipsychotics to date, have been shown to modulate AKT signaling by recruiting a signaling complex that results in the inactivation of AKT. Upon D2 receptor activation and phosphorylation by G-protein coupled receptor Kinase (GRK), β-arrestin and PP2A are recruited to the cell membrane, where they interact with and dephosphorylate AKT. Antipsychotics inhibit this process, allowing AKT to remain active(Beaulieu et al., 2005; Freyberg et al., 2010).

Lithium, a treatment for bipolar disorder, is thought to exert its action by acting to activate AKT activity on GSK3β. Lithium has been proposed to solubilize AKT from the β-

arrestin/PP2A complex(Beaulieu et al., 2004). Thus, both these treatments secondarily affect the wnt pathway via affecting AKT activity.

PLC-PKC pathway

Phospholipase C is an enzyme that is activated downstream of GαoGβywhich cleaves phosphatidylinositol 4, 5 bisphosphate (PIP2) into Inositol-3-phosphate (IP3) and diacylglycerol (DAG). IP3 binds to receptors that regulate the release of calcium from intracellular stores, such as the smooth endoplasmic reticulum, leading to elevated levels of intracellular calcium, and subsequently activating calcium-dependent signaling cascades such as those involving calcium/calmodulin-dependent protein kinase II (CaMKII), calcineurin and PKC.

Map Kinase/ ERK pathway

The mitogen associated protein kinase (MAPK)/ ERK pathway refers to a general pathway in which a receptor-linked tyrosine kinase activation by ligand activates the GTPase Ras. Ras then activates a MAP kinase kinase kinase (MAP3K), which activates, MAP kinase kinase, which activates MAP kinase (MAPK). MAPK's activate transcriptional activity of pro-survival and proliferative genes via several mechanisms. One mechanism is the phosphorylation and activation of RSK, a 40s ribosomal protein S6 kinase. This results in the alteration of RNA translation. RSK also phosphorylates CREB as well as c-Myc, c-Fos, NfκB, altering transcription of survival genes. Notably, RSK also phosphorylates GSK3β.

ERK2 is known to be essential for neuronal progenitor proliferation. ERK2 (MAPK1) knockouts(Satoh et al., 2011) die during embryogenesis. Conditional knockout of ERK2 in neuronal progenitors results in mice with decreased cortical thickness, and have a pool of undifferentiated neuronal progenitors (Samuels et al., 2008).

The Wnt signaling pathway

Wnts are secreted lipoproteins that act as morphogens and play important roles in the development and patterning of various limbs and organs. The Wnt ligands bind to their cell surface receptor, the Frizzled (FZ) family of seven transmembrane G-protein coupled receptors. The Wnt ligands were identified by their homology to Wnt, which was named from a combination of the drosophila gene wingless (Wg) and its mammalian homolog Integration 1 (Int1). To date, nineteen WNTs and ten FZD family members have been identified in humans. The various Wnts as well as the FZDs have distinct, yet overlapping expression patterns throughout the body, which is thought to account for the variability of phenotypes that result upon loss of function (http://www.tcd.ie/Zoology/research/WntPathway/wnt.php, accessed April 17, 2012). Furthermore, it has also been shown that different Wnts have different affinities for the different FZs, and that different Wnts activate different downstream signaling cascades (Kikuchi et al., 2009).

Though none of the Wnts have been crystallized for structural analysis, much is known about their general structure. The Wnts are all about 320-400 amino acids long. They all have an N-terminal signaling sequence, which is required for their secretion, followed by a conserved domain consisting of 22-24 cysteines, the first of which is palmitoylated. The secreted form of the protein is highly hydrophobic (Mikels and Nusse, 2006). The loss of palmitoylation reduces Wnt 's potency, which can be overcome with high concentrations of Wnt, suggesting that palmitoylation is required for Wnt localization(Nusse, 2003; Mikels and Nusse, 2006). Numerous glycosylated forms of Wnt proteins have been detected, supporting the posttranslational modification of Wnts as a means to regulate Wnt signaling.

The ten members of the frizzled family of seven transmembrane G-protein coupled receptors share an N-terminal extracellular domain, three extracellular and three intracellular loops, and an intracellular C-terminal tail, like the classical G-protein coupled receptors; however many features make the FZD family unique. The N-terminus contains a domain rich in

cysteines, and Wnts bind to this region (Schulte and Bryja, 2007). The extracellular loops have putative glycosylation sites, a posttranslational modification that is known to play a role in both receptor localization and ligand binding. The intracellular C-terminal region of the receptor contains a PSD-95/DISC Large/POZ-1-homologous (PDZ) binding domain that is crucial for protein-protein interactions needed to mediate its downstream effects. Furthermore, the intracellular loops have numerous consensus sites for serine/threonine as well as tyrosine phosphorylation, reminiscent of receptor tyrosine kinases. There is evidence to suggest that FZD receptors exist as dimers which is facilitated by the cysteine-rich domain, and Wnt ligand binding. Moreover, FZD dimerization appears to be sufficient for activation of downstream pathways (Carron et al., 2003).

Extensive work in the past 20 years has led to the identification of several Wnt signaling pathways downstream of Wnt ligand binding to FZD or one of its co-receptors:lipoprotein related protein 5/6 (LRP5/6) (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000), receptor-tyroine kinase-like orphan receptor 2 (Ror2) (Saldanha et al., 1998), and related to receptor tyrosine kinase (Ryk) (Inoue et al., 2004).

Canonical Wnt signaling

Binding of Wnt to LRP5/6- associated frizzled activates a pathway known as the canonical Wnt signaling pathway. The canonical Wnt signaling pathway is defined by their dependence on the transcription co-activator β-catenin and the downstream signaling cascades that are associated with it. Briefly, Fzd proteins have a conserved KTxxxW domain that is required for interaction with dishevelled (DVL) proteins. In the absence of Wnt stimulation, DVL is part of a destruction complex, in conjunction with AXIN, adenomatous polyposis coli (APC), casein kinase 1 alpha (CK1α), and glycogen synthase kinase 3 (GSK3β). This destruction complex binds and sequentially phosphorylates β-catenin at multiple sites, thus increasing the

affinity of β-catenin to β-TrCP. β-TrCP serves as one of the F-box proteins in the Skp1-Cullin1-F-box-protein (SCF) E3 ubiquitin ligase complex. Binding of β-TrCP to β-catenin leads to the polyubiquitination and subsequent degradation of β-catenin, preventing the accumulation of β-catenin and limiting its downstream function. Upon Wnt stimulation of the FZD-LRP5/6 complex, DVL is recruited to the complex, where it polymerizes, and through its DIX domain binds to the DIX domain of AXIN. This, in turn, leads to the recruitment of AXIN towards the FZD-LRP5/6 complex, where it mediates interaction with of CK1α and GSK3β to LRP5/6. Next, the kinases phosphorylate LRP5/6 and increases its affinity for AXIN. The LRP5/6 interaction directly inhibits GSK3β phosphorylation of β -catenin (Piao et al., 2008), one of the priming phosphorylations of β-catenin, disrupting the ability of the destruction complex to target β-catenin for ubiquitination and subsequent degradation. Thus, the activation of the Wnt pathway leads to an increase in cytosolic β-catenin.

β-catenin is a transcriptional co-activator of TCF/LEF transcription factors, which control the dynamics of the cell cycle via regulation of cyclinD1. The accumulation of β-catenin in the cytosol leads to its increase in the nucleus and the binding of β-catenin and it's associated transcriptional activators to the T-cell factor and lymphoid enhancer-binding protein (TCF/LEF) DNA binding protein family. TCF/LEF has three identified domains, a high mobility group (HMG) box domain that is crucial for DNA interaction, a caspase activated deoxyribonuclease (CAD) domain necessary for binding to Groucho/TLE, and an N-terminal β-catenin binding domain. In the absence of nuclear β-catenin, the TCF/LEF occupying TCF/LEF DNA binding sites is bound to tetrameric Groucho/TLE, which recruits transcriptional repressors, such as histone deacetylases (HDACs), keeping the DNA in a condensed, repressed state. β-catenin directly competes with, and displaces Groucho/TLE (Daniels and Weis, 2005), and recruits general transcriptional activators such as the histone acetyltransferase CBP, the SWI/SNF complex protein Brg-1, and TATA- binding protein, as well as a more specific core complex consisting of

Pygopus and Legless/BCL9 (Belenkaya et al., 2002; Kramps et al., 2002; Thompson et al., 2002). CBP acetylates histone residues in the promoter region, and the recruitment of Pygopus and Legless/BCL9 leads to the methylation of H3K9 residues by SET-1 methyltransferases, altering the chromatin structure from a condensed, transcriptionally repressed one, to a more relaxed and open state, thus allowing transcription (Fiedler et al., 2008).

The canonical Wnt pathway and psychiatric disease gene candidates

Several players in the Wnt signaling pathways have been associated with psychiatric disease. CHD8, which is also recruited during this process, acts to counter the activating activity of β-catenin, by binding to β-catenin and also Histone1, and thus limiting the relaxation of chromatin. CHD8 SNPs are associated with autism, and BCL9 is one of the genes in the 1q21.1 recurrent CNV, with microdeletions associated with schizophrenia and microcephaly and microduplications associated with autism and macrocephaly. Furthermore, ADA2A, in the 17q12 microdeletion associated with autism, is known to be an acetyltransferase required for transcriptional activity and proliferative effects mediated by β-catenin (Yang et al., 2008).

Both the wnt ligands and the receptor are glycosylated, and perturbations of glycosylation are known to affect the function, localization and signaling through them. SNPs in genes that are involved in glycan synthesis (hsa01030) associate with schizophrenia. Ankyrin 3, which has noncoding SNPs within its gene that associate with both schizophrenia and bipolar disorder, is known to be a component of the Cadherin complex, and disruption of the cadherin complex is known to lead to mislocalization of β-catenin (Orsulic et al., 1999).

DLG1 (Sap97) is one of the interactors of Fz and is known to signal downstream of wnt binding by binding to Adenomatous Polyposis Coli (APC), affecting cell proliferation: this gene is found in the 3q29 CNV deletion. ErBB4/Neuregulin, another well-studied candidate that involves a secreted protein ligand and surface receptor, is a receptor tyrosine kinase, and its

family members have been shown to activate cascades that phosphorylate and lead to the intracellular localization of β-catenin: overexpression of ErbB4 Cyt2 incresed nuclear β-catenin and TCF-LEF transcriptional activity (Muraoka-Cook et al., 2009). PAK2, also in the 3q29 CNV, also serves to phosphorylate and promote the nuclear localization of β-catenin (Zhou et al., 2011).

In addition to the recruitment of DVL, Wnt activates the G-proteins associated with Fz proteins. The FZDs are coupled to the $G\alpha_0$ Gβγ trimeric G-protein complex (Katanaev et al., 2005), and Wnt binding to Fz liberates the Gβγ subunits to activate a calcium-dependent pathway that involves the activation of phospholipase C cascade. The perturbation of these signaling pathways can have consequences beyond this pathway, and can impact the canonical Wnt pathway as well. Neurogranin, implicated in bipolar disorder and schizophrenia, is a key regulator of calmodulin, limiting its activity by binding to it; its binding to calmodulin is dependent on its PKC phosphorylation. The association of voltage-gated calcium channel, CACNA1C,with bipolar and schizophrenia is another intriguing risk gene related to calcium signaling.

Binding of Wnt to Fz leads to activation of pathways involved in planar cell polarity, acting through dishevelled, activating RhoA and RAC1 (Gao et al., 2011; Shafer et al., 2011) (Bo et al 2011 Developmental Cell). RAC1 activation then activates c-Jun N-terminal kinase (JNK) downstream of Wnt5a (Oishi et al., 2003). JNK activation has been shown to be crucial for the proper development of axons and dendrites (Rosso et al., 2005)Calderon et al 2012), and to enhance or inhibit canonical Wnt activation, depending on the Wnt ligand (Billiard et al., 2005). Tao Kinase 2, in the 16p11.2 region, which has CNV's that associate with both autism and schizophrenia (deletion and duplication, respectively), also plays a role in the activation of JNK.

One of the alternative, β-catenin independent pathways that is downstream of LRP5/6-Frizzled signaling, depends on the inhibition of GSK3β. GSK3β is known to destabilize

microtubules by phosphorylating microtubule proteins such as TAU, MAP1B, and MAP2. Inhibition of GSK3β stabilizes microtubules, promoting axon growth and growth cone remodeling. In this manner, the wnt pathway is able to exert effects on neuronal morphology.

Binding of Wnt to Ryk has been shown to directly activate the Src pathway, similar to the activation of many other receptor tyrosine kinases such as EGR. The activation of Src, in turn, has been shown to be crucial for axon guidance. Ryk has also been shown to interact directly to FZD8 XXXXXX), as well as DVL, and to potentiate the canonical Wnt signaling pathway, suggesting that the presence of Ryk in conjunction with Frizzled may either stabilize the DVL-Fz interaction, or enhance AXIN interaction with Fz. The convergence of neuropharmacological and genetic evidence makes the wnt pathway(s), if not a promising target, at least a convenient framework within which the function of psychiatric risk gene polymorphisms and rare variants can be tested.

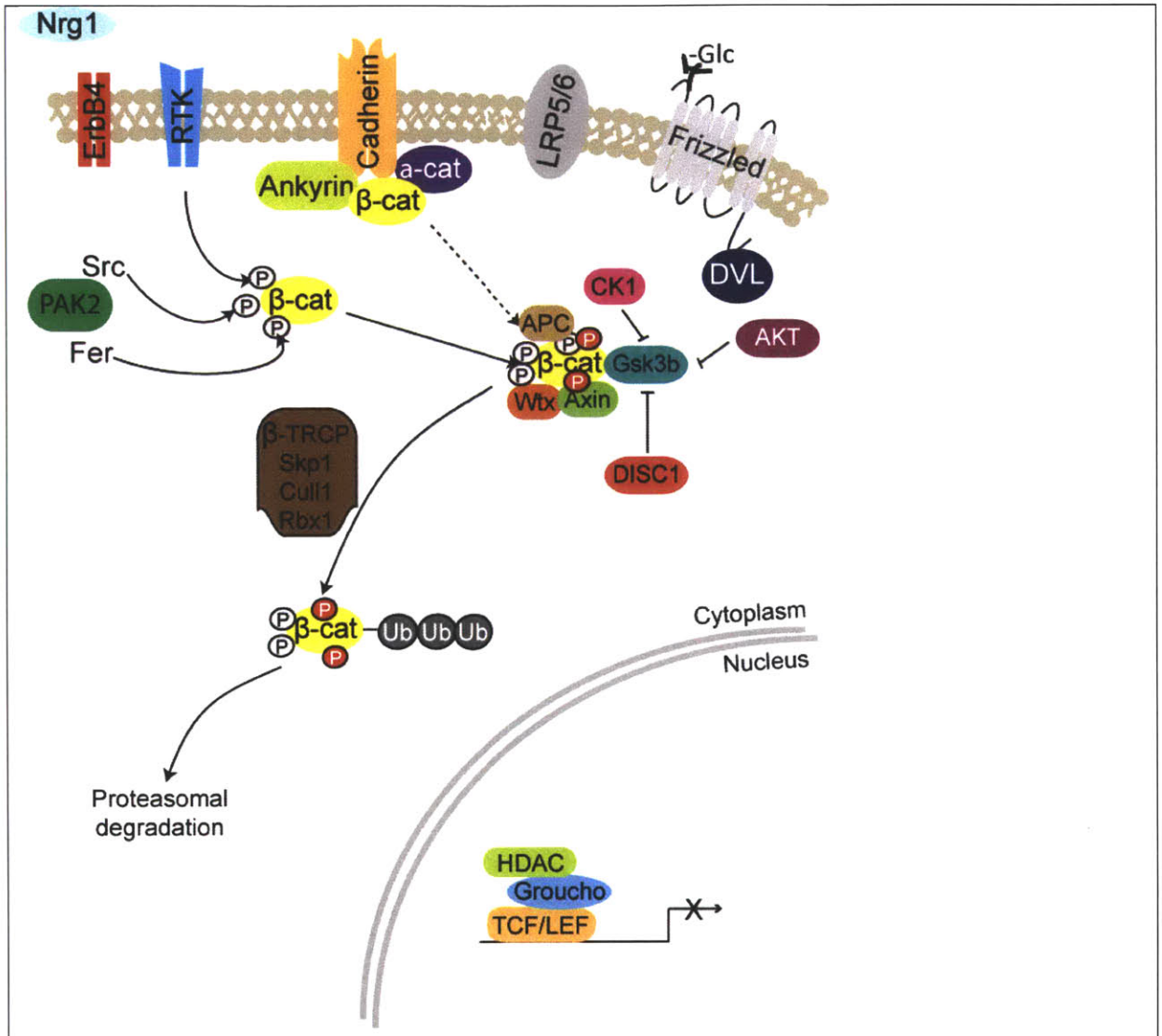

Figure 1. Canonical WNT pathway in inactive state. Tyrosine phosphorylation of β-catenin results in dissociation of β-catenin from its membrane interactors and results in cytosolic localization of β-catenin. And binding to destruction complex. Sequential serine phosphorylation by CK1 and GSK3β (represented as red P's). β-catenin is subsequently polyubiquitinated and degraded by the proteasome.

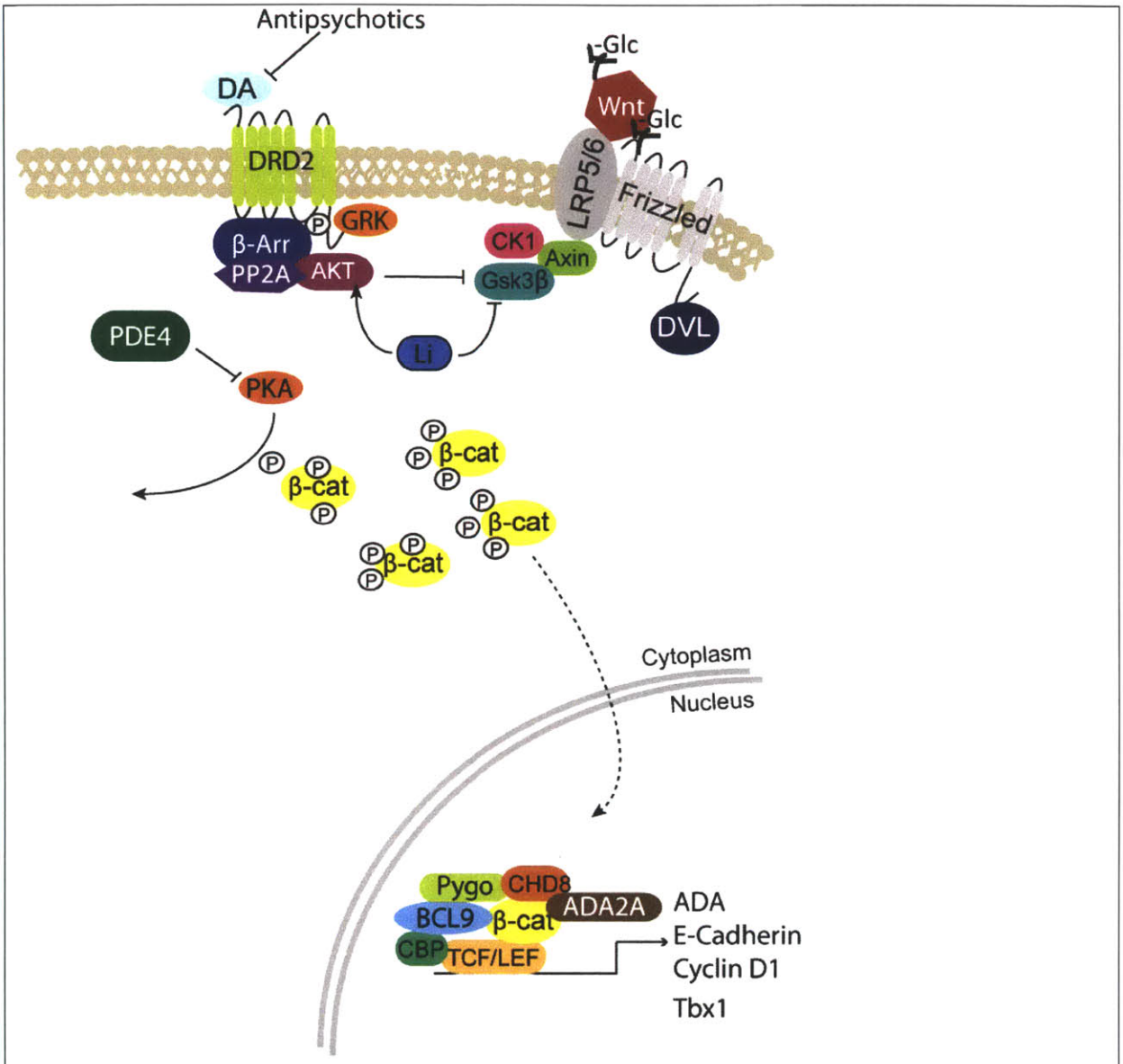

Figure 2. Activated canonical Wnt pathway, and select interactions of pathway with psychiatric disorder therapeutics and risk genes. Wnt-Fz/LRP5/6 interaction recruits destruction complex to the cell surface, and CK1/GSK3β is inactivated. Cytosolic β-catenin bind does not get degraded, gets phosphorylated by PKA, translocates to nucleus where it binds to TCF/LEF transcription factors, recruiting BCL9, Pygo, CHD8, ADA2A, CBP to initiate polII mediated transcription. Lithium, antipsychotics increase AKT activity, which increases its inhibition of GSK3β, enhancing the activity through this pathway. Phosphodiesterase inhibitors would increase PKA activity, which enhances this pathway by increasing nuclear β-catenin translocation and by inactivating GSK3β.

Disrupted in Schizophrenia 1 (DISC1): a single gene to which many pathways converge

Disrupted in Schizophrenia-1 (DISC1) was identified as the gene that was disrupted on chromosome 1 in a Scottish family with a high concordance of major psychiatric disorders and a balanced translocation between chromosomes 1 and 11 (Millar et al., 2000). Karyotyping on five generations of this family revealed 18 of the 29 members with this translocation have schizophrenia, recurrent major depression, or bipolar disorder. This type of near Mendelian segregation for psychiatric disorders had not been previously observed. Mouse models for DISC1 have been generated, and the mice display a variety of phenotypes that can be considered schizophrenia-like behavioral deficits. Mouse models expressing a transgene of human DISC1 mimicking the Scottish translocation mutant exhibit increased ventricle size, decreased gray matter volume, and changes in dendritic arborization in cortical and hippocampal neurons (Hikida et al., 2007; Li et al., 2007; Pletnikov et al., 2008; Shen et al., 2008). These mice also exhibit behavioral abnormalities such as hyperactivity (Hikida et al., 2007; Pletnikov et al., 2008), increased immobility in the forced swim test (Hikida et al., 2007), decreased sociability (Pletnikov et al., 2008), and working memory (Kvajo et al., 2008; Pletnikov et al., 2008). Clapcote et al characterized ENU induced mouse DISC1 exon 2 mutants and reported that the Q31L mutant exhibits depression-like and the L100P mutant exhibits schizophrenia like behavior (Clapcote et al., 2007). In addition, reduced embryonic and adult hippocampal neurogenesis have been reported in a DISC1 BAC transgenic mouse model and DISC1 exon 7-8 deletion mutant mice (Kvajo et al., 2008; Shen et al., 2008). Finally, DISC1 is implicated in the proper integration of adult born neurons in the dentate gyrus circuit (Duan et al., 2007). Collectively, the neuropathology and behavioral phenotypes exhibited by the various DISC1 mouse models strongly supports a role for DISC1 in mental health.

DISC1 binding partners

Since its identification as a psychiatric disease risk gene, a large number of DISC1 binding partners have been identified. Some validated interacting partners for DISC1 include NDE1/NDEL1, important for neuronal migration, Kalirin-7, important for glutamatergic signaling, ATF4/5, important for neuronal progenitor proliferation, the PDE4family of phosphodiesterases, implicated in depression, and GSK3β, a target of Lithium used in bipolar disorder treatment. These interactions will be described in more detail below.

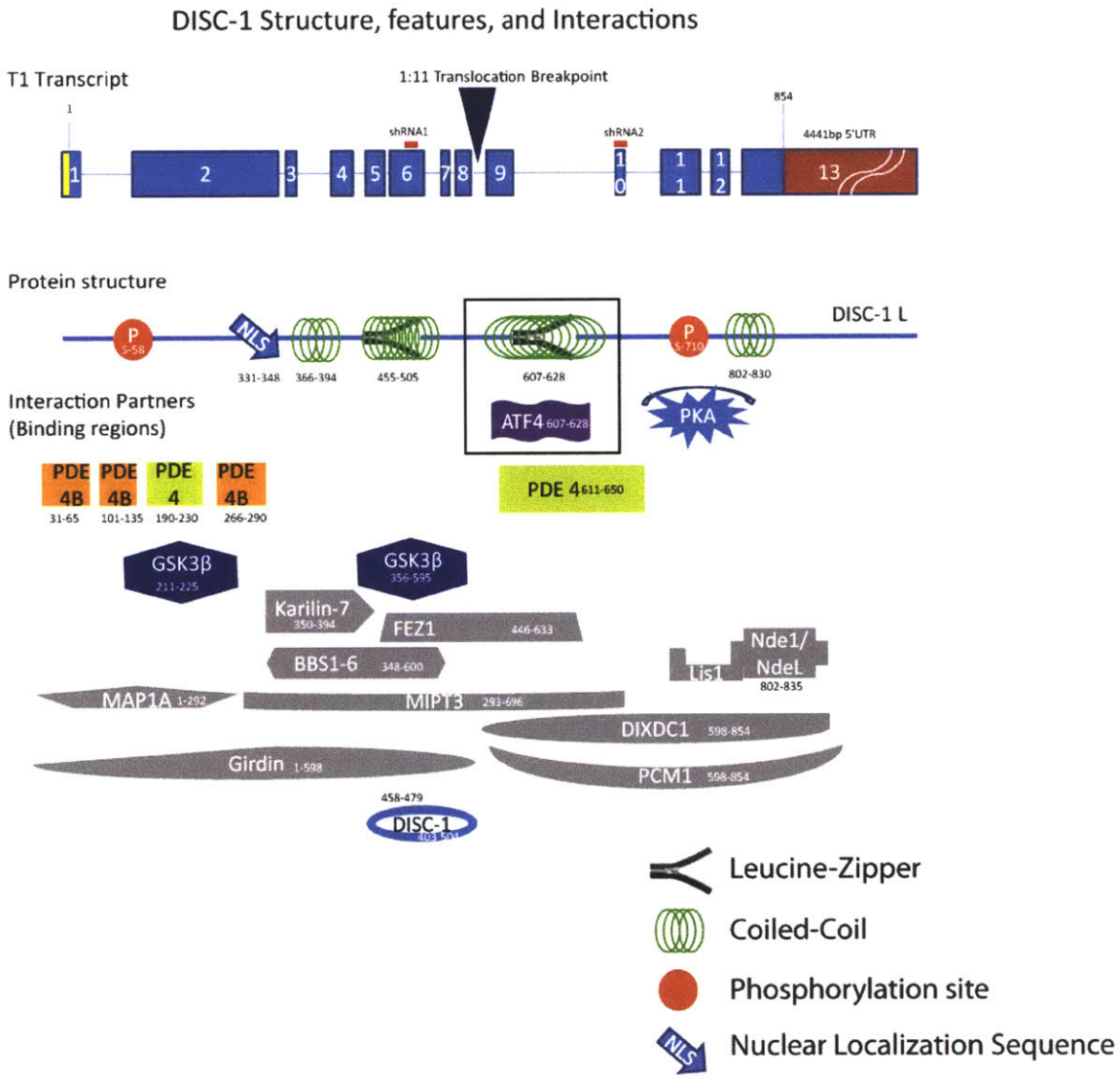

Figure 3. Schematic of validated DISC1 partners. The interactions relevant for further chapters are colored.

DISC1 and neuronal migration

DISC1 binds to nudE nuclear distribution gene E homolog-like 1 or NDE1/NDEL1, which is known to play a role in neuronal migration, proper neuronal localization and neurite and axonal development (Kamiya et al., 2005). Disrupting DISC1/Ndel1 interaction disrupts neurite outgrowth in PC12 cells (Pletnikov et al., 2007), and also prevents the colocalization of DISC1 and Lis1, which localizes with the dynein heavy chain, indicating that DISC1 plays an important role in the function of Ndel1/Lis1 complex by regulating its localization. Our lab has shown that DISC1 also interacts with this complex via its interaction with DIX domain containing-1 (Dixdc1), the third mammalian gene discovered to contain a Disheveled-Axin (DIX) domain (Dixdc1). DISC1, Dixdc1 and Ndel1 form a tripartite interaction, and the presence of either DISC1 or DIXDC1 seems to be able to properly localize Ndel1, indicating that these two proteins may have overlapping/ redundant effects on neuronal migration (Singh et al., 2010). This also indicates that a partial loss of function of either of these two genes can be offset by the function of the other, potentially explaining why differences in the ability of DISC1 snps to bind to NDEL does not result in an appreciable difference in human disease burden.

DISC1 and the WNT signaling pathway

DISC1 has also been shown to bind to, and inhibit the activity of GSK3-β, one of two forms of an enzyme that is known to lead to the phosphorylation of many downstream proteins that are crucial for the pathophysiology of many nervous system disorders. One GSK3-β target is TAU, which forms aggregates in many nervous system disorders including Alzheimer's (TAU forms the neurofibrillary tangles that are used in the Braak and Braak postmortem staging of Alzheimer's), Parkinson's, and tuberous sclerosis: nervous system disorders in which there is TAU aggregation are collectively referred to as tauopathies. Another GSK3-β target is β-catenin. β-catenin is a crucial component of the canonical WNT signaling pathway. β-Catenin is bound

to α-catenin and the cadherin proteins at the cell membrane, as part of the adherens junction (AJ) complex. β-catenin that is not bound to cadherins at the membrane binds to axin, which, in conjunction with APC, complexes β-catenin with GSK3β. The formation of this complex leads to the phosphorylation of β-catenin, which promotes its poly-ubiquitination and subsequent proteasome-mediated degradation. When WNT binds to its receptor, frizzled, disheveled is recruited to the membrane and activated. Activated disheveled inhibits GSK3-β, which subsequently inhibits the phosphorylation and degradation of β-catenin, leading to its accumulation in the cytosol and the activation of downstream pathways, such as the transcriptions of genes which bind TCF/LEF transcription factors. The activation of this pathway has been shown to be crucial for neuronal progenitor proliferation and the inhibition of the premature differentiation of these neuronal progenitors, and deficits in this pathway have been shown to result in microcephaly(Salcedo-Tello et al., 2011). By inhibiting GSK3β, DISC1 promotes neuronal progenitor proliferation, and the loss of DISC1 leads to decreased neuronal progenitor proliferation (Mao et al., 2009; Ming and Song, 2009).

A molecular switch between the wnt signaling and neuronal migration function of DISC1 has been recently uncovered. DISC1 was found to be a target of PKA-mediated phosphorylation, downstream of cAMP activation. The study identified two putative PKA mediated phosphorylation sites on DISC1, and discovered that one of the sites, at serine 710, is responsible for the differential localization and binding properties observed in neuronal progenitors versus migrating neurons. DISC1 in neuronal progenitors is unphosphorylated at this site, and binds to and inhibits GSK3β activity: upon phosphorylation at this site, DISC1 increases its affinity for BBS1 and localizes at the centrosome. Crucially, the authors demonstrated that the phosphor-dead form of DISC1 (S710A) rescued the proliferative but not the migrational phenotype observed in DISC1 knockdown conditions, while the phosphor-

mimetic form of DISC1 (S710E) rescued the migrational aspects of DISC1 function(Ishizuka et al., 2011).

DISC1 and Kalirin-7: regulation of glutamatergic synapses

DISC1 plays a role in the regulation of dendritic morphology via its interaction with Kalirin-7 a GDP/GTP exchange factor for Rac1. The DISC1/Kalirin-7 interaction is dependent on NMDA-receptor mediated signaling, and acts to inhibit Kalirin-7 interaction with Rac1. The loss of DISC1 function results in a short-term increase in dendritic spine size, and long-term shrinkage of dendrites and a concurrent loss of surface AMPA receptors, accompanied by a reduced frequency of miniature EPSC's (Hayashi-Takagi et al., 2010).

DISC1 and ATF4

DISC1 also binds to Activating transcription factors 4 and 5 (ATF4 and ATF5, respectively), basic-region-leucine zipper domain containing transcription factors of the CREB/ATF family (Morris et al., 2003). ATF4, also known as CREB2 is both a transcriptional activator and repressor that binds to CRE elements throughout the genome and is known to play a role in hematopoiesis, osteoblast differentiation, neuronal progenitor proliferation, learning and memory, and behavior (Ameri and Harris, 2008; Frank et al., 2010). Disrupting ATF4 interaction results in an alteration of CRE mediated gene transcription (Sawamura et al., 2008), which is known to have a critical role in memory formation and affective behavior (Bartsch et al., 1995; Chen et al., 2003; Green et al., 2008).

Previous work examining the interaction of ATF4 and DISC1 was conducted using overexpression studies in cell lines, and in the nervous system of drosophila. Sawamura and colleagues found that DISC1 overexpression resulted in a decrease in transcription as assayed using a luciferase assay that implemented the consensus CRE sequence as the promotor to drive luciferase expression, and examining the effect of DISC1 overexpression on the activity of

this promotor (Sawamura et al., 2008). However, the endogenous function of the DISC1-ATF4 interaction remains unanswered, as knockdown of DISC1 was not assayed in this study. The effect of endogenous DISC1 on behavior was likewise not addressed in this past study, as drosophila has no endogenous DISC1 homologue (Sawamura et al., 2008). A more recent study has also undertaken the study of DISC1 on ATF4 mediated transcription (Malavasi et al., 2012), yet the study has still relied on overexpression assays, leaving the question of endogenous DISC1 function unaddressed.

DISC1 and the PDE4 phosphodiesterases

DISC1 has also been shown to bind to, and inhibit the activity of several isoforms of a rolipram-sensitive, cAMP specific phosphodiesterase, grouped together as the phosphodiesterase 4 (PDE4) family of genes. DISC1 has been shown to directly bind to, and inhibit, at least some variant(s) of each of the four, A, B, C and D, PDE4 isoforms (Millar et al., 2005; Cheung et al., 2007; Millar et al., 2007; Murdoch et al., 2007). The PDE4's, in particular PDE4D, will be discussed in greater detail below.

PDE4D

The phosphodiesterase 4D gene is one of 4 genes that make up the PDE4 family of phosphodiesterases (table X). The different genes that comprise the PDE4 family encode distinct isoforms of a cyclic adenosine monophosphate (cAMP) specific and rolipram sensitive phosphodiesterase. The gene locus that encodes each isoform contain multiple exons that span dozens of kilobases of the genome, and each isoform has many distinct transcripts, known as variants. All variants from an isoform contain a unique 5' exon which is believed to confer distinct subcellular localization to the variants, and a common catalytic domain, which, as the name suggests, converts cyclic AMP into non-cyclized AMP through a catalytic reaction (Ref).

The regulation of distinct variants within this gene is a subject of intense study ((Dlaboga et al., 2006; Li et al., 2009; De Arcangelis et al., 2010; Li et al., 2011b)refs).

In addition to the distinct functions conferred by the unique 5' exons, the phosphodiesterase activity of this family of enzymes is carefully regulated through numerous mechanisms intrinsic to the protein itself. The catalytic domain itself has a serine site that, when phosphorylated by ERK/MAP kinase, inhibits the catalytic activity. The short variants (PDE4D1/2) have the catalytic domain immediately following their unique exons, but the long variants have an additional common regulatory domain that binds to and inhibits the activation of phosphodiesterase activity by the catalytic domain (Houslay et al., 2005; Houslay, 2010)A serine residue within the regulatory domain is phosphorylated by PKA, and this phosphorylation triggers a release of the catalytic domain from binding to the regulatory domain(Zhang et al., 2006a). This makes the long-isoforms of PDE4D's a PKA-dependent cAMP specific phosphodiesterase.

DISC1 interacts with the cyclic AMP specific, rolipram sensitive phosphodiesterase 4 (PDE4) family isoforms through multiple interaction sites. Rolipram is effective as an antidepressant in mice, but is not used in the United States for this purpose because of its side effect profile, which includes nausea. Mice lacking PDE4B and 4D display a phenotype reminiscent to mice on antidepressants (Zhang et al., 2002; Zhang et al., 2008). Previous work has shown that DISC1 has multiple interaction domains with phosphodiesterase 4, and interactions have been reported with isoforms A, B, C and D (Millar et al., 2005; Murdoch et al., 2007). Elevated levels of cAMP activity is able to disrupt the interaction of full length DISC1 with isoform C variant 2 and isoform D variant 3, but does not affect binding to isoform A variant 5 and isoform B variant 1. It is presumed that the binding characteristics are consistent within the different variants within the isoforms described. A peptide array binding characterization utilizing various fragments of DISC1 and PDE4 B and D have shown that compared to PDE4D, PDE4B

has multiple unique binding sites and it is believed that these additional binding sites confer stability to PDE4A and B compared to C and D. In contrast to full-length DISC1, a 71kb form of DISC1 dissociates from PDE4B under cAMP/PKA stimulation, and the dissociation of PDE4B from DISC1 results in an increase in its phosphodiesterase activity.

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CHAPTER 2:

DISC1 acts as a co-repressor to inhibit ATF4 mediated transcription at the PDE4D loci

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Sangki Park and Li-Huei Tsai.

ABSTRACT

Disrupted in schizophrenia-1 (DISC1) is the gene on chromosome 1 that is disrupted due to a balanced translocation between chromosomes 1 and 11. The LOD score for having this translocation and having schizophrenia, bipolar disorder, or major depressive disorder is 7.1. DISC1 has been shown to interact with numerous protein partners and to affect multiple biochemical signaling pathways that impact neuronal development and function. One such protein is activated transcription factor 4 (ATF4), also known as cyclic adenosine monophosphate response element binding 2 (CREB2), a transcription partner that is crucial for the maintenance of the neuronal progenitor pool. DISC1 has been shown to inhibit ATF4 mediated transcription from CRE binding sites, as assayed by artificial luciferase expression assays; however the role of DISC1 on endogenous ATF4 function remains unclear. In this study we identify a novel ATF4 and DISC1 binding region within the phosphodiesterase 4D gene (PDE4D). PDE4D is itself a gene implicated in psychiatric disorders. Here, we demonstrate that DISC1 interacts with this region to repress the expression of a unique variant of the gene, PDE4D9. We further show that PDE4D9 expression is also regulated via G_s G-protein coupled receptor signaling, and that this regulation is mediated by a novel mechanism in which DISC1 dissociates from this locus upon G_s stimulation. Our results suggest that a fraction of DISC-1 dysfunction can be accounted by the increase in PDE4D9 and that PDE4D9 may serve as a target for novel therapeutic approaches.

INTRODUCTION

Disrupted in schizophrenia-1 (DISC1) was identified in 2000 (Millar et al., 2000) as a gene product that becomes disrupted on chromosome 1 due to a balanced translocation between chromosomes 1 and 11. Seventy percent of the family members that inherit this translocation have major depression, bipolar disorder, or schizophrenia, with a combined LOD score of 7.1 (Porteous et al., 2011). This type of Mendelian inheritance pattern had not been

previously observed in psychiatric disorders, and set off a flurry of investigations into the function of this gene. The identification of numerous DISC1 binding partners (Millar et al., 2003; Brandon, 2007; Camargo et al., 2007) has uncovered not only the function of DISC1 in several signaling pathways, but has also implicated these pathways in psychiatric disorders. DISC1 has served as a crucial pathfinder for psychiatric disorder risk genes; much as the genes identified in rare neurodevelopmental disorders have served for the understanding of the molecular players important for neuronal development. This has helped to set up a framework for researchers to identify how newly identified risk alleles may contribute to disease and the manifestation of disorder phenotypes.

Transgenic mice expressing the human DISC1 Scottish translocation mutation exhibit increased ventricle size, decreased gray matter volume, and changes in dendritic arborization in cortical and hippocampal neurons (Hikida et al., 2007; Li et al., 2007; Pletnikov et al., 2008; Shen et al., 2008). These mice also exhibit behavioral abnormalities such as hyperactivity (Hikida et al., 2007; Pletnikov et al., 2008), increased immobility in the forced swim test (Hikida et al., 2007), decreased sociability (Pletnikov et al., 2008), and working memory (Kvajo et al., 2008; Pletnikov et al., 2008). Clapcote and colleagues characterized ENU induced mouse DISC1 exon 2 mutants and reported that the Q31L mutant exhibits depression-like and the L100P mutant exhibits schizophrenia like behavior (Clapcote et al., 2007). In addition, reduced embryonic and adult hippocampal neurogenesis have been reported in a DISC1 BAC transgenic mouse model and DISC1 exon 7-8 deletion mutant mice (Kvajo et al., 2008; Shen et al., 2008). Studies using acute knockdown of DISC1 products in mice have identified the role of DISC1 in neuronal progenitor proliferation (Mao et al., 2009; Ishizuka et al., 2011), the proper integration of adult born neurons in the dentate gyrus circuit (Kamiya et al., 2005; Duan et al., 2007; Ishizuka et al., 2011), and neuronal migration (Ishizuka et al., 2011). Collectively, the

neuropathology and behavioral phenotypes exhibited by the various DISC1 mouse models strongly supports a role for DISC1 in mental health.

Since its identification as a psychiatric disease risk gene, a large number of DISC1 binding partners have been identified. DISC1 interaction with GSK3β has been shown to be crucial for its effects on neuronal progenitor proliferation, secondary to its effect of Wnt signaling (Mao et al., 2009). DISC1 binds to centrosomal proteins, such as NDE1/NDEL1(Brandon et al., 2004; Bradshaw et al., 2008), Lis1, BBS1 and 4, and these interactions are important for proper neuronal migration (Duan et al., 2007; Kamiya et al., 2008). The interaction between DISC1 and Girdin/KIAA1212, an actin interaction protein, has been shown to be crucial for axonal development, through the regulation of the AKT signaling pathway (Enomoto et al., 2005; Enomoto et al., 2009; Kim et al., 2009). DISC1 modulates Rac1 GTPase activity by binding to Kalirin-7, anchoring it to promote proper glutamatergic signaling (Hayashi-Takagi et al., 2010). DISC1 has also been shown to regulate the activity of several isoforms of a cAMP specific, rolipram-sensitive family of phosphodiesterases, referred to as the PDE4s (Millar et al., 2005; Millar et al., 2007; Murdoch et al., 2007; Bradshaw et al., 2011; Carlyle et al., 2011). In summary, DISC1 seems to be required for the proper localization and activity of its binding partners, and the loss of DISC1 liberates its binding partners and allows for their unhindered activity.

DISC1 has been shown to associate with activating transcription factors 4 and 5 (ATF4 and ATF5), basic-region-leucine zipper domain containing transcription factors of the CREB/ATF family of proteins (Morris et al., 2003). ATF4, also known as CREB2, is both a transcriptional activator and repressor that binds to CRE elements throughout the genome and is involved in diverse processes, including hematopoiesis, osteoblast differentiation, neuronal progenitor proliferation, learning and memory, and behavior (Ameri and Harris, 2008). Overexpression of DISC1 has been shown to decrease CRE-mediated activity, while loss of DISC1 has been shown increase in the transcriptional activity of ATF4 (Malavasi et al., 2012).

However, we know very little about the effect of DISC1 dysfunction on endogenous targets of ATF-4 mediated transcription. In this study, we identified a novel endogenous target of DISC-1 mediated transcriptional repression of ATF-4. This target region lies within the PDE4D gene locus, and importantly, the effect of DISC1 mediated transcriptional repression is exquisitely specific to a unique variant of PDE4D. Furthermore we identified a novel physiological mechanism by which DISC-1 mediated transcriptional repression is regulated. Our results suggest that a fraction of DISC-1 dysfunction can be accounted by the increase in PDE4D9 and that PDE4D9 may serve as a target for novel therapeutic approaches.

RESULTS

Identification of ATF4-regulated Gene regions

DISC1 has been shown to lead to changes in gene transcription by modulating CRE mediated transcription (Sawamura et al., 2008). Since DISC1 has no known DNA binding domains, we hypothesized that DISC1 does this by binding to ATF4. To identify the targets of DISC1 mediated transcription we conducted an unbiased chromatin-immunoprecipitation based cloning strategy using a polyclonal ATF4 antibody developed in our lab (Frank et al., 2010) (Figure 1A). Using this strategy, 100 loci were detected, and transcripts in the vicinity were then identified by using the UCSC genome browser Blat. 12% of the binding loci lie within 1kb and 5' of a nearest transcription start site, 10% within 1kb and 3' of the last known encoded exon, 46% are located within introns, and 33 % are positioned more than 1kb away from any currently identified transcripts, comparable to results obtained by studies using this method (Chen et al., 2010) (Figure 1B). The identified regions were analyzed using Matinspector to search for potential ATF4 binding sites. We subsequently confirmed three ATF4 binding regions through ChIP-PCR from wildtype mice, PDE4D, TrpC, Sema4a, (Figure 1C). Importantly, these regions could not be detected using ChIP-PCR on samples from ATF4 knockout mice (data not shown).

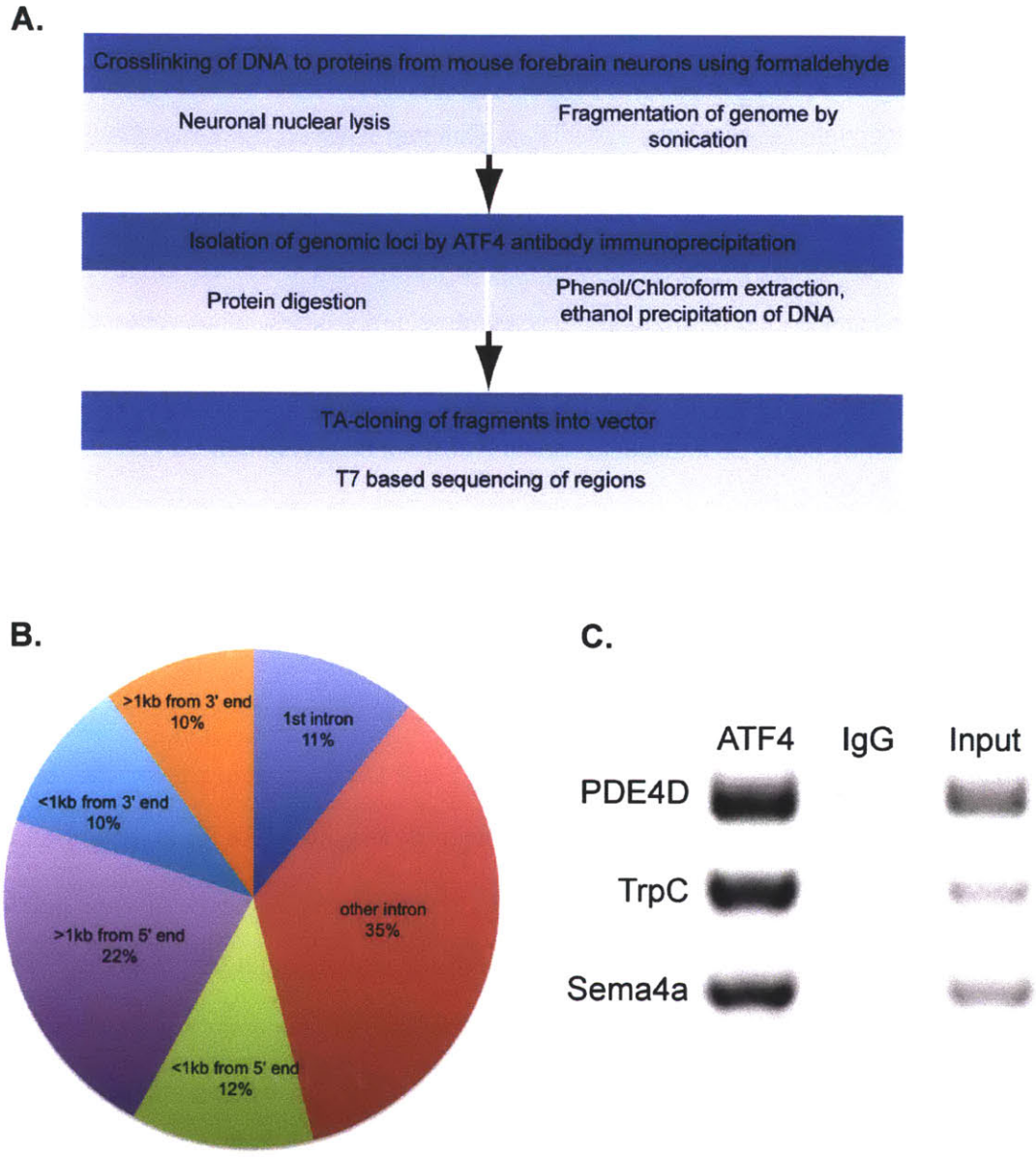


Figure 1: Identification of PDE4D loci as an ATF4 binding site. **A**. Schematic summarizing our cloning strategy. **B**. Summary of the distribution of ATF4 binding sites relative to nearest known coding region, identified by Blat search on the UCSC genome browser, mouse genome mm9, based on NCBI build 37. **C**. Confirmation of several ATF4 binding regions. PDE4D-Phosphodiesterase 4D, TrpC-Transient receptor potential channel C, Sema4a-Semaphorin 4a.

DISC1 binds to PDE4D loci

We chose to further characterize the ATF4 binding site within the PDE4D gene. PDE4D is one of four genes encoding a variety of cyclic adenosine monophosphate (cAMP)-specific, rolipram-sensitive phosphodiesterases (Jin et al., 1998; Zhang et al., 1999; Houslay, 2010). Animal models of PDE4D loss of function exhibit a phenotype similar to mice under antidepressant treatment; they have a decreased immobility time in the forced swim test (FST) (Zhang et al., 2002; Li et al., 2011b; Schaefer et al., 2012) and have increased neurogenesis (Li et al., 2011b). Furthermore, PDE4D activity is known to be directly modulated by DISC1 through direct protein-protein interaction (Millar et al., 2005; Murdoch et al., 2007; Bradshaw et al., 2011). To test our hypothesis that ATF4 mediated gene transcription is modulated by DISC1 via ATF4, we conducted a ChIP-PCR using a polyclonal DISC1 antibody targeted to the N-terminus of DISC1, using samples from 8-week old wild-type (WT), ATF4 heterozygous ($ATF4^{+/-}$) and ATF4 knockout ($ATF4^{-/-}$) littermate mice. DISC1 binding to this locus is dependent on ATF4 in a dosage-dependent manner (Figure 2A). Our results suggest that the association with DISC1 and the PDE4D loci is mediated by ATF4.

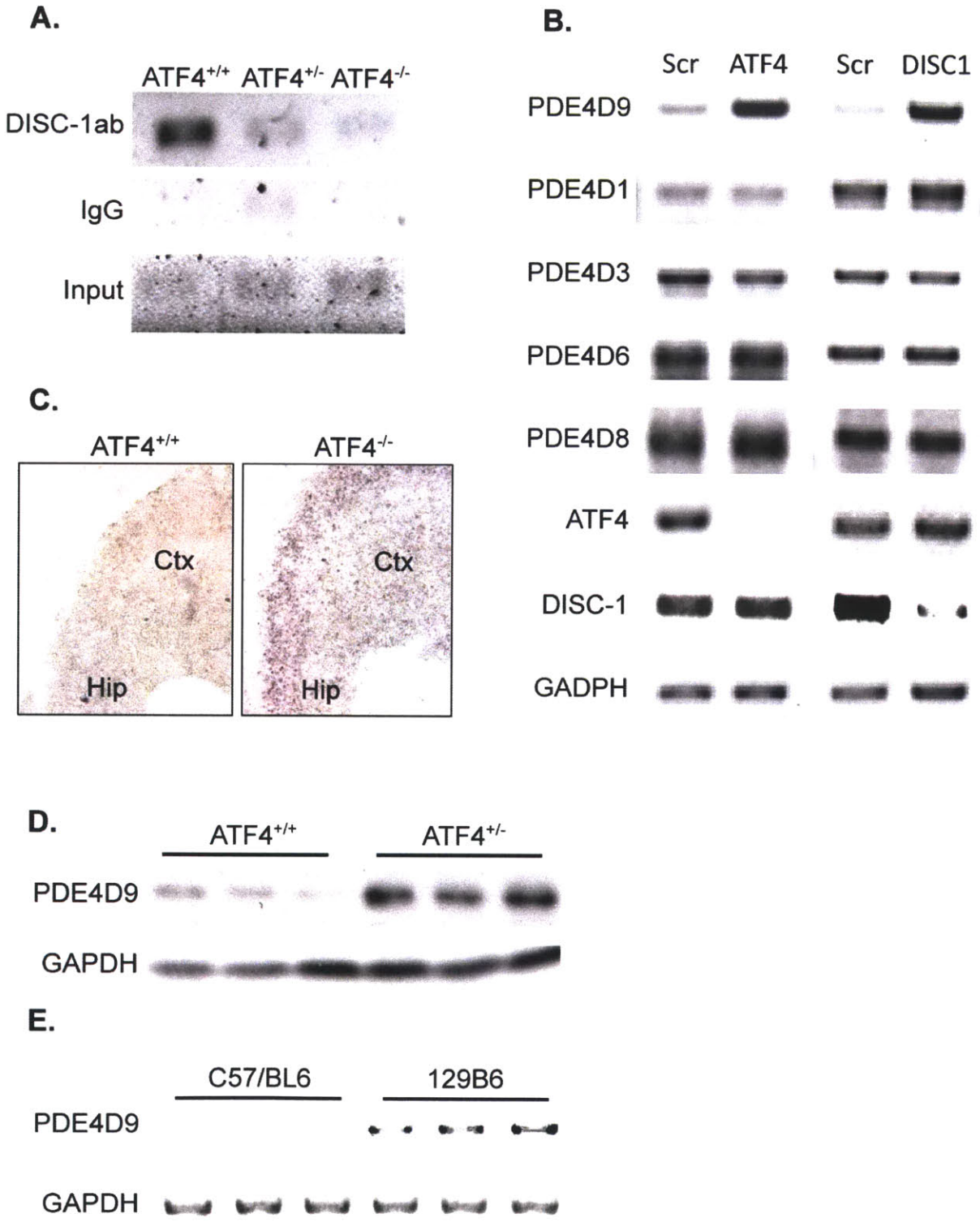


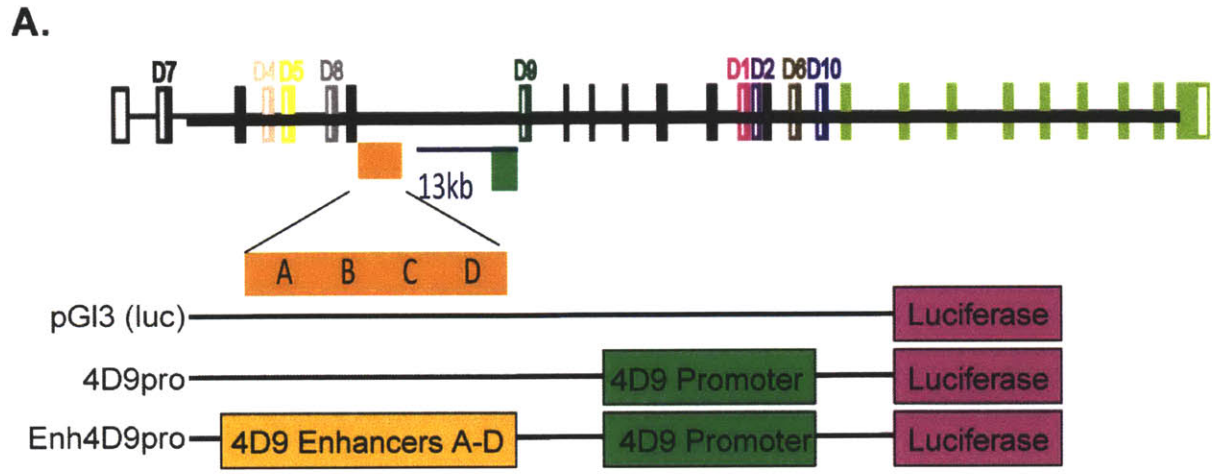
Figure 2: **DISC-1 binds to ATF4 binding site within PDE4D region to regulate PDE4D9 expression**. **A.** DISC-1 binding to region within PDE4D gene is ATF4 dependent. **B.** Infection of cortico-hippocampal neurons with lentiviri expressing ATF4 or DISC1 shRNA results in the

specific increase of PDE4D9 transcripts. **C**. *In situ* hybridization of E15 WT or $ATF4^{-/-}$ demonstrates elevated levels of PDE4D9 in the absence of ATF4. **D**. PDE4D9 protein levels are elevated in 12-week old $ATF4^{+/-}$ mice compared to WT mice. **E**. mRNA levels of PDE4D9 are increased in the 129/B6 congenic mouse model relative to C57/B6 littermates. 129 mice were backcrossed over 10 generations, selecting for mice that retained the deletion within the DISC1 gene at each generation. RNA was isolated from forebrain and subjected to reverse transcription, and the unique region of PDE4D9 was amplified from the cDNA. These mice also display higher levels of PDE4D9.

DISC1 and ATF4 both specifically regulate PDE4D9

The PDE4D gene locus encodes numerous variants of PDE4D, all of which share a common catalytic domain, but are differentiated from each other by their unique N-terminal fragment encoded by specific N-terminal exons that are thought to confer a definite subcellular localization, and thus distinct functions, to each variant. We sought to determine which, if any, of these variants were regulated by the ATF4-DISC1 binding site, using semiquantitative PCR with primers previously optimized to determine relative PDE4D levels (Nemoz et al., 1996; Jin et al., 1998; Richter et al., 2005). mRNA obtained from days *in vitro* (DIV) 14 cultured cortico-hippocampal neurons infected at DIV 4 with control, ATF4, or DISC1 shRNA expressing lentiviri (Mao et al., 2009) revealed a marked and specific increase in the levels of PDE4D9 transcript, but not others (Figure 2B). *In situ* hybridization using an antisense probe targeted to the unique region of PDE4D9 at embryonic day 15 (E15; when ATF4 levels are at their peak) reveal that there is a substantial increase in the levels of PDE4D9 transcript in $ATF4^{-/-}$ mice compared to widtype littermates (Figure 2C). PDE4D9 protein levels are significantly upregulated in hippocampal brain lysates from 8-week old $ATF4^{+/-}$ mice compared to WT littermates (Figure 2D). Collectively, these results indicate that loss of ATF4 or DISC1 results in an increase in PDE4D9 mRNA and protein levels. Thus, at baseline, ATF4 and DISC1 occupancy of the PDE4D locus act as transcriptional repressors specifically for the PDE4D9 isoform.

To determine whether this increase in transcript can be exclusively accounted for by transcriptional activity, and not by other factors such as altered transcript stability, we created several luciferase constructs. We placed the 1000bp region immediately upstream of PDE4D9 coding region in front of the luciferase gene and compared it to the baseline activity of the pgl3-luciferase construct, which does not have a promoter driving luciferase activity. The 1000bp region upregulates luciferase activity indicating that this region acts as a promoter. We then assayed whether the ATF4/DISC1 binding locus had any effect on the transcriptional activity by the promoter by taking the ChIP region (~450bp), dividing it into four fragments, and putting each fragment upstream of the 1000bp PDE4D9 promoter. The effect on transcriptional activity for fragments A, B and C was minimal, while fragment D significantly altered the baseline activity of the construct (Figure 3A,B); we chose fragment D for further evaluation. When we subjected cells overexpressing this fragment to ATF4/ DISC1 knockdown, the luciferase activity increased dramatically when exposed to DISC1 knockdown (Figure 3D). Moreover, when a putative ATF4 binding site within this fragment was subsequently mutated, the ability of DISC1 shRNA to enhance transcription was diminished (Figure 3D). Together, these results indicate that the ATF4/DISC1 binding site acts as an enhancer to the endogenous PDE4D9 promoter, and that the loss of DISC1 significantly increases the transcriptional activity downstream of the PDE4D9 promoter under the influence of this enhancer.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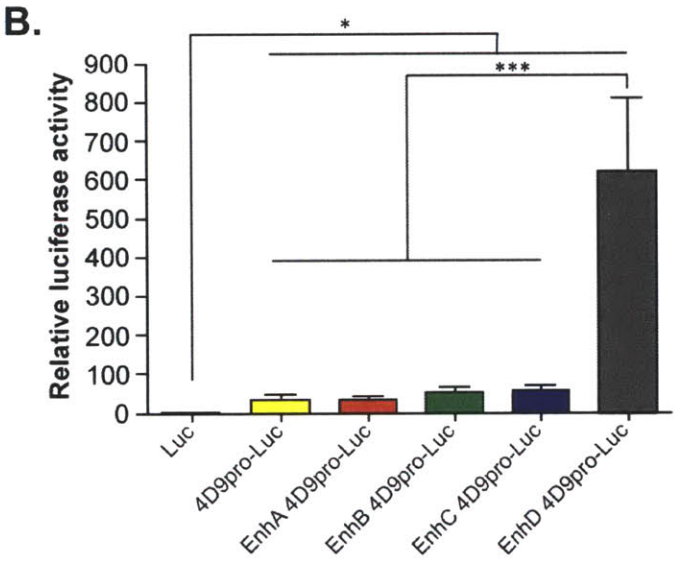
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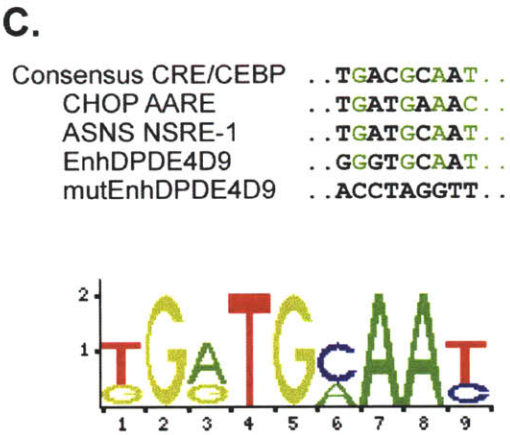
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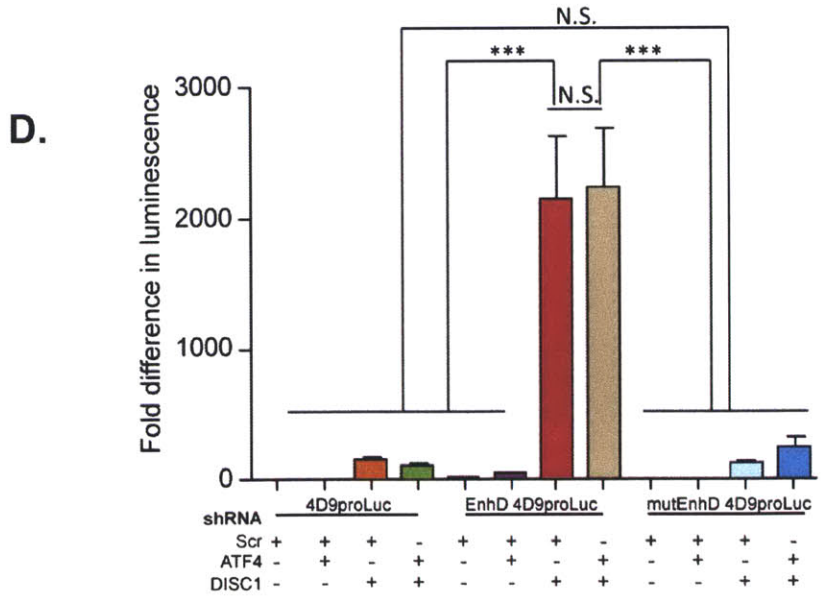
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Figure 3: The ATF4-DISC1 binding region acts as a transcriptional repressor to endogenous PDE4D9 promoter. A. Schematic of constructs used to study the transcriptional effects of the ATF4-DISC1 binding region. **B.** Placement of the 1000bp region immediately upstream of PDE4D9 coding region in front of the luciferase gene upregulates luciferase activity indicating that this region acts as a promoter. Fragment D significantly altered luciferase activity relative to the others (n=5 ANOVA $p<0.0001$. * Dunnett's test $p<0.05$ for comparison of luc to all others, ***Tukey's test $p<0.001$ for EnhDPDE4D9 compared to other fragments, PDE4D9 promoter only). **C.** A putative CRE/CEBP binding site was identified within EnhD. The site was mutated to eliminate sequence similarity to CRE/CEBP binding site. **D.** Comparison of the effects of control, ATF4 or DISC1 shRNA combinations on luciferase activity on EnhDPDE4D9The presence of enhancer fragment D (EnhD) upstream of the 4D9 promoter is critical for the dramatic elevation in signal seen after ATF4, DISC1 shRNA expression (3E)(n=5 ANOVA $p<0.0001$, ***Tukey's test $p<0.001$ for comparisons between EnhDPDE4D9 DISC1 shRNA and DISC1/ATF4 shRNA compared to all others).

Cocaine and amphetamines are known to act through the excitation of dopaminergic signaling: they are abused for their euphoric effects, and used in animals as a model for psychosis. Amphetamine is also used in the treatment of psychiatric symptoms in attention-deficit and hyperactivity disorder (ADHD), implicating the importance of their action on psychopathology. ATF4-dependent transcription is known to be mediated by several factors, including oxidative/nutrient stress, and cAMP-induced increases in PKA activity; notably, CART, or cocaine-amphetamine response transcript, is a product of ATF4 mediated transcription known to be upregulated upon treatment with cocaine and amphetamines. This led us to hypothesize whether PDE4D9 levels may also be regulated by similar mechanisms. Treatment with dopamine (100um) as well as isoproterenol (100um), a beta-adrenergic receptor agonist, but not potassium chloride (KCL, 55mM), which induces generalized depolarization, or Prostaglandin E, (PGE, 10mM, not shown) which has been reported to increase phosphodiesterase activity in other systems, in DIV14 cultured cortico-hippocampal neurons lead to increases in PDE4D levels as assayed by observable changes in mRNA levels by semi-quantitative qPCR (Figure 4A). These results suggested that agonists of D1-type or $G_{\alpha}s$ coupled G-protein coupled receptor stimulation lead to increases in PDE4D9. We further validated this hypothesis by using qPCR to assay for changes in PDE4D9 levels, comparing vehicle,

dopamine (10um), D1-type receptor agonist SKF81297 (3uM), and D2-type receptor agonist quinpirole (10uM) treatment. Both dopamine and SKF81297, but not quinpirole, lead to elevations of PDE4D9 transcript, indicating that D1-type receptor stimulation increases levels of PDE4D9 (Figure 4B). Amphetamine treatment (0.35mg/kg) of 8-week old mice resulted in elevated PDE4D9 mRNA levels as well as detectable changes in PDE4D9 protein in mouse hippocampus (Figure 4C, D). These results indicate that PDE4D9 levels are mediated physiologically by dopamine via a D1-type receptor signaling mechanism, and that neuropharmacologically relevant doses of amphetamine can increase levels of PDE4D9.

Figure 4

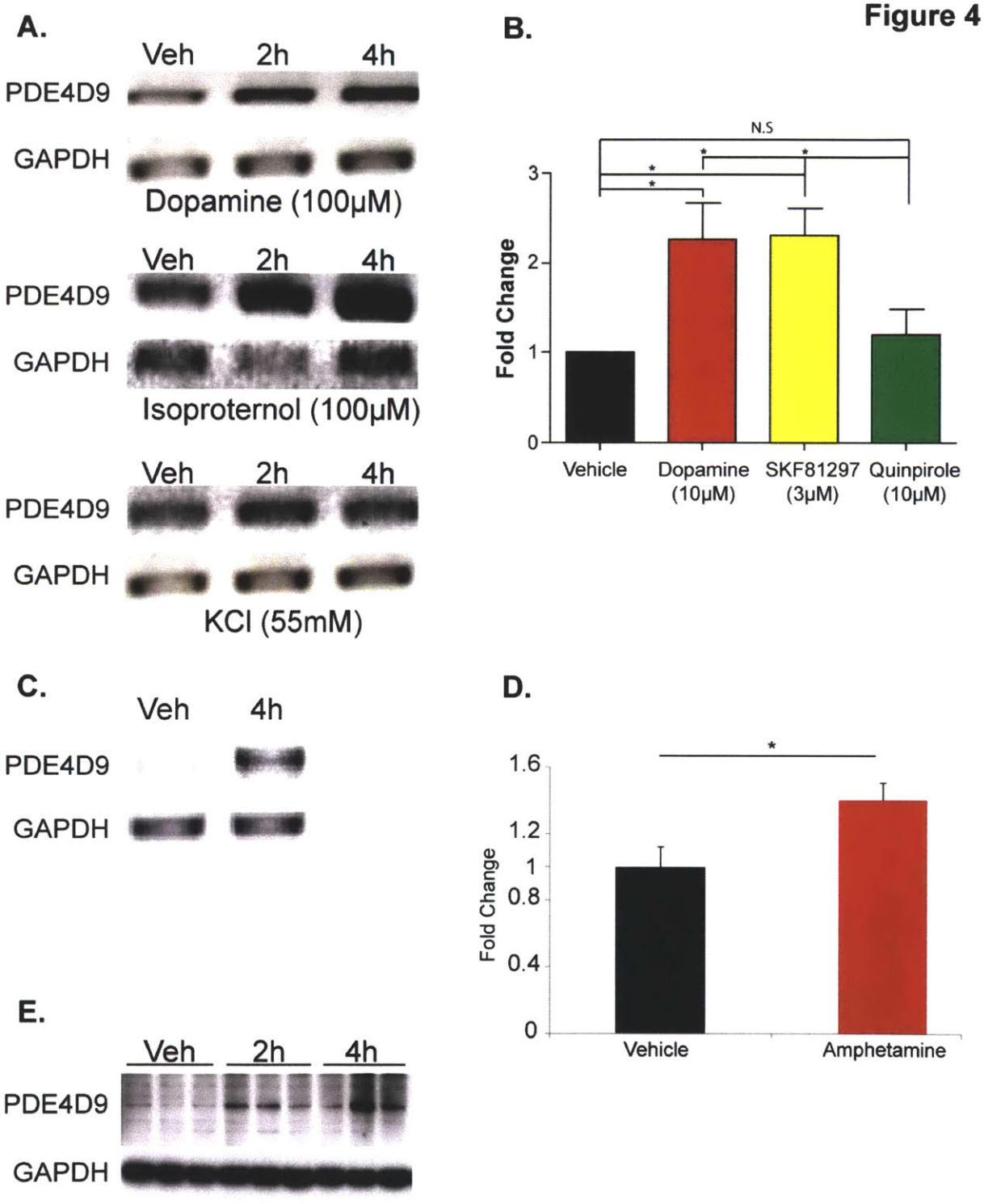

Figure 4: G_s-coupled G-protein coupled receptor signaling leads to increases in PDE4D9 levels. A. Dopamine and Isoproternol (β-adrenergic receptor agonist), but not KCl (general activator of neuronal activity via depolarization), leads to increases in PDE4D9 transcript. **B**. D1-

type, but not D2-type dopamine receptor agonists, lead to detectable changes in PDE4D9 transcript. Dopamine (10μM), SKF81297 (3μM), but not quinpirole (10μM) treatment lead to detectable increases in PDE4D9 levels, assayed by qPCR for unique region of PDE4D9.N=3 ANOVA $p<0.05$, *$p<0.05$ Dunnett's test. **C**. 0.35mg/kg amphetamine treatment of C57Bl6/J mice leads to detectable increases in PDE4D9 transcript. N=4, student's T-test $p<0.05$). **D**. 0.35mg/kg amphetamine treatment leads to detectable increases in PDE4D9 protein levels.

To examine the role of DISC1 and ATF4 in the aforementioned phenomenon, we conducted the ChIP experiments described previously in mice treated either with amphetamine (0.35mg/kg) or with vehicle. PDE4D loci occupancy for DISC1 was significantly diminished either two or four hours after treatment, compared to vehicle or untreated mice (Figure 5A); vehicle treatment at these time-points, were similar to untreated mice (data not shown). Interestingly, we observed a detectable, but non-statistically significant, increase in the levels of ATF4 occupancy at this locus with this treatment (Figure 4E). Increases in the levels of ATF4 in the hippocampus after amphetamine treatment have been reported elsewhere in the literature (Green et al., 2008). Taken together, our results indicate that PDE4D9 levels are repressed by the interaction between ATF4, DISC1 and the PDE4D loci, and that the dopaminergic stimulation of D1 type receptors leads to the dissociation of DISC1 from the loci, leading to increases in PDE4D9 transcription.

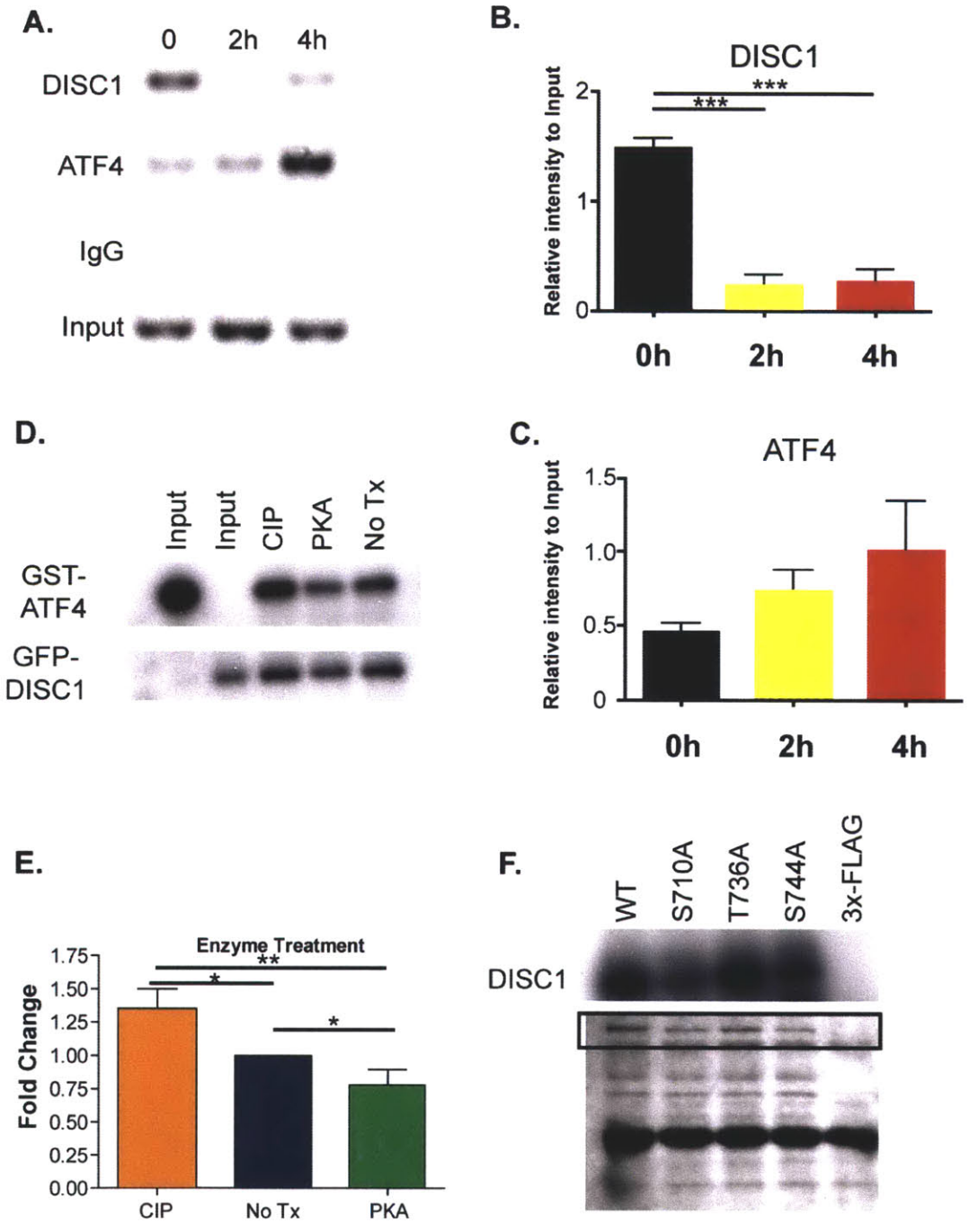

Figure 5: Gs-Coupled and downstream effects lead to dissociation of DISC1 from ATF4. **A**. ChIP using DISC1 and ATF4 antibodies after amphetamine treatment (0.35mg/kg) from C57/Bl6J hippocampus reveals that there is loss occupancy of DISC1 at the PDE4D9 locus compared to vehicle injected mice. **B**. Quantification of DISC1 occupancy at PDE4D9 locus. (n=6, ANOVA $p<0.05$, Dunnett's test $p<0.001$ for comparisons between vehicle injection and 2 and 4 hours after amphetamine injection). **C**. Quantification of ATF4 occupancy at the PDE4D9 locus. ANOVA $p=0.13$, n=6) (D)Purified GST-tagged ATF4 protein and GFP-DISC1 protein, from overexpression in bacteria and HEK 293T cells, respectively, decrease interaction *in vitro*

after the addition of the catalytic unit of PKA. **E**. Quantification of (D). N=4, ANOVA $p<0.05$, *$p<0.05$, **$p<0.01$ by Neuman-Kewls post hoc analysis.

Both ATF4 and DISC1 have known PKA phosphorylation sites (Elefteriou et al., 2005; Ishizuka et al., 2011). ATF4 has been shown to turn from a repressor to a transcriptional activator after PKA phosphorylation (Elefteriou et al., 2005), and DISC1 localization has been reported to be altered by PKA phosphorylation (Ishizuka et al., 2011). We sought to determine whether phosphorylation sites have an effect on DISC1-ATF4 interaction. To do this, full-length GFP-tagged DISC1 and GST-ATF4 proteins were purified and recombined in an *in vitro* context. The DISC1 and ATF4 interaction was substantially diminished upon the addition of the catalytic subunit of PKA, which was not observed with the addition of equimolar concentrations of BSA; the addition of alkaline phosphatase increased the observed interaction, compared to BSA control (Figure 5F). Neither the mutation of the DISC1 S710 site, nor the ATF4 S254 site resulted in significant changes in DISC-1 ATF4 interaction (data not shown). Also, contrary to previously published results (Malavasi et al., 2012), the ATF4/ DISC1 interaction did not seem to be significantly altered between L607 and 607F DISC1 proteins (data not shown). Our results suggest that in response to G_s coupled GPCR stimulation DISC1 leaves from the ATF4/DISC1 binding locus, and that this effect is likely mediated through direct phosphorylation events on ATF4 and/ or DISC1 by PKA.

DISCUSSION

The DISC1 gene has been clearly implicated in the manifestation of psychiatric disorder phenotypes. Here we uncover a novel transcriptional mechanism by which DISC1 regulates 1 specific variant of one of the four genes that encode the rolipram-sensitive, cAMP specific phosphodiesterases, genes that have been implicated in psychiatric disorder phenotype and antidepressant efficacy.

DISC1 binds to and represses an ATF4 transcriptional target

Here we report that DISC1 is, in fact, bound to mammalian ATF4 targets in a manner that is dependent on ATF4. This is an important result because previous work has been conducted under the assumption that this is the case (Sawamura et al., 2008; Malavasi et al., 2012). Our finding that endogenous DISC1, immunoprecipitated by antibodies that bind directly to DISC1 can be found at ATF4 regulated loci, gives much credence to the work conducted using transfected luciferase constructs that contain canonical CRE sites as readout of DISC1 function. Our data suggests that mutating a putative CRE/CEBP site renders the binding site null of its enhancer function, which further adds to the literature that has documented similar sites as sites of ATF4 action (Siu et al., 2002; Fujita et al., 2007; Gombart et al., 2007). It is tempting to speculate that endogenous ATF4 prefers to bind to this type of genetic site, rather than the traditionally reported CRE site. Our results also indicate that, in addition to ATF4 interaction, DISC1 has other mechanisms by which it regulates transcriptional activity, because DISC1 lacks any enzymatic activity. The role of DISC1 interaction with other transcriptionally relevant partners, such as the SWI/SNF proteins, is lacking in the literature and further studies in this area would add to our understanding of DISC1 function in the modulation of transcription.

DISC1 ATF4 interaction is physiologically regulated

Interestingly, numerous psychotherapeutic agents impact cAMP levels via secondary actions. Amphetamine result in increased dopaminergic release and is used in the treatment of ADHD. Improper administration leads to euphoria. MAOi's are classical antidepressants and act by elevating dopamine, serotonin and norepinephrine. New-generation antidepressants elevate cAMP via increases in 5-HT and norepinephrine via selective serotonin or serotonin-norepinephrine reuptake inhibition (SSRIs and SNRIs). Our study provides further clues into the development of psychiatric phenotypes secondary to the dysregulation of this pathway. An

increase in PDE4D9 secondary to the activation of cAMP, is a form of transcriptional feedback regulation, one normally predicted to be necessary for the proper maintenance of baseline cAMP levels and the maintenance of activity (Figure 6). Our results indicate that, in the absence of DISC1, this feedback loop would be not just inactivated, but rather usurped. Under DISC1 knockdown conditions cells already have a higher baseline level of PDE4D9, which is normally increased secondary to G_s coupled dopamine D1-type and beta-adrenergic receptor stimulation. A baseline increase in phosphodiesterases would lead to perturbed signal propagation downstream of these receptors (Figure 6). This is in addition to its previously reported direct interaction and inhibition of its catalytic activity, which has shown that DISC1 is able to regulate some, but not all, gene products of the four PDE4 genes in an activity dependent manner (Millar et al., 2005; Bradshaw et al., 2011).

There is evidence suggesting that PDE4D levels are regulated by both chronic antidepressants and rolipram administration, further implicating the cAMP pathway in the regulation of this genetic locus. Our study provides a biochemical basis for this regulation. A previous study reported that chronic antidepressant or rolipram treatment resulted in the increase of PDE4D3 variant, based on the band size of the increased PDE4D variant(Dlaboga et al., 2006). This study was published just after the identification of PDE4D9. Another study which characterized the expression of the different PDE4D's, noted that tissues previously thought to express predominantly PDE4D3, in fact expressed three PDE4D's of very close molecular size, PDE4D3, PDE4D8 and PDE4D9 (Richter et al., 2005). It is likely that the study from Dlaboga and colleagues had documented the increase in PDE4D9 but miscategorized it as PDE4D3.

Figure 6

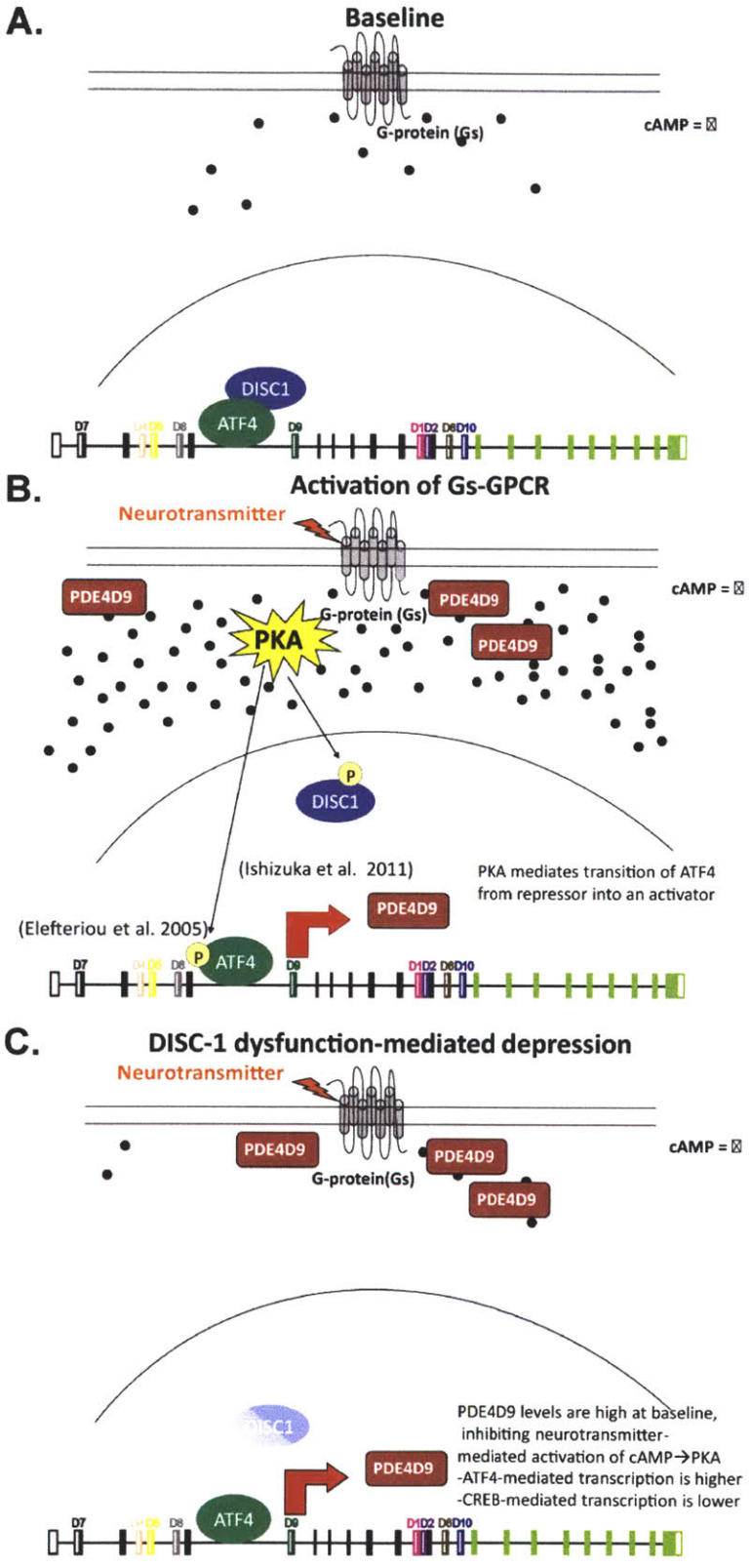
A.
Baseline
G-protein (Gs)
cAMP = ⊠
DISC1
ATF4
D7
D8
D9
D1D2
D8D10
B.
Activation of Gs-GPCR
Neurotransmitter
PDE4D9
PDE4D9
PDE4D9
cAMP = ⊠
G-protein (Gs)
PKA
P
DISC1
(Ishizuka et al. 2011)
PKA mediates transition of ATF4
from repressor into an activator
(Elefteriou et al. 2005)
PDE4D9
P
ATF4
C.
DISC-1 dysfunction-mediated depression
Neurotransmitter
PDE4D9
PDE4D9
PDE4D9
cAMP = ⊠
G-protein(Gs)
PDE4D9 levels are high at baseline,
inhibiting neurotransmitter-
mediated activation of cAMP→PKA
-ATF4-mediated transcription is higher
-CREB-mediated transcription is lower
PDE4D9
ATF4

Figure 6: **Model of endogenous DISC1/ATF4 function at the PDE4D binding site**, at baseline (**A**), after Gs-coupled G-protein coupled receptor stimulation (**B**), or in DISC1 loss of function (**C**).

Our results may also offer interesting insights on further therapeutic approaches targeting PDE4's in the treatment of psychiatric disorders. DISC1 disrupts cAMP signaling by perturbing the relative levels of the PDE4 variants, in addition to selectively affecting the activity of some, but not all of the PDE4 variants. The perturbation of this pathway may disrupt the ability of neurons to respond appropriately to neurotransmitter signaling, ultimately affecting the neuronal circuitry and resulting in abnormal behavioral responses that may manifest as psychiatric disease.

Our lab has previously demonstrated that DISC1 effects on behavior can be rescued by the use of GSK3β inhibitors. The current finding is consistent with previous findings that a loss of DISC1 function would lead to increased phosphodiesterase activity. Further experiments are ongoing to determine if there will be synergistic effects of the combined use of GSK3β and PDE4 inhibitors on depressive phenotypes as well as their effect on neurogenesis. Preliminary results, as well as evidence from previous literature suggest that this is indeed the case (Zhang et al., 2006b; Taurin et al., 2008).

Methods

Generation of antibodies

Polyclonal antibodies against the unique portion of PDE4D9 was raised by the generation of a peptide identical to the unique sequence of PDE4D9(PDE4D9unq), amino acid sequence MSIIMKPRSRSTSSLRTTEAVC. Briefly, the peptide was generated and purified by HPLC, then 500 µg was injected, along with Freund's complete adjuvant (FCA) into 2 rabbits after prebleeding. The rabbits received a booster dose of 500µg PDE4D9unq and Freund's

Incomplete adjuvant (FIA) 20 days after initial injection. 4 additional booster doses of 250μg PDE4D9unq and FIA were given at 20 day intervals. At 52 days post initial injection, a test bleed was obtained to assay antibody titer. Two production bleeds were obtained at 94 and 118 days after initial antigen injection. Sera obtained from the bleeds were then subjected to affinity column purification to obtain purified antibodies. Antibodies used for this study were from the first bleed. All in vivo work was done by Covance Inc. in accordance with MIT CAC guidelines. Antibody purification from sera was done in Tsai lab.

Polyclonal antibodies against the N-terminal portion of DISC-1 was generated in a protocol similar to that described above, save for the dose of injected antigen: 250μg DISC-1 N-term was initially injected, followed by 125μg booster doses at 20 day intervals. 6-His-DISC-1 N-term was generated by cloning the N-terminal portion of mouse DISC1, amino acids 1-220 into the pET-32b+ vector. The plasmid was then transformed into BL21(DE3) Competent E.Coli. Protein expression was driven by IPTG stimulation. The protein was purified using column affinity purification, and the his tag was removed by thrombin cleavage by the addition of 1 unit of thrombin per column overnight. The supernatant was collected and delivered to Covance for in vivo work.

Commercial Antibodies used for the study

Antibody	Source	Dilution
ATF-4 (C-20)	Santa Cruz	2.5μg per sample ChIP
GAPDH(6C2)	Santa Cruz	1:5000
FLAG M2 (mouse)	Sigma-Aldrich	1:2000 for western, 2μg per sample IP
GFP (Rb)	Injitrogen	1:2000
Myc tag	Cell Signaling Technology	1:1000, 2μg per sample IP

Compounds used for the study

Name	Concentration	Source
KCl	55mM	Sigma-Aldrich

Forskolin	10μM	Tocris
Dopamine	Variable	Sigma-Aldrich
SKF81297	3μM	Sigma-Aldrich
Quinpirole	10μM	Tocris
Prostaglandin E	10μM	Tocris
Isoproternol	100μM	Sigma-Aldrich
Protein Kinase A, catalytic subunit	2500 units/reaction	NEB
D-Amphetamine	0.35mg/kg	Tocris
poly-D-lysine	50μg/ml	BD life sciences
Laminin	30μg/ml	BD life Sciences
Trypsin	0.25%	Gibco
BSA	Variable	Invitrogen
PKA, catalytic subunit	2500000units/ml	NEB
Calf Alkaline Phosphatase (CIP)	10,000 units/ml	NEB

Primers used for the study

NAME OF TRANSCRIPT	FORWARD	REVERSE
PDE4D1/2 RT	TATGAAGGAGCAGCCCTCATG	CCAGGACATCTTCCTGCTCTG
PDE4D3 RT	CAGAAGGCATTCCTGGATATG	TGGCCAGTTTCTGGTAGGCCTC
PDE4D6 RT	CACATTTTAGAACTTGCTGTCAC	CCAGGACATCTTCCTGCTCTG
PDE4D8 RT	GCCACAAGTGCCTCTTGCAGC	TCCAGACACCAGTCCAGCTCCTCC A
PDE4D9 RT	ATGAGCATTATTATGAAGCCG	TGGCCAGTTTCTGGTAGGCCTC
ATF4 RT	GGAATGGCCGGCTATGG	TCCCGGAAAAGGCATCCT
DISC-1 RT	TTGCTGGAAGCCAAGATGCTGG	CTTCACGCCTATGGCTTCGC
GAPDH RT	TCACCACCATGGAGAAGGC	GCTAAGCAGTTGGTGGTGCA
3XMYCPDE4D1	GCCACCATGGAGAGGGACACTTG TG	TTACGTATCAGGACAGCAGTCATCC CTAGGGCG
3XMYCPDE4D2	GCCACCATGGCCTCCAACAAGTT	TTACGTATCAGGACAGCAGTCATCC CTAGGGCG
3XMYCPDE4D5	GCCACCATGGCTCAGCAGACGAC AAGC	TTACGTATCAGGACAGCAGTCATCC CTAGGGCG
3XMYCPDE4D6	GCCACCATGCCTGAAGCAAACTA TTTATTG	TTACGTATCAGGACAGCAGTCATCC CTAGGGCG
3XMYCPDE4D7	GCCACCATGCTCAAACCAGAGTG TTGGGAT	TTACGTATCAGGACAGCAGTCATCC CTAGGGCG
3XMYCPDE4D9	GCCACCATGAGCATTATTATGAA GCCG	TTACGTATCAGGACAGCAGTCATCC CTAGGGCG
3XMYCPDE4D3	GCCACCATGGAGGCAGAGGGCA GCAGCGTG	TTACGTATCAGGACAGCAGTCATCC CTAGGGCG
4D CHIP	CAATACCGAATGCCCCTTTA	CAGAGTGTGGCAAACAGGAA

4D9 PROMOTOR	GCGCTCGAGCTGTATAATGGAAA TGACTTTCTG	GCGAGATCTGGGAAAGGGAAATGT TCAAGTCTTCTGATC
4D9 ENHANCER FRAGMENT A	TACATACGTACCTTATATCATGGC ACTGAAGTACTTTACCCCACACCT ATGTGTACTTAACATCCCTGTACT TAGCAGCAACTCTGTAGCTGTAC AACGTTAGTTTCTTGGG	CCCAAGAAACTAACGTTGTACAGCT ACAGAGTTGCTGCTAAGTACAGGG ATGTTAAGTACACATAGGTGTGGG GTAAAGTACTTCAGTGCCATGATAT AAGGTACGTATGTA
4D9 ENHANCER FRAGMENT B	AACCTTTGCCATGGGCCAATACC GAATGCCCCTTTATTATTGAGTAA ACCTGCATCTGGCCTTCCCCCAT CGCTGCAGACTAATCTTACCCTTT GTTCGCCTCGTCCATGTTT	AAACATGGACGAGGCGAACAAAGG GTAAGATTAGTCTGCAGCGATGGG GGAAGGCCAGATGCAGGTTTACTC AATAATAAAGGGGCATTCGGTATTG GCCCATGGCAAAGGTT
4D9 ENHANCER FRAGMENT C	GGTATGGTTATAGACTCTCTGTGT ATCTCTTGGAGGGGACACAAACA ATGGACGCTGTTTGTCTTTTCCTG TTTGCCACACTCTGTGGAGTTCTT	AAGAACTCCACAGAGTGTGGCAAA CAGGAAAAGACAAACAGCGTCCAT TGTTTGTGTCCCCTCCAAGAGATAC ACAGAGAGTCTATAACCATACC
4D9 ENHANCER FRAGMENT D	GACACACTCTAGAGGGTGCAATA GCATTCTGTAATATTGTGGTCCAA TCACGTTTCTTCATACTCTGAATG ATACTACATTGAATTGGTTTTTCC ACGGCCTG	CAGGCCGTGGAAAAACCAATTCAAT GTAGTATCATTCAGAGTATGAAGAA ACGTGATTGGACCACAATATTACAG AATGCTATTGCACCCTCTAGAGTGT GTC
Δ4D9 ENHANCER FRAGMENT D	GACACACTCTAGAACCTAGGTTG AGCATTCTGTAATATTGTGGTCCA ATCACGTTTCTTCATACTCTGAAT GATACTACATTGAATTGGTTTTTC CACGGCCTG	CAGGCCGTGGAAAAACCAATTCAAT GTAGTATCATTCAGAGTATGAAGAA ACGTGATTGGACCACAATATTACAG AATGCTCAACCTAGGTTCTAGAGTG TGTC
CONTROL SHRNA1	TCGGCTGAAACAAGAGTTGGTTC AAGAGACCAACTCTTGTTTCAGC CGCTTTTTTC	TCGAGAAAAAACGGCTGAAACAAG AGTTGGTTCAAGAGACCAACTCTTG TTTCAGCCGCA
DISC1SHRNA1	TGGCAAACACTGTGAAGTGCTTC AAGAGAGCACTTCACAGTGTTTG CCTTTTTTC	TCGAGAAAAAAGGCAAACACTGTG AAGTGCTCTCTTGAAGCACTTCACA GTGTTTGCCA
DISC1SHRNA2	TGCAGGAGGTCAGCAAGGCCTTG TTCAAGAGACAAGGCCTTGCTGA CCTCCTGC TTTTTTC	TCGAGAAAAAAGCAGGAGGTCAGC AAGGCCTTGTCTCTTGAACAAGGC CTTGCTGACCTCCTGCA
ATF4SHRNA1	TGAGCATTCCTTTAGTTTAGTTCA AGAGACTAAACTAAAGGAATGCT CTTTTTTC	TCGAGAAAAAAGAGCATTCCTTTAG TTTAGTCTCTTGAACTAAACTAAAG GAATGCTCA
ATF4SHRNA2	TGAGAAGAGAGTTCCGTAATATTC AAGAGATATTACGGAACTCTCTTC TG TTTTTTC	TCGAGAAAAAAGAGAAGAGAGTTC CGTAATATCTCTTGAATATTACGGA ACTCTCTTCTGA
PDE4D9 SHRNA1	TGCCAAGATCCAGGTCTACATTC AAGAGATGTAGACCTGGATCTTG GCTTTTTTC	TCGAGAAAAAAGCCAAGATCCAGG TCTACATCTCTTGAATGTAGACCTG GATCTTGGCA
PDE4D9 SHRNA2	TGTCCGTTGTTATGGAATTGTTCA AGAGACAATTCCATAACAACGGA CTTTTTTC	TCGAGAAAAAAGTCCGTTGTTATGG AATTGTCTCTTGAACAATTCCATAA CAACGGACA

4D primer sequences adapted from Plasmids used for the study adapted from (Richter et al., 2005) to account for mouse, not rat, sequences

Plasmids used in study

Name	Insert	Vector	Tag	Expression (promotor)
3xFLAG vector control		pcDNA3.1	3x-FLAG	Mammalian (CMV)
3x-FLAG ATF4	mATF4	pcDNA3.1	3x-FLAG	Mammalian (CMV)
pEGFP-C1		pEGFP-C1	GFP	Mammalian (CMV)
GFPmDISC1-Fl	mDISC1-T1	pEGFP-C1	GFP	Mammalian (CMV)
GFP-mDISC1 S710A	mDISC1-T1	pEGFP-C1	GFP	Mammalian (CMV)
GFP-mDISC1 T736A	mDISC1-T1	pEGFP-C1	GFP	Mammalian (CMV)
GFP-mDISC1 S744A	mDISC1-T1	pEGFP-C1	GFP	Mammalian (CMV)
GFP-hDISC1 WT	hDISC1	pEGFP-C1		Mammalian (CMV)
GFP-hDISC1 S704C	hDISC1	pEGFP-C1		Mammalian (CMV)
GFP-hDISC1 L607F	hDISC1	pEGFP-C1		Mammalian (CMV)
pGEX-4T2		pGEX-4T2	GST	Bacterial (Ptac)
GST-mATF4	mATF4	pGEX-4T2	GST	Bacterial (Ptac)
pRK5 HA-mDISC1	mDISC1	pRK5	HA	Mammalian (CMV)
pRK5 HA-mDISC1 S710A	mDISC1	pRK5	HA	Mammalian (CMV)
pRK5 HA-mDISC1 S710E	mDISC1	pRK5	HA	Mammalian (CMV)
pll3.7	GFP(tag)	pll3.7	GFP	Mammalian (CMV for GFP, U6 for shRNA), lentiviral
pll3.7-mCherry Scr	mCherry(tag)	pll3.7-mcherry	mCherry	Mammalian (CMV for GFP, U6 for shRNA), lentiviral
pll3.7-mCherry-DISC1N1	DISC1shRNA1	pll3.7-mCherry	mCherry	Mammalian (CMV for GFP, U6 for shRNA), lentiviral
pll3.7-mCherry-DISC1N8	DISC1shRNA2	pll3.7-mCherry	mCherry	Mammalian (CMV for GFP, U6 for shRNA), lentiviral
pll3.7-ATF4-2	ATF4shRNA2	pll3.7	GFP	
pll3.7-ATF4-4	ATF4shRNA4	pll3.7	GFP	

pGl3		pGl3	Luciferase	None
4D9pro-luc	1013bp upstream of PDE4D9 transcription initiation	pGl3	Luciferase	4D9 promoter
EnhA4D9pro-luc	Enhancer region A	pGl3	Luciferase	4D9 promoter, Enh A
EnhB4D9pro-luc	Enhancer region B	pGl3	Luciferase	4D9 promoter, Enh B
EnhC4D9pro-luc	Enhancer region C	pGl3	Luciferase	4D9 promoter, Enh C
EnhD4D9pro-luc	Enhancer region D	pGl3	Luciferase	4D9 promoter, Enh D
ΔEnhD4D9pro-luc	mutant Enhancer region D	pGl3	Luciferase	4D9 promoter, mutEnh D
HSVLT-4D9	mPDE4D9	HSVLT	IRES-GFP	Mammalian, Proprietary (Dr.Rachel Neve)
HSVLT-GFP	GFP	HSVLT	IRES-GFP	Mammalian, Proprietary (Dr.Rachel Neve)
TK-Renilla Luciferase	Renilla Luciferase	TKRL	Renilla Luciferase	Mammalian, Constitutive (Thymidine Kinase)
pSP6T7		pSP6T7		In Vitro
pSP6-4D9uniqueT7	4D9 unique	pSP6T7		In Vitro

GFP-mDISC1 and 3xFLAG-mDISC1 has been described previously (Mao et al., 2009). Briefly, the full-length human DISC1 transcript was isolated from cDNA, then subcloned into the pEGFP-C1 vector (Clontech, Takara). S710A, T736A, S744A mutants were generated by site-directed mutagenesis using the quickchange lightning site directed mutagenesis kit. HA-mDISC1wt, S710A, S710E, were kind gifts from Dr. Ishizuka. GST-ATF4 has been described previously: full length mATF4 was cloned into the pGEX-4T-2 vector. 3x-myc PDE4D2,3,5,6,7,8, and 9 were generated by PCR amplification of the full-length cDNA fragment from a cDNA library generated from RNA extraction and cDNA synthesis, as described below, using 5' primers flanked by a Kozak (GCCACCATG) sequence and 3' primers flanked by a XhoI restriction site, and then cloned into a pcDNA(3.1)+ vector modified to add 3 myc tags at the C-terminal end of the insert. The vector was digested using EcoRV and XhoI, and the inserts were annealed to the vector.

Production of plentilox3.7 ATF4 shRNA

ATF4 and DISC1 shRNA was cloned into the plentilox 3.7 vector (Addgene plasmid 11795) as previously described (Mao et al., 2009). The DISC1 shRNA sequences used are the same as those described. Briefly, complimentary 5' phosphorylated oligonucleotides for ATF4, DISC1, and PDE4D9, provided above, were annealed, digested with XhoI and ligated into the plentilox 3.7 vector that had been digested with HpaI and XhoI. Proper insertion and orientation of the sequence downstream of the U6 promoter was confirmed using a sequencing primer with the sequence cagtgcaggggaaagaatagtagac. To create DISC1shRNA and scr-shRNA mCherry, the GFP cDNA sequence contained within pll3.7 was excised and replaced with the mCherry cDNA sequence, obtained by amplification using from pRK5-mCherry (addgene).

Chromatin Immunoprecipitation

ChIP was conducted from either neurons isolated from adult (12 week-old) hippocampus or cortex by loose dounce, by modifying the conditions provided in the EZ-MagnaChIP kit (Millipore). Briefly, cells were isolated by loose dounce, then crosslinked for 10 minutes in 1ml 1% formaldehyde-PBS with nutation in a 1.5ml eppendorf tube. Formaldehyde was then quenched for 5 minutes by the addition of glycine, accompanied by nutation. Cells were washed twice with ice-cold PBS, then, after removal of supernatant, lysed using 1mL ice-cold cell lysis buffer with protease inhibitors. The supernatant was removed, and 0.5mL nuclear lysis buffer was added to the cell pellet to lyse the nucleus. The lysate was sonicated to shear the crosslinked DNA in conditions optimized to obtain ~500bp fragments using a Branson 250-D sonifier. Insoluble cell debris was spun down at 15,000xg. 50µL lysate was used for each subsequent reaction. Lysate was diluted into 450µL dilution buffer, then 5µg of antibody or Normal Rabit IgG and 20µL suspended protein A magnetic beads were added to the diluted lysate. The mix was then incubated for 2 hours at 4°C with rotation. Magnetic beads were isolated, then washed twice with low salt immune complex wash buffer, twice with high salt

immune complex wash buffer, once with LiCl immune complex wash buffer, and once with TE buffer. DNA was isolated from the beads by adding 100µl ChIP elution buffer with 1µl proteinase K, and incubating for 2 hours at 62 degrees in a shaking incubator. Proteinase K was inactivated by incubation at 95°C for 10 minutes. The beads were removed, and 0.5ml bind reagent A was added to each DNA sample. The mixture was then put through a spin filter in a collection tube, and the spin filter was further washed wish Wash reagent B. Purified DNA was eluted from the spin filter column with the addition of 30µl Elution buffer C. For all subsequent reactions, 1µl of DNA was used for analysis.

Amphetamine treatment

Animals were injected with 0.35 mg/kg Amphetamine resuspended in 0.9% saline at 70ng/ul, or with saline only in accordance with CAC procedures, then placed in a novel cage for 2 or 4 hours before being sacrificed by CO_2 euthanasia, followed by rapid isolation and processing of brain samples. Briefly, after animals were sacrificed, the head was quickly dissected and rapidly frozen by placement for 20 seconds in liquid nitrogen. Following rapid freezing, brains were dissected out and separated by subregions on ice. Brains remained frozen in liquid nitrogen until enough samples had been collected for parallel processing for Chromatin IP, protein, and RNA isolation.

Primary cortico-hippocampal cultures

Cortico-hippocampal cultures were prepared from embryonic E15-16 swiss-webster mice. Hippocampi and cortices were dissected, trypsinized, and plated in cell culture plates coated with poly-D-lysine and laminin (BD Biosciences) at a density of 5.0 x 105 cells/ml in neurobasal media (Gibco/Invitrogen) containing 10% horse serum (Invitrogen), penicillin/streptomycin (Invitrogen), and glutamax (Invitrogen). Plating media was exchanged for neuronal media (neurobasal media supplemented with B27) 2-8 hours after plating. Experiments were performed on 10 to 17 days *in vitro* (DIV) primary cultures.

Lentiviral mediated knockdown of targets.

Production and titration of virus.

Lentiviral particles were made as previously described 51. Briefly, HEK-293Tcells were plated on 10 cm dishes in 10% FBS-DMEM and transfected at 95% confluency with 18 μg plenti 3.7, 6ug pCMV-ΔR8.9, and 5 μg pCMV-VSV-G per dish. The culture medium was switched to 30% FBS DMEM 12 hours after

transfection and viral supernatant was collected 48 and 96 hours later. Viral supernatant was filtered through a 0.45μm cellular acetate vacuum filter (Corning 431155), and concentrated by ultracentrifugation at 25,000 x g for 90 minutes. Viral pellets were resuspended in DPBS+0.1% glucose and stored at -80 ˚C.

Viral titers were determined on HEK-293T cells plated at 2x105 cells/well in 6-well plates, and serial dilutions of 1:200, 1:2000, and 1:20,000 were used to determine viral titer. After 48 hours of viral supernatant application, % infected cells were determined by determining % fluorescent cells/total # cells by visual inspection. Four fields of view were counted per well, and three wells were inspected per dilution. Infection was performed with MOI =10.

mRNA isolation, analysis

RNA was isolated using the RNEasy plus mini kit. Briefly, neurons were isolated by either aspiration of the media in the case of cultures, or by isolation, in the case of brain tissue. Buffer RLT plus, supplanted with 10μl β-mercaptoethanol/1ml was added to the cells, and the lysate was homogenized using a 28 gauge needle and syringe. Genomic DNA was cleared from the solution by passage through a gDNA binding column. RNA was isolated by passing the gDNA cleared lysate through a RNA spin column after the addition of equal part 70% ethanol. The bound RNA was purified with a series of wash steps: once with 700μl buffer RW1 and twice

with 500µl buffer RPE before isolation of RNA from the column using 30µl RNAse-free ddH_2O. Subsequent analysis was done using 1µl of isolated RNA per reaction.

cDNA synthesis, Semiquantitative PCR, quantitative PCR

equivalent amounts of RNA was used for cDNA synthesis using the first-strand cDNA synthesis kit (Invitrogen). Briefly, RNA concentration was measured and 0.8ug total RNA was used per reaction. The RNA was diluted to 8ul, and 1ul 50µM oligo dT and 1µl 10mM dNTP mix was added. The reaction was incubated at 65°C for 5 minutes, then placed on ice. 2µl 10x RT buffer,4µl 25mM $MgCl_2$, 2µl 100mM DTT, 1µl RNaseOUT (40U/µl), and 1µl Superscript III reverse transcriptase per reaction was premixed, and 10µl of the mixture was added to each RNA/primer/dNTP mix. The mixture was incubated for 50 minutes at 50°C, and the reaction was terminated by incubating at 85°C for 5 minutes. 1µl RNase H was added to all mixes, and incubated at 37°C for 20 minutes. 1µl cDNA synthesis reaction was used for subsequent analysis.

Semiquantitative PCR was conducted using the primers described above, at optimized temperatures. Briefly, 1µl cDNA was added into a mixture of 9µl dnase-free H_2O and 10µl 2x PCR mastermix, a mixture of TAQ DNA polymerase and optimized enzyme buffer. Reactions were terminated at 20 cycles for GAPDH, and multiple cycles were collected for other primers: the cycle indicating the biggest difference between conditions was used for data collection: this was typically between 25-32 cycles.

Quantitative PCR was conducted using the primers described above, at optimized temperatures. Briefly, 1µl cDNA was added into 9µl dnase-free H_2O and 10µl SsoFast Evagreen Supermix (Bio-Rad). The qpcr optical reaction was conducted in a T100 thermal cycler fitted with a CFX96 Touch real-time PCR system (Bio-Rad). All experiments were conducted with triplicated samples for each cDNA sample per primer pair. All comparators, as well as loading controls

were loaded onto the same 96-well and normalization was conducted within each plate before statistical analysis.

Luciferase Activity Assay

CAD or N2A cells were plated at a density of 2.5 x 10^5 /ml in 24 well plates one day prior to transfection with a combination of a plasmid expressing renilla luciferase under the control of a human thymidine kinase 1 promotor (TK-RI, addgene), firefly luciferase within the pgl3 backbone under the control of numerous promotors and enhancers, as described, at a ratio of 1:10. For assays comparing ATF4 and DISC1 knockdown, they were also infected at an MOI=5 with lentivirus that transduce expression of shRNA driven by a mouse U6 promotor 2 hours after transfection. Assays were conducted two days post transfection/viral transduction using the dual-luciferase reporter assay (Promega) in conjunction with the Spectramax-L luminescence microplate reader (Molecular Devices). Briefly, an hour before assaying, cells were lysed in 100µl 1x passive lysis buffer and rocked at room temperature for 20 minutes. Following cell lysis, 30ul of each lysate was loaded onto a 96-well plate. 25ul of LARII was dispensed onto each lysate to measure the firefly luciferase activity, followed by 25ul of Stop & Glo Reagent to measure the Renilla luciferase activity. The ratio of firefly:renilla activity was then determined to normalize the value of firefly luciferase activity to cell viability and transfection efficiency, as assessed by the constitutive expression of renilla. A minimum of three wells' values were then averaged to count as the normalized value for the experiment. This was further normalized to the values of the control conditions, present on each plate, before combining them for statistical analysis.

Immunoblot Analysis

Unless otherwise indicated, at the end of the indicated treatments, neurons or cells were lysed in RIPA buffer (50 mM Tris,pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1%

SDS) containing protease and phosphatase inhibitors, plus SDS sample buffer (2% SDS, 0.6M DTT, 62.5mM Tris, 10% glycerol). Equal quantities of neuronal lysates were subjected to SDS-PAGE and Western blot analysis, and probed with the indicated antibodies at the concentrations included in table 3.1. Forebrains or hippocampus were dissected and Dounce homogenized in RIPA buffer. Lysates were spun at 13,000RPM for 15 min, after which supernatants were removed and analyzed for protein concentration (Bio-Rad Protein Assay). SDS buffer was added to equal amounts of protein. Immunoblot analysis of hippocampal or forebrain lysates was performed using 25-200 µg of protein.

Co-immunoprecipitation studies

HEK293T cells were transfected with various constructs using Lipofectamine 2000. 24 hours post-transfection, cells were lysed with IP buffer (0.4% Triton X-100, 200mM NaCl, 50mM Tris 7.5) containing protease and phosphatase inhibitors. Equal amounts of lysates were incubated with anti-antibody-conjugated beads (Sigma, Santa Cruz Biotechnology) in IP buffer overnight, then washed three times in IP buffer. Immunoprecipitations from PSD fractions were performed using equal amounts of protein and rocking the lysates with the 1 µg of the indicated antibodies plus IP buffer overnight at 4°. Protein A or protein G sepharose beads were then added and allowed to rock with the antibody/protein complexes for ~2hrs at 4°. Beads were then washed 3-5 times with IP buffer. Immune complexes were eluted by addition of sample buffer and boiling and analyzed by SDS-PAGE.

***In Vitro* kinase-IP assay of GST-ATF4 and GFP-mDISC1**

The generation of GST-ATF4 is described previously (Frank et al., 2010). Briefly, bacteria containg the pGEXT-4T2-WTATF4, which places the mouse ATF4 cDNA under control of a LacZ promotor was grown to an O.D. of 0.3 before being stimulated to produce ATF4 by 0.1µM

IPTG. The bacteria were lysed and the GST-ATF4 was collected using glutathione conjugated sepharose beads and then eluted with elution buffer. The protein concentration of the elutate was determined. .5ug of protein was resuspended in 250 µl PKA kinase reaction buffer supplemented with 200 µM ATP.

HEK293-T cells were transfected with GFP-mDISC1T1 and lysed 24 hours post transfection in IP buffer (0.4% Triton X-100, 200mM NaCl, 50mM Tris 7.5) with phosphatase and protease inhibitors. After spinning down the cellular debris and GFP-DISC1T1 was isolated by binding to 50µg GFP-agarose beads (B2, Santa Cruz) for 1 hour. The beads were washed 4 times with the IP buffer, then twice with PBS: for the last wash, the beads were resusupended in 5ml PBS with protease inhibitor, and 1.2ml of the resuspended PBS-beads were aliquotted into 4 eppendorf tubes. After the PBS was removed from the GFP-agarose beads, 50 µl GST-ATF4 in PKA kinase buffer was added to each reaction condition. 2500units of PKA, 2500units of CIP, or .5ug BSA was added into the reaction mixture and allowed to incubate at room temperature for 7 minutes before washing again 5 times with IP buffer. For the GFP-only input, the beads were washed after the addition of BSA in PKA kinase reaction buffer without GST-ATF4. 2% input of GST-ATF4 is shown.

In vitro-transcription- Hot Kinase assay.

3xFLAG empty vector, mDISC1 WT, 710A, 736A, and 744A were subjected to in-vitro transcription/translation using the TnT Quick Coupled Transcriptoin/Tanslation System (Promega). Briefly, the system is a reticulocyte lysate based system in which RNA polymerase, nucelotides, saltes, and RNAsin RNAse inhibitor is combined with the reticulocyte lysate. The 3xFLAG vector can be driven by T7 RNA polymerase. 1µg of 3xFLAG-mDISC1 was added into 40ul TnT master mix supplemented with 1ul1mM methionine, mixed, spun down at 1000xg for 10 seconds and incubated at 30°C for 1 hour. The lysate was then added to 20µg anti-FLAG (m2)-conjugated agarose beads resuspended in 500µl IP buffer, washed 5 times using IP buffer,

then resuspended in 20µl PKA kinase buffer described above supplanted with 2.5 µCi γ-32ATP. The reaction was incubated for 20 minutes at RT, then stopped by placing on ice for 5 min, followed by the addition of 4µl 6xSDS-PAGE buffer, and boiling. The samples were subjected to SDS-PAGE, and subjected to commassie staining to confirm equal loading of mDISC1 protein. The gel was the dried and subjected to film exposure to ascertain the differential incorporation of γ-32ATP.

Data Analysis

All data was analyzed using Prism software (GraphPad Software, Inc, La Jolla, CA).

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CHAPTER 3

Investigation of the role of BCL9, a key regulator of canonical Wnt signaling-mediated transcription, in neuronal development

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ABSTRACT

BCL9 is a key regulator of Wnt signaling and is required for TCF/LEF transcription. It is also one of the nine genes in the critical region of the 1q21.1 copy number variant (CNV). A microdeletion of the 1q21.1 region is associated with schizophrenia and autism, while a microduplication of the region is associated with macrocephaly and autism. To assess the role of BCL9 in neuronal development and psychiatric disorder phenotype, we crossed mice with the flank-loxed BCL9 allele to Nestin-CRE transgenic mice, creating mice with a targeted deletion of BCL9 in neuronal progenitors. To identify novel functions of BCL9 that can account for its transactivational activity, we conducted a yeast-two-hybrid screen to identify novel interactors.

INTRODUCTION

Copy number variation of the 1q21.1 region has been identified to associate with a number of neurodevelopmental disorders (2008; Brunetti-Pierri et al., 2008; Mefford et al., 2008; Stefansson et al., 2008a). The 1q21.1 microdeletion has been associated with microcephaly and schizophrenia, while the 1q21.1 microduplication has been associated with macrocephaly and autism, suggesting the presence of a gene, or a set of genes, within this region that are a necessary part, and a driver, of head and brain size, as well as neuropsychiatric disorder manifestation.

Many of the causative genetic perturbations that have been identified in familial cases of microcephaly and macrocephaly perturb genes that are crucial for proper neuronal development. We were interested in identifying potential regulators of neuronal development within the CNV for further evaluation. To this end, we focused on the genes present within the critical region of the CNV and conducted literature investigations to see if any of them were in pathways known to be crucial for neuronal development. Of the 9 genes in the critical region, PRKAB2, PDIA3P, FMD5, CHD1L, BCL9, ACP6, GJA5, and GJA8, BCL9 stood out in terms of it's known role in the canonical Wnt signaling pathway (Kramps et al., 2002; Brembeck et al., 2004).

BCL9 and its homologue BCL9L are key components of the canonical Wnt signaling pathway (Kramps et al., 2002; Adachi et al., 2004; Brembeck et al., 2004). In a baseline state, cytosolic β-catenin associates with the destruction complex, consisting of Axin, Adenomtous polyposis coli (APC), dishevelled (DVL), casein kinase α, and Gsk3β (Moon et al., 2004). The destruction complex phosphorylates multiple residues on β-catenin that facilitate its interaction to β-TRCP, Skp1-Cullin1-F-box-protein (SCF) E3 ubiquitin ligase complex, which ubiquinates it and targets it for degradation. Wnt binding to Frizzled and LRP5/6 recruits the destruction complex to the cell surface, and concurrently Gsk3β activity is repressed by LRP5/6. This leads to the accumulation of β-catenin in the cytosol, and the recruitment of β-catenin and its binding partners into the nucleus.

Nueclear β-catenin binds to the TCF/LEF family of transcription factors that are bound to TCF/LEF binding sites throughout the genome. β-catenin binding to TCF/LEF recruits histone acetyltransferases such as CBP as well as other co-transcriptional activators, which leads to the relaxation of the chromatin and the initiation and maintenance of RNA polymerase II (Pol II) mediated transcription. BCL9 is known to interact directly with β-catenin (Krieghoff et al., 2006; Sampietro et al., 2006) and this interaction is crucial for the enhancement of TCF/LEF transcriptional activity by Wnt stimulation.

BCL9 and its homolog BCL9L share five homology domains, HD1-5. HD1 binds to, and is essential for the recruitment of Pygopus (Pygo1/2), a TCF/LEF transcriptional co-activator to TCF/LEF binding sites (Hoffmans and Basler, 2007). HD2 has been shown to bind directly to β-catenin. BCL9 appears to aid in the retention of β-catenin within the nucleus, indicating that it may maintain the β-catenin level necessary for transcriptional activity. HD1 and HD2 also appear to be necessary for BCL9's function in the facilitation of β-catenin-mediated TCF/LEF transcription. While the function(s) of HD3-5 are yet unknown, a fragment that contains the

region between HD4 and HD5 was shown to have transactivation activity (Sustmann et al., 2008).

A crystal structure has been made that analyzed the Pygo-BCL9 interaction with methylated histone H3K4 (Fiedler et al., 2008), as well as the β-catenin-BCL9-TCF4 complex (Sampietro et al., 2006). These complexes seem to be quite discrete, and this has prompted an interest in BCL9/BCL9L as molecular targets for novel therapeutics. Presumably, modulation of BCL9's interaction with β-catenin, Pygo, and the histones may be a mechanism to target Wnt-mediated transcription in a manner that is more specific than targeting Gsk3β or other upstream kinases.

The known roles of BCL9 in the Wnt pathway lead us to hypothesize that BCL9 may play a role in the microcephaly/macrocephaly observed in 1q21.1 CNV deletions and duplications. It also led us to hypothesize that the perturbation of BCL9 levels may recapitulate some of the phenotypes observed in schizophrenia and autism. To assess the role of a reduction of BCL9 levels specifically in neurodevelopment, we set out to create a conditional mutant of BCL9. We also hypothesized that BCL9 may interact with other genes that are either implicated in neuronal development or psychiatric disorders, or open up new avenues for exploration. To do this, we conducted both biased and unbiased binding assays of BCL9 protein fragments and have started to characterize a BCL9 conditional knockout mouse.

METHODS AND PRELIMINARY DATA

Generation of a conditional BCL9 mouse

We followed the steps listed in the recombineering website, adapted from Liu and colleagues (Figure 1) (Liu et al., 2003). Briefly, we decided to add flanking loxP (flox) sites around exon 2 of BCL9, as the resulting transcript, even if transcribed consisting of exons 1-3 would result in a stop codon:

Exon 1, exon 2, exon 3 of mouse BCL9- translated

Met H P S N P K V R S S P S G N T Q S S P K S K Q E V M V R P P T V M S P S G N P Q L D S K F S N Q G K P G G S A S Q S Q P S P C D S K S G G H T P K A L P G P G G S M G L K N G A G N G A K G K G K R E R S I S A D S F D Q R D P G T P N D D S D I K E C N S A D H I K S Q E S Q H T P H S M T P S T A T A P R S S T P S H G Q T P A P E P I S A Q K T P A K V V Y V F S T E M A N

Exon1, exon3 predicted translation

Met H P S N P K V R S S P S G N T Q R M Stop

BAC constructs RP23-242D21 and RP23-134D1 were ordered from the bacpac at CHORI (Frengen et al., 2000), and the region containing the first five coding exons of BCL9 were transformed into the pl253 vector through homologous recombination. Briefly, ~400 base pair fragments flanking these coding exons were amplified out of DNA obtained from RP23-242D21 and RP23-134D1 and chosen for the presence of a single BamHI site. The fragment isolated that was homologus to the ~400bp's upstream of BCL9's first coding exon was amplified using primers that added a NotI restriction site. The fragment was digested using NotI/BamHI enzymes, then ligated into the PL253 vector. The ~400bp fragment downstream of BCL9's fifth coding exon was amplified using a 5' primer that added an XbaI site. The fragment was digested using XbaI and BamHI, then ligated into the PL253 vector that already contained the first fragment. After verification of proper insertion of both arms, the PL253-ArmA-ArmB vector was linearized using BamHI and dephosphorylated using calf-intestine phosphatase, then purified. EL350 cells, that had been previously transformed to contain RP23-242D21 or Rp23-134D1, were grown to optimal optical density (0.2-0.35 λ) and induced for exo, bet, and gam recombination enzymes, were then electroporated with 100ng of linearized PL253-ArmA-ArmB. The bacteria were then plated on ampicillin plates for selection of colonies that had successfully

recircularized PL253. Colonies were then sent to sequencing to identify the ones that had recombined (i.e. recircularized with the BCL9 sequence in the plasmid) instead of religated. They will be referred to as PL253-242D21 or PL253-134D1. PL253-242D21 and PL253-134D1 were retransformed into electrocompetent DH10B cells to select against multimeric plasmids.

To insert the first loxP site we utilized a similar recombination approach, this time using the PL452 vector. This vector contains a floxed Neomycin cassette (Neo). To recombine this neomycin cassette into the PL253 construct, we created two recombination arms distal to the second exon of BCL9. We amplified these two recombination arms using primers flanked by 5'EcoRI and 3' SalI, and 5'BamHI/3'NotI, respectively, for the arms proximal and distal to floxed Neo, and sequentially ligated them into the construct. The flox Neo cassette with BCL9 homology arms C and D was then subsequently isolated from PL452 by digestion with NotI and EcoRI, dephosphorylated, and recombination was induced by combining 100 ng of the fragment into PL253-242D21 and PL253-134D1 containing EL350 cells that had been grown to optimal density and induced for recombination, as described above. Bacteria that had recombined PL253-242D21/134D1 to include the Neo cassette were isolated by selection on kanamycin plates. Colonies were isolated and PL253-242D21/134D1 flox neo was purified. Cre-mediated excision of the neomycin cassete was induced by transforming the DNA back into EL350 bacteria grown in arabinose-supplanted media. The DNA from this was subsequently sequenced to identify the clones that had incorporated the single LoxP site.

To add the second LoxP site, the PL451 plasmid was used to insert the second loxP site in a manner similar to that described above, using homology arms E and F that targeted the region in the intron between the first and second exons. The PL451 plasmid features a Neo cassette that is flanked by frt sites, which can also act to excise DNA placed in between the sites. The region downstream of the Neo cassette contains a single LoxP site that will remain

after the expression of flippase (flpe), which will result in the excision of frt-flanked regions. Proper insertion of the second loxP site was also confirmed by sequencing.

The targeting constructs were sent to the MIT transgenic facility for electroporation into mouse ES cells. Two days after receiving the electroporated ES cells to analyze for recombination, a paper was published that characterized a targeted deletion of BCL9/9L in mouse muscle progenitors (Brack et al., 2009) and our efforts were discontinued. We have established a collaboration with the group that generated the mice, and have subsequently obtained the line. Rederivation, isolation of the flank-loxed allele from BCL9l flank-lox, and backcrossing of the line has been in progress for some time, and we have started analyzing the effects of a targeted deletion of BCL9 in neuronal progenitor cells (Nestin-CRE targeted excision).

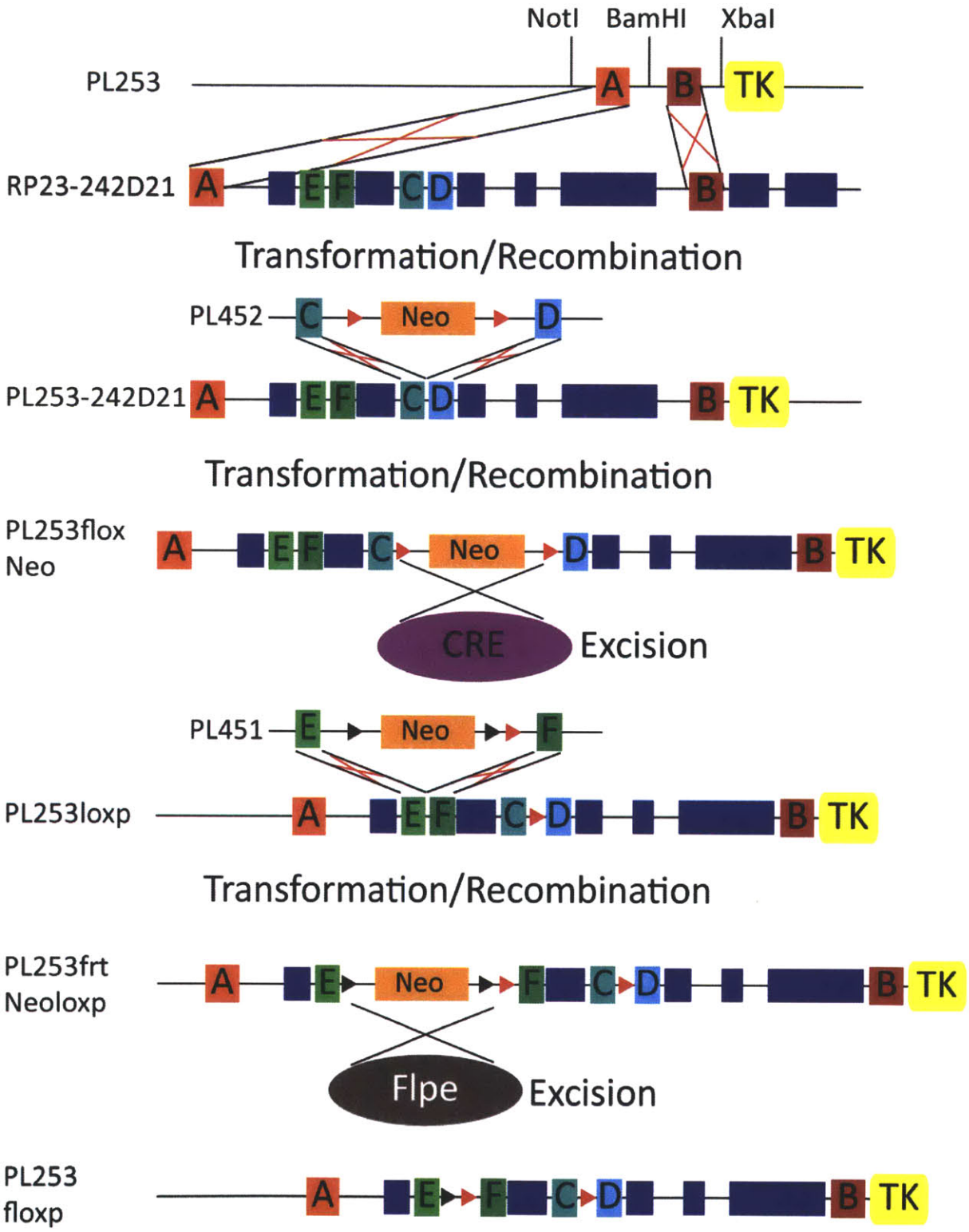

Figure 1: Schematic of the insertion scheme for flank-lox sites for generation of the conditional BCL9 knockout mouse. Dark Blue boxes represent BCL9 coding exons.

Yeast-2-Hybrid screen to identify novel interactors of BCL9

The interaction of BCL9 with its known binding partners, Pygo and β-catenin, are relatively well characterized. The exact armadillo repeat within β-catenin required for the interaction (Kramps et al., 2002), as well as the region within Pygo that binds BCL9 has been determined (Stadeli and Basler, 2005). As mentioned previously, the region between HD4-5 has also shown transcription enhancing ability, although its function has yet to be elucidated. To identify potential mechanisms of transcriptional enhancement, we sought to determine what, if any, proteins interact with Homology Domains 4, 5 and its connecting segment. We chose to carry out a yeast-2-hybrid screen to identify potential binding partners targets for further validation. For this, we implemented the Matchmaker system developed by Clontech. Briefly, we constructed a fusion protein between the GAL4-DNA binding domain and the BCL9 HD4-5 fragment by cloning the fragment into the pGBKT7 construct. BCL9 HD4-5 was amplified from mouse cDNA isolated from adult mouse cortex using the primers HD4-5 Forward (TAGCGAATTC- TATCCCATGCCTCCAGAGCCC) HD4-5 Reverse (TATCGTCGAC-TGAAAACCCAGGCCCAGGACC) to generate a fragment that corresponds to amino acids 992 to 1270 in mouse BCL9. The fragment and pGBKT7 vector were digested using EcoRI and SalI, purified, and then ligated. The ligation product was transformed into DH5α chemically competent cells, then plated onto Luria broth-agar plates with kanamycin (50μg/ml). Several colonies were picked and sequenced using the T7 primer for verification.

Once the correct insert had been verified, pGBKT7 was transformed into Y2H gold yeast cells. Transformed yeast were plated on SD/-trp, SD/-trp/X-α-gal and SD/-trp/X-α-gal/AbA to test for autoactivation. After verifying that autoactivation did not occur, the bait strain was grown overnight in 50ml SD/-Trp liquid media at 30°C and 250rpm overnight. When the OD_{600} reached 0.8, yeast cells were pelleted by centrifugation at 1000g for 5 minutes. The cells were resuspended in 5ml SD/-trp, and combined with 1ml of embryonic mouse brain library

(Clontech) in Y187 yeast strain. Then, 45 ml of 2xYPDA medium with 50ug/ml Kanamycin (YPDA/Kan) was added to the mixture, placed in a 2L flask, and incubated for 24 hours at 50 rpm. After veryfing the presence of Zygotes, cells were pelleted by centrifugation at 1000g for 5 minutes. The cells were then resuspended in 0.5x YPDA/Kan. 100 µl of cells were plated onto control (SD/-Trp, SD/-Leu, SD/-Leu/-Trp (DDO) at 1/10, 1/100, 1/1000, and 1/10,000 dilutions to evaluate the necessary number of clones to screen. The rest of the cells were plated on fifty-five 150mm DDO/X/A plates. These plates were incubated for 4 days. Viability of the diploids was calculated to be around 2%. All the blue colonies were then re-streaked on higher stringency QDO/X/A plates. The plasmids contained within the colonies that remained were then purified by yeast miniprep, transformed back into DH10B, and plated on ampicillin plates. Three colonies were selected from each transformation reaction, grown overnight, and sent for sequencing. A total of 300 colonies were screened (Table 1).

Cloning of BCL9 fragments for verification of interactors, *in vitro* work, and rescue.

Because of the relatively large size of BCL9, we set out to identify the roles of various fragments of BCL9. To achieve this, we generated multiple fragments of human BCL9 tagged to an N-terminal GFP by the insertion of these fragments into the pEGFP-C1 Vector (Clontech) (Figure 2). Interestingly, we found that DISC1 binds to a region of BCL9 that binds to Pygopus, as well as the region of BCL9 that is known to have transactivational activity. This suggests that DISC1 may have a more direct role in TCF/LEF transcription than it has been currently ascribed.

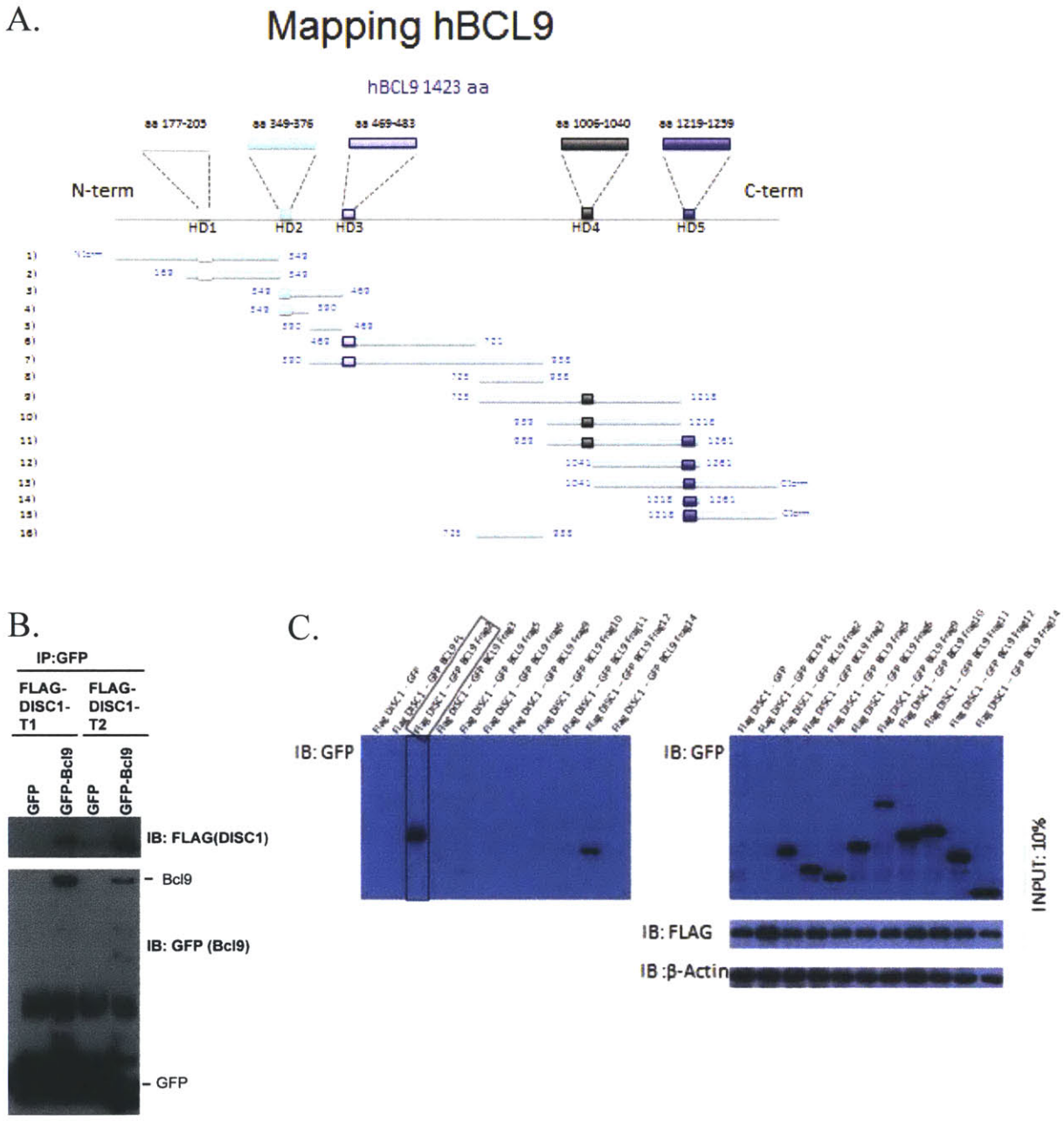

Figure 2: BCL9 interacts with DISC1 through several regions. **A**. Schematic for the design of hBCL9 fragments tagged with GFP. **B**. Full-length DISC1 shows interaction with BCL9. Immunoprecipitation (IP) using anti-FLAG (M2) conjugated agarose beads after overexpression of FLAG-tagged mouse DISC1 T1 or T2 and Full length GFP tagged hBCL9. BCL9 showed stronger interaction with mouse DISC1 T1 than T2. **C**. DISC1 interacts with several BCL9 regions. Immunoprecipitation using anti-FLAG (M2) conjugated agarose beads after overexpression of FLAG-tagged mouse DISC1 T1 and various GFP-tagged BCL9 fragments. Fragment2, which contains the HD1 domain of BCL9, showed consistent enrichment after immunoprecipitation.

Discussion

The importance of the Wnt signaling cascade in brain development is well established (Chenn and Walsh, 2003). BCL9 knockdown has been shown in previous studies to greatly reduce Wnt-mediated increase in TCF/LEF response (de la Roche et al., 2008) and BCL9 is known to be expressed in the fetal brain (Willis et al., 1998). It is one of the genes in the critical region of the 1q21.1 CNV that is associated with a increase in the risk for schizophrenia (International Schizophrenia, 2008; Stefansson et al., 2008b), microcephaly, macrocephaly, and autism (Brunetti-Pierri et al., 2008; Mefford et al., 2008) that have been replicated multiple times (Levinson et al., 2011; Grozeva et al., 2012). SNPs within the gene are associated with an increased risk for schizophrenia in the Han Chinese population (Li et al., 2011a). These evidence make it quite likely that BCL9 will play some role in neuronal development; yet to date no study of its role in the nervous system has been published.

We have created the necessary reagents to conduct a through investigation of the role of BCL9 in neuronal development as well as its effect on the behavior of adult mice. The conditional knockout model is preferable to full-body knockouts because of the specificity, and to viral injection models because of its reproducibility of effect. Moreover, the mice are much more likely to be amenable for behavioral testing because we can avoid the effects of the loss of gene function on the rest of the body. We can also test the effects of gene dosage by comparing Nestin-CRE^{+} BCL9 $^{flox/flox}$ mice to Nestin-CRE^{+}, BCL9 $^{+/flox}$ and Nestin-CRE+ BCL9 $^{+/+}$ as well as Nestin-CRE^{-} mice.

Comparing the phenotype of the Nestin-CRE^{+} BCL9$^{flox/flox}$ mice to those phenotypes seen in the β-catenin and GSK3β loss of function models because this will allow us to discern which aspects of the phenotypes observed in the mice are due to TCF/LEF mediated transcription, as opposed to perturbations in other aspects downstream of these molecules. Our current

understanding for the role of BCL9 would indicate that the noncanonical Wnt signaling pathways would largely remain intact, and processes such as axonal guidance and neuronal migration would not be expected to be affected by the loss of BCL9, as the role of the noncanonical Wnt signaling cascade is more established for these functions; however whether this is the case remains to be seen.

Recently, a SNP in the Chromodomain containing protein 8 (CHD8) gene was identified to be significantly associated to autism (Neale et al., 2012). CHD8, like BCL9, is known to also form a tripartite complex with β-catenin and methylated histone H3K4 residues. However, unlike BCL9 which is required for transactivation, CHD8 acts to inhibit transcription at TCF/LEF sites. Though the presence of CHD8 in the same complex with BCL9 has not been shown directly, it is likely that these two proteins are part of the same complex that acts to regulate TCF/LEF transcription. It is also possible that these two proteins are actively competing to enhance, or inhibit, transcription from the same genetic regions. These are testable hypothesis and is an area of active investigation.

Also quite likely to be present in the same complex is transcriptional adaptor 2A (ADA2A), encoded by TADA2, present in the 17q12 CNV. ADA2A is the namesake of the ADA-2-A-containing complex (ATAC/PAF) double histone acetyltransferase (HAT) complex that has both HAT as well as histone deubiquitination activity, and helps drive the initiation of PolII mediated transcription (Nagy et al., 2010). It is also known to be required for TCF/LEF mediated transcription (Yang et al., 2008). Genetic abnormalities in another component of this complex, CBP/p300 is the cause of Rubenstein-Taybi syndrome, a syndrome that includes mental retardation and behavioral abnormalities (Petrij et al., 1995). It is worth noting that the top hit within our Yeast-2-hybrid screen B945IP1 is likely to aid this process, and is an active area of investigation as well. That perturbations in the genes that encode proteins within the same

complex adds to the likelihood that TCF/LEF mediated transcription plays a role in the risk for, and/or development of psychiatric disorders.

The characterization of the Nestin-CRE BCL9$^{flox/flox}$ mice should inform us how much to pursue this line of reasoning. It should also provide interesting insights on the functional relevance of TCF/LEF mediated transcription on the efficacy of antidepressant treatment. Should antidepressants be ineffective in these mice, for example, it will be insightful both from a mechanistic as well as therapeutic standpoint, as it may narrow down a more specific pathway, from the myriad of pathways activated by receptor signaling. As Nestin-CRE BCL9$^{flox/flox}$ mice would be predicted to have aberrant neurogenesis, in addition to dramatically reduced TCF/LEF mediated transcription in all neurons, a comparison with mice with subgranular zone irradiation will inform one the role of TCF/LEF mediated transcription in neurons that have already been born. The role of TCF/LEF transcription in the efficacy of antidepressants can be tested in this manner. It is also possible to test many other compounds and treatments to ask a similar question. In summary I believe that this mouse line will be an important reagent to address a host of questions relevant to psychiatric disorders, pharmacological mechanisms, and basic molecular neuroscience.

Candidate	Repeat?	Chr, strand, start, end, span	Function
HD45IP1	Y 8		
HD45IP5	y 3		
TIMP4	Y 3	3 - 12169575 12173350 3776	Tissue inhibitor of metalloproteinases 4,netrin domain-containing protein encoded by this gene is involved in regulation of platelet aggregation and recruitment and may play role in hormonal regulation and endometrial tissue remodeling, highly expressed in heart, endomertium and brain
TSPAN7	y 2	X + 38305741 38432410 126670	tetraspanin 7, cell-surface proteins characterized by the presence of four hydrophobic domains. Mediate signal transduction events that regulates of cell development, activation, growth and motility. Cell surface glycoprotein, role in the control of neurite outgrowth. Complex with integrins. This gene is associated with X-linked mental retardation and neuropsychiatric diseases such as Huntington's chorea, fragile X syndrome and myotonic dystrophy
?	Y 2	9 + 80448773 80449713 941	
?	y 4	2 + 116319295 116320282 988	
?	y 3	10 - 93796828 93797837 1010	
?	Y	2 + 116319295 116320279 985	
AHCYL1	Y 2	1 + 110362538 110366379 3842	S-adenosyl homocysteine hydrolase homolog, intracellular Ca2+ regulation, binds to IP3
FAM35A,	y 2	10 + 88940232 88941201 970	
HD45IP6	y ?	7 - 44368022 44368702 681	
RAF1	Y 2	3 - 12600115 12601055 941	raf proto-oncogene serine/threonine protein kinase, MAP kinase kinase kinase (MAP3K), Activates MEK1 and MEK2Mutations in this gene are associated with Noonan syndrome 5 and LEOPARD syndrome 2.
SPATA2	y 2	20 - 47954097 47954885 789	spermatogenesis associated protein 2
YLPM1	Y 2	14 + 74318208 74358571 40364	The nuclear PP1 interacting protein ZAP3 (ZAP) putative nucleoside kinase that complexes with SAM68, CIA, NF110/45, and HNRNP-G.

CLSTN2		3 + 141456983 141457843 861	may modulate calcium mediated posysynptic signals, Highest levels in GABAergic neurons, association with episodic memory recall task difference (snp rs6439886)
HD45IP2			
HD45IP3			
IGF1R		15 + 97324315 97325281 967	Insulin-like growth factor I receptor, tyrosine kinase, essential for hippocampal growth NMDAactivity attenuates signaling via receptor
KLHL1		13 - 69173683 69212582 38900	MRP2, a target of Nurr1, involved in differentiation of mesolimbic dopamine neurons, oligodendrocite elongation, neurite outgrowth (gsk3 dependent), actin binding
?		10 - 93796792 93797837 1046	
?		10 - 93796793 93797837 1045	
?		X + 64287007 64287852 846	
?		7 - 44367884 44368784 901	
?		1 + 144401069 144402014 946	
AF338195		8 + 43104650 43105475 826	
ANGEL1		14 - 76324395 76324734 340	
ARRDC3		5 - 90700350 90701383 1034	TBP-2-like inducible membrane protein,
BC036485		3 - 180218821 180219825 1005	
HD45IP4			
CGI-150		17 + 852171 853012 842	protein, AF151908
C1orf216		1 - 35952269 35953340 1072	
CTSB		8 - 11739597 11742639 3043	Cathepsin B
DDX21		10 + 70407363 70413053 5691	RNA helicase II, found in nucleoli, c-Jun mediates its nucleolar localization
DDX58		9 + 32449381 32450391 1011	RIG-1, cell receptor for RNA, regulates IFN production after virus infection, suppresses RAS
EDNRB		13 - 77367625 77368579 955	Endothelin receptor type B, GPCR, activates PIP3 signaling involved in neuronal progenitor proliferation hirschprung's disease
FAM153C		5 + 177368594 177406666 38073	
FAM171B		2 + 187337894 187338703	

		810	
FBLN-5		14 - 91405648 91413783	Secreted, extracellular matrix protein containing anArg-Gly-Asp (RGD) motif and calcium-binding EGF-like domains.
FLJ35848		17 - 40105846 40106495 650	
GAD1		2 + 171425125 171425907 783	Glutamic Acid decarboxylase 1.
Galc		14 + 87491112 87492095 984	galactosylceramidase-->Krabbe disease(myelin loss)
GNAL		18 + 11842584 11843552 969	Golfalpha gene.. Candidate for schiz
GUCY2C		12 + 14683720 14690806 7087	
IPO7		11 + 9423818 9424822 1005	Importin 7, ERK phosphorylated at SPS NTS site interacts with it, required for translocation
IRF2BP2		1 - 232806734 232807832 1099	p53 signaling, reduces apoptosis if overexpressed after actinomcin D treatment
JMJD7		15 + 39907588 39916755 9168	Has jumonji domain, may have fusion protein with pplA2
KBTBD11		8 + 1941403 1942495 1093	Kelch repeat and BTB domain containing 11
KCNH1		1 - 209097623 209098625 1003	Pottasium channel hEAG related, voltage-gated non-inactivating delayed rectifier
KDM3B		5 + 137799299 137800280 982	Lysine-specific demethylase 3B
KIAA0408		6 - 127804060 127805118 1059	
KIAA1434		20 - 5473261 5474287 1027	
LYST		1 - 233942980 233961015 18036	chediak-higashi syndrome 1, lysosomal trafficking regulator, May be required for sorting endosomal resident proteins into late multivesicular endosomes by a mechanism involving microtubules
Mal2		8 + 120325950 120326928 979	T-cell differentiation protein2, component of lipid rafts, localizes to endosomal structures beneath the apical membrane. Required for transcytosis, an intracellular transport pathway used to deliver membrane-bound proteins and exogenous cargos from the basolateral to the apical surface

NAPEPLD		7 - 102528524 102529509 986	N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D
NFI-B		9 - 14076780 14077475 696	Nuclear factor 1/B, Recognizes and binds the palindromic sequence 5'-TTGGCNNNNNGCCAA-3' Capable of activating transcription and replication, affects brain development
ODC1		2 - 10497961 10499493 1533	ornithine decarboxylase 1,
PALLD		4 - 170056052 170057073 1022	NCAM, FGF interactor
PCDH9		13 - 66698405 66699345 941	Protocadherin 9, cadherin-related neuronal receptor that localizes to synaptic junctions and is putatively involved in specific neuronal connections and signal transduction. Has large exon that encodes 6 cadherin domains and a transmembrane region. Two alternatively spliced transcript variants encoding distinct isoforms have been found for this gene.
PI4KA		22 - 19392025 19397046 5022	phosphatidylinositol (PI) 4-kinase which catalyzes the first committed step in the biosynthesis of phosphatidylinositol 4,5-bisphosphate. The protein encoded by this gene is a type III enzyme that is not inhibited by adenosine
PI4KAP1		22 - 18763559 18768572 5014	
PI4KAP2		22 - 20157333 20162351 5019	
PIP4K2A		10 - 22944727 22945751 1025	
PLG		6 - 161081425 161082351 927	(plasminogen)
PPPR2B		5 - 146221613 146222477 865	intron
PPWD1		5 + 64916572 64917930 1359	peptidylprolyl isomerase, found in splicosome
PSD3		8 - 18640974 18641957 984	Pleckstrin homology and SEC7 domain-containing protein 3, guanine nucleotide exchange factor
PURA		5 + 139475455 139476417 963	DNA binding, PURalpha
RNF111		15 + 57174351 57175716 1366	Arkadia, amplifies TGF-beta signaling by degrading SMAD7
SEPT13		7 - 45729916 45731092 1177	

SET		9 + 130494968 130497301 2334	inhibitor-2 of protein phosphatase-2A, Isoform 1 and isoform 2 are potent inhibitors of protein phosphatase 2A. Isoform 1 and isoform 2 inhibit EP300/CREBBP and PCAF-mediated acetylation of histones (HAT) and nucleosomes,
SLC29A2		11 - 65886845 65890068 3224	
SLC38A1		12 - 44867836 44868786 951	
SLC39A5		12 - 54916711 54916736	
SMBT2		10 + 7276076 7276381 306	Scm-related gene containing four mbt domains 2, upregulated in response to valproic acid
STAMBP		2 + 73931027 73943507 12481	STAM binding protein, involved in cell-cycle progresison
TNS3		7 - 47281923 47282373 451	actin remodeling, integrin-tensin-actin
UBE2V2		8 + 49136759 49137716 958	ubiquitin-conjugating enzyme E2 variant 2, Has no ubiquitin ligase activity on its own. In The UBE2V2/UBE2N heterodimer. Makes non-canonical poly-Ub chains linked through 'Lys-63'. Does not lead to protein degradation by the proteasome. Mediates transcriptional activation of target genes. Plays a role in the control of progress through the cell cycle and differentiation. Plays a role in the error-free DNA repair pathway and contributes to the survival of cells after DNA damage
UFSP2		4 - 186557712 186563979 6268	Thiol protease which recognizes and hydrolyzes the peptide bond at the C-terminal Gly of UFM1.
UTRN		6 - 144835142 144835656 515	utrophin (homologous to dystrophin),
WWOX tanscript variant 1		16 + 77233895 77234945 1051	WW domain-containing oxidoreductase, Probable oxidoreductase, which acts as a tumor suppressor.
ZNF33A		10 + 38386017 38387039 1023	
ZNF567		19 + 41902522 41903609 1088	
ZNF573		19 + 42975494 42976530 1037	
LOC401397 (LOC401397), transcript variant 1, non-coding RNA.	Y 4	7 - 112544015 112545845 1831	
Table 1. BCL9 HD4-5 Yeast-2-Hybrid results			

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Chapter 4

Discussion, future directions

Follow-up studies to: *DISC1 acts as a co-repressor to inhibit ATF4 mediated transcription at the PDE4D loci*

The role of PDE4D9 in particular in the DISC1 knockdown phenotype, as well as the function of PDE4D9 in the nervous system is an area of active investigation. PDE4D9 has been shown to interact with β_2-adrenergic receptors in cardiac myocytes (De Arcangelis et al., 2009). We have conducted a yeast-2 hybrid screen in which we identified two novel interactors for the unique portion of PDE4D9. We have confirmed one of these interactors by immunoprecipitation studies. Interestingly, this hit it is a protein known to play a role in the degradation of dopamine D2 receptors. It is hard to believe that this is purely due to serendipity, given the importance of dopamine D2 receptor signaling in antipsychotic action. A characterization of the effects of PDE4D9 knockdown on D2 receptor surface expression and regulation, will likely yield interesting results.

PDE4D9 overexpression, and assaying for similarities in its phenotype relative to the existing DISC1 mouse models, is one method in which we may ascertain the role of PDE4D9. However, overexpression of PDE4D9 could have a confounding effect, such as ectopic subcellular localization of PDE4D9, potentially resulting in a general PDE4D overexpression phenotype. To control for this, the overexpression of another long-variant of PDE4D (or another PDE4 member) may also be overexpressed, and the nonoverlapping phenotypes may be an indication of the variant's unique roles.

Rolipram, as well as DISC1, are known to inhibit both isoforms of PDE4s. Past studies have reported a decrease in immobility in the forced swim test using acute doses of GSK3β (Mao et al., 2009), and rolipram (Zhang et al., 2002). Rolipram's antidepressants effects are diminished in PDE4D loss of function mice (Zhang et al., 2012), while the antipsychotic-like effects of rolipram are diminished in PDE4B loss of function mice (Siuciak et al., 2007). Synergy

between tricyclic antidepressants and rolipram has been demonstrated in rodents (Itoh et al., 2004; Marchetti et al., 2010). A recent study has also demonstrated that schizophrenic and depressive phenotypes seen in mice with missense mutations in the DISC1 gene can both be rescued by GSK3β and PDE4 in a synergistic manner (Lipina et al., 2012). It is interesting to note that the synergy was not seen in wildtype mice for some assays, including the forced swim test. We are assaying whether a similar effect can be seen in the wildtype context, as this may address an issue of applicability to humans, as equivalent DISC1 mutations to the ones used for this study have not been reported in humans. However, since their mechanism seems to be dependent on the differential binding affinities of DISC1 to PDE4B and GSK3β, it may turn out to be that DISC1 SNPs confer differential response profiles to these compounds, and may be predictive of response to this form of combination treatment. Nonetheless, we still do not know just how much of this effect is mediated by PDE4D9.

We attempted double-knockdown of DISC1 and PDE4D9 in mouse dentate gyrus. Unfortunately, the co-localization of the markers for PDE4D9 and DISC1 shRNA viral transduction was low, making the interpretation of the results difficult (results not shown). We are currently taking advantage of a system developed in the Sudhof lab (Pang et al., 2010) in which two different shRNAs are expressed from a single lentiviral construct, tagged with a single fluorophore (GFP), yielding a virus that can simultaneously knock down DISC1 and PDE4D9. Cell culture based characterization of the effects of dual-knockdown, and the pharmacological synergy of the inhibition of GSK3β and PDE4's are ongoing. The construct also leaves room for the insertion of an overexpression rescue construct, which can be either short hairpin resistant PDE4D9, or another PDE4 variant (to assay for specificity of effect on decreasing particular variant versus lowering overall PDE4 levels).

Discussion regarding approach to assaying the effect of genetic perturbations that confer risk to neuropsychiatric disorders

Our current framing of these disorders assumes that schizophrenia, bipolar disorder, and autism represent distinct categories in the domain of genetic risk, and that this genetic risk will give rise to specific perturbations in biochemical pathways that will bring about these disorders. These disorders are defined by a constellation of symptoms and the diagnosis itself is a hypothesis. It presumes that categorizing patients in this manner will help guide treatment, because the disorders result from different causes. The initial genome-wide association studies (GWAS) applied the disorder criteria in order to have a workable hypothesis. However, the ensuing biochemical and molecular studies to uncover the functional effect of the identified genetic risks and whatever new therapeutic approaches these yield, need not rely on diagnosis. It is entirely possible that numerous perturbations in different biochemical pathways underlie a constellation of symptoms that end up being categorized as the same disorder. It is also entirely possible that these different manifestations arise from the same etiology and what is beneficial for one diagnosis will be useful for all. Evidence from the GWAS conducted comparing patients with these disorders to the general population, have revealed that these disorders share common genetic risk perturbations. The potential impact of uncovering the consequences of the genetic risk factors that are common to all these disorders seem more important, than focusing on efforts that seek to identify which genetic risk factors are specific to each disorder.

Genome-wide association studies have yielded two general classes of perturbations that confer risk for neuropsychiatric disorders; common alleles that contribute a small risk, and rare alleles that contribute a significant degree of risk. There is much contention within the field in how much these alleles contribute to the overall prevalence of these disorders, one model

assuming that the common alleles account for much of the prevalence, and the other assuming that the rare alleles can account for much of the prevalence. The implication of assuming one model or the other as the causative reason behind these psychiatric disorders are discussed in greater detail elsewhere (Moskvina et al., 2009; Ripke et al., 2011; Rodriguez-Murillo et al., 2012; Sullivan and Psychiatric Genetics, 2012) and is the source of much tension in the field today. Suffice it to say that the genetic differences driving both hypotheses are statistically significant and reflect true genetic differences that associate more or less with disorder. Importantly, for the first time, these true genetic associations can be tested by biologists, to determine *how* these differences lead to disorder association. Many of the functions of the putative transcripts and proteins which these genetic differences are thought to affect, are themselves not known, and it would certainly be beneficial to attribute some function to these transcripts and proteins proximal to or within the areas of association.

Although not necessary, it would also be advantageous to have some testable hypothesis to assess the function of these transcripts of unknown function. The Wnt pathway is a useful framework in which to think about the pathways not only because of the apparent convergence of proteins identified, but also because the reagents and techniques, which can be used to probe the pathway, are well established.

From the viewpoint, "one must carefully choose which perturbation to study, as the findings (or lack thereof) will determine the rest of one's career", the advantages of choosing to study a CNV, or a gene within a CNV is that the effect of the perturbation on the gene dosage is known and the increase in risk is higher, giving one more confidence that creating a construct-valid model will produce a phenotype. In a deletion, there is one less copy of that gene/region, and in duplication, there are more copies. Studying the genes in a gain-or loss of function model has face validity just based on this fact.

The use of single-gene deletion/overexpression animal models also assumes that genetic dosage of the gene being studied will result in an observable biological phenomenon in and of itself. The disadvantage from the trainee's perspective is that there is more than one gene in a single CNV, and the loss of function of a single gene within the CNV may not yield a phenotype. Most of the CNV critical regions identified in human patients also have systemic regions within the mouse genome. An ideal animal model to test the effect of the CNV would be to generate CNV models in which the systemic region homologous to the critical region has been deleted or duplicated. Indeed, mice that mimic the 22q.11, 16p, and 15p CNV's have already been created (Prescott et al., 2005; Takumi, 2010; Horev et al., 2011). Our lab has attempted, without success thus far, to create the 1q21.1 CNV deletion mouse. A cross of a CNV deletion mouse with overexpression of a single gene (preferably using the endogenous promotor), or of a CNV duplication mouse with a deletion of a single gene, would be extremely insightful as to the contribution of that gene to the CNV phenotype.

Many of the SNPs identified as conferring risk are in noncoding regions of the genome. The study of the effect of common SNPs in disorder requires an extra step, which is in determining what the effect of these SNPs are on the expression of genes near the region. The advantage of studying these SNPs is that they are common, and obtaining samples that harbor the risk alleles is relatively simple. Conversely, because these SNPs are so common, it is quite likely that the chromosomes from each sample will harbor multiple risk-conferring SNPs. Another issue is the relatively low increase in risk conferred by each risk SNP. However, I believe the same approach must be taken for these SNPs as the CNVs: engineer these SNPs in the same identical genetic background, and assess differences at the biochemical level. One must be patient, thorough, and potentially make multiple SNP mutations, but the increase in risk is multiplicative. If these SNPs are affecting a common pathway, the addition of multiple risk alleles will, after a certain threshold, elicit an observable biochemical effect. Having the right

assays in place will be critical to the analysis of this type of work; a pathway driven approach can be taken here as well. From a practical standpoint, by biasing the initial mutations to create onto those genes that encode proteins in a single testable pathway with a highly sensitive assay, one may reduce the number of mutations needed to be created before an effect can be seen (and become publishable). Eventually, a repository of psychiatric disorder associated SNPs generated on a common human background can be created through the work of multiple groups.

For both types of genetic perturbations, it would be beneficial to have access to patient postmortem brain samples, or patient-derived neurons/neuronal progenitors to be able to assay transcript levels and further strengthen the validity of these models. This can either be done at the genome-wide level or by assaying genes nearby the SNPs and contained in, or near the CNVs. Because patient samples are not of the identical genetic background, a large number of patient samples will need to be assayed for the results to bear significant meaning.

It is evident that these approaches will take the commitment and effort of a large number of people collaborating with each other, and involves a lot of trust: though one may be pessimistic in thinking that such collaborations simply do not happen in biology, one might look towards the consortium studies conducted by geneticists that yielded the genetic data with which the field is about to conduct studies. I hope to be able to contribute, along with many other people, to the understanding of these disorders, and, with hope, to uncover therapeutic targets that will improve the lives of those that suffer from them.

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