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Transcriptional Regulation of *GAD1* GABA Synthesis Gene in the Prefrontal Cortex of Subjects with Schizophrenia

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Abstract

Expression of *GAD1* GABA synthesis enzyme is highly regulated by neuronal activity and reaches mature levels in prefrontal cortex not before adolescence. A significant portion of cases diagnosed with schizophrenia show deficits in *GAD1* RNA and protein levels in multiple areas of adult cerebral cortex, possibly reflecting molecular or cellular defects in subtypes of GABAergic interneurons essential for network synchronization and cognition. Here, we review 20 years of progress towards a better understanding of disease-related regulation of *GAD1* gene expression. For example, deficits in cortical *GAD1* RNA in some cases of schizophrenia are associated with changes in the epigenetic architecture of the promoter, affecting DNA methylation patterns and nucleosomal histone modifications. These localized chromatin defects at the 5' end of *GAD1* are superimposed by disordered locus-specific chromosomal conformations, including weakening of long-range promoter-enhancer loopings and physical disconnection of *GAD1* core promoter sequences from cis-regulatory elements positioned 50 kilobases further upstream. Studies on the 3-dimensional architecture of the *GAD1* locus in neurons, including developmentally regulated higher order chromatin compromised by the disease process, together with exploration of locus-specific epigenetic interventions in animal models, could pave the way for future treatments of psychosis and schizophrenia.

GABAergic Dysfunction in Schizophrenia – A Brief Chronology

Schizophrenia (SCZ)— a major psychiatric disorder with symptoms of delusions, hallucinations disorganized thought and affect, social withdrawal and apathy— lacks unifying neuropathology (Catts et al., 2013; Dorph-Petersen and Lewis, 2011), or narrowly defined genetic risk architectures and disease etiologies (Andreassen et al., 2014; Rodriguez-Murillo et al., 2012). Yet, clinical and translational research conducted over the last 40 years is beginning to identify major building blocks within the complex pathophysiology of SCZ.

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Contributors

Amanda Mitchell and Schahram Akbarian edited and drafted the manuscript. Yan Jiang and Cyril Peter drafted TALE and CRISPR images for the final figure. All authors reviewed the final manuscript.

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As highlighted in the various articles in this Special Issue of *Schizophrenia Research*, one such building block is the inhibitory GABAergic circuitry in the cerebral cortex. While the primary focus of our review will be on the transcriptional dysregulation of the *GLUTAMIC ACID DECARBOXYLASE 1 (GAD1)* gene, encoding the 67 KDa GABA synthesis enzyme, we will begin with a brief synopsis of past studies in pursuit of the ‘GABAergic hypothesis of SCZ’, which proposes that GABAergic systems could play a key role in the pathophysiology of SCZ. This idea is not new. Thus, 25 years after the first reports described the relatively large amounts of GABA and high levels glutamic acid decarboxylase (GAD) in brain (Roberts and Frankel, 1950, 1951), the role of inhibitory inputs to midbrain dopaminergic neurons was hypothesized to be the key mechanism responsible for excessive and dysregulated dopaminergic activity in psychosis (Smythies et al., 1975; Stevens et al., 1974). While it was quickly recognized that a generalized deficit in GABA signaling is not a characteristic of SCZ—as the symptoms of psychosis remained unresponsive to GABA agonists in early clinical trials (Tamminga et al., 1978)—the idea of region-specific dysfunctions of GABA systems nonetheless continued, up to the present day, to maintain significant traction and in fact, emerged as one of the most popular hypotheses in SCZ research. For example, several studies reported a decrease in GABA levels and GAD activity in the medial temporal lobe, thalamus and ventral striatum of the SCZ postmortem brain (Bird et al., 1977; Perry et al., 1979; Spokes et al., 1980) albeit other investigators reported negative findings (Cross et al., 1979). There were also reports on low GABA levels in the cerebrospinal fluid in at least a subset of patients diagnosed with SCZ (Lichtshtein et al., 1978; van Kammen et al., 1982). Following these early studies on GABA and GAD quantifications in the schizophrenic brain, several papers explored alterations in the inhibitory system in the context of abnormal circuitry. This type of work was mainly focused on the cerebral cortex and hippocampus, starting with Benes’ model proposing excessive excitatory and insufficient inhibitory signaling in the upper layers of the cerebral cortex due to a possible loss of GABAergic neurons and/or supranormal numbers or densities of glutamatergic afferent input into the same cortical layers (Benes et al., 1992a; Benes et al., 1992b). There is also an ongoing discussion if and how decreased expression of GABAergic markers genes in the cerebral cortex could be related to neurodevelopmental alterations (Benes, 2012), including the excessive numbers and densities of subcortical white matter neurons that seem to affect some cases with SCZ (Akbarian et al., 1993a; Akbarian et al., 1995; Akbarian et al., 1993b; Anderson et al., 1996; Benes, 2012; Eastwood and Harrison, 2005; Joshi et al., 2012; Kirkpatrick et al., 1999; Rioux et al., 2003; Yang et al., 2011).

However, the circuitry model with the greatest impact in the field to date was put forward by Lewis and colleagues (Lewis et al., 2005). At the core of this model is a deficit in perisomatic inhibition of cortical pyramidal neurons that involves their axon initiating segments (AIS) as a key control point for the output of cortical information processing (Lewis et al., 2005). The AIS is innervated by a specialized subtype of GABAergic interneuron, the chandelier cell, which is thought to be one of the interneuron types that are dysfunctional in SCZ cerebral cortex as evidenced by molecular alterations both on the pre- and postsynaptic site of the AIS (Pierri et al., 1999; Volk et al., 2002; Woo et al., 1998). Importantly, these postmortem studies, together with related work in preclinical model

systems, paved the way for clinical trials and novel treatment approaches aimed at alleviating GABAergic deficits at the AIS and other key nodes of the cortical inhibitory system (Geffen et al., 2012; Lett et al., 2013; Lewis et al., 2008; Radhu et al., 2012; Rowland et al., 2013; Rudolph and Mohler, 2014; Stan and Lewis, 2012). For example to test the concept that reduced GABA signalling from chandelier cells to pyramidal neurons contribute to working memory dysfunction via parvalbumin-positive GABA neurons, a benzodiazepine-like compound, MK-0777, a selective agonist of GABA_A α -2 and α -3 subunits, was tested in clinical trials. It was associated with improved performance on cognitive tasks (N-back, AX Continuous Performance Test, and Preparing to Overcome Prepotency tasks) (Lewis et al., 2008). Similarly, a GABA agonist and dopamine antagonist (BL-1020) showed greater improvements on cognitive tasks than risperidone and better than placebo effects on positive and negative symptoms assessed via PANSS and CGI (Geffen et al., 2012). While it is fair to state that none of these studies so far have resulted in a therapeutic breakthrough, they reflect a remarkable advancement for the field. We view them as evidence-based clinical trials in pursuit of drug treatment targets beyond the dopamine receptors that had been the focus of this field for more than half a century (Kapur and Mamo, 2003).

Altered Expression of GABA Synthesis Enzyme GAD1 is one of the most frequently reported molecular alterations in SCZ brain

The function of multiple subtypes of GABAergic neurons, including the aforementioned Chandelier cells, is thought to be compromised, at least in part, due to altered expression of *GAD1* GABA synthesis enzyme (Lewis et al., 2005). *GAD1* (GAD67) accounts for 80–90% of overall brain GABA, while 10–20% reflects the activity of a related gene, *GAD2* (GAD65) (Asada et al., 1997; Condie et al., 1997). To date, there are at least 20 reports in the literature, conducted by multiple groups of investigators on postmortem tissues collected in the U.S., Europe and Australia, reporting downregulated RNA and protein expression specifically of *GAD1* in multiple brain regions of SCZ subjects, including prefrontal, medial temporal and occipital cortex and cerebellar cortex and basal ganglia (Akbarian et al., 1995; Benes et al., 2007; Bullock et al., 2008; Curley et al., 2011; Fatemi et al., 2005; Gilabert-Juan et al., 2012; Guidotti et al., 2000; Hashimoto et al., 2008a; Hashimoto et al., 2008b; Hashimoto et al., 2003; Huang et al., 2007; Impagnatiello et al., 1998; Mirnics et al., 2000; Sheng et al., 2012; Thompson Ray et al., 2011; Torrey et al., 2005; Veldic et al., 2005; Veldic et al., 2007; Volk et al., 2000; Volk et al., 2012). Furthermore, in a postmortem cohort comprised of elderly subjects, increased *GAD1* expression in SCZ brain has been reported (Dracheva et al., 2004). These studies—each conducted with a sample size typically in the range of 20–120 brains—when taken together, leave little doubt that dysregulated *GAD1* expression, primarily manifesting as a decrease in RNA levels, is a type of molecular alteration that is representative for a significant portion of SCZ brains. It has been suggested that approximately one out of three disease cases are affected by a more robust, perhaps more than > 30% downregulation in expression when compared to unaffected controls (Curley et al., 2011; Volk et al., 2012). Interestingly, decreased levels of *GAD2*, a paralogue of *GAD1* resulting from an ancestral gene duplication (Bu and Tobin, 1994), have been reported somewhat less frequently in the SCZ postmortem literature, and

could affect the hippocampus of the same cases on the mood or psychosis spectrum (Heckers et al., 2002; Todtenkopf and Benes, 1998).

Given that SCZ is a disorder of complex etiology and substantial subject-to-subject heterogeneities as it pertains to individual genetic risk architectures, environmental exposures and psychiatric manifestations, any type of molecular and cellular abnormality affecting a substantial subset of cases, such as decreased *GAD1* expression, is likely to represent adaptive pathophysiology resulting from a fairly broad range of potential factors operating further upstream. Indeed, as outlined in a previous review (Akbarian and Huang, 2006) and in the next chapters, this hypothesis receives strong support from preclinical studies, with a steadily increasing list of animal models and cell culture systems providing insights into the molecular and cellular systems governing neuronal *Gad1* expression.

Activity-dependent regulation of GABAergic gene expression in rodent and primate brain

Given that various portions of cerebral cortex show functional hypoactivity during various neuropsychological task performance in many cases with SCZ (Snitz et al., 2005; Streit et al., 2001), it is possible that some of the molecular and cellular changes observed in SCZ brain could reflect secondary changes in response to alterations in neuronal activity. Indeed, a broad range of studies, from *in vivo* work with non-human primates to *ex vivo* studies in the cell culture dish, are in support of this hypothesis. For example, 48 hours after monkey visual cortex had been deprived from sensory input in one eye, a marked reduction in GABA and GAD protein levels was found in the ocular dominance columns associated with a deprived eye (Hendry and Jones, 1986), followed by a robust downregulation of *Gad1* RNA levels after a period of 15 days (Benson et al., 1994). These and other changes in *Gad1* expression in the cerebral cortex of non-human primates are viewed as adaptive responses aimed at restoration of the excitation/inhibition (E/I) balance after afferent activity in the network has changed (Hendry and Jones, 1988; Jones et al., 1994). This hypothesis is also supported by some independent observations in rodents. For example, in rats, kainic acid-induced loss of hippocampal pyramidal neurons leads to a permanent downregulation of *Gad1* expression in the same brain region, which could contribute to the rewiring and hyperexcitability of the postlesion hippocampus (Shetty and Turner, 2001). In cultured hippocampal neurons, synaptic inactivity results in reduced GABA levels and *Gad1/Gad67* expression, and furthermore, *Gad1/Gad67* emerged as a critical regulator for cytosolic levels and vesicular filling of GABA, thereby affecting synaptic homeostasis (Lau and Murthy, 2012). Whether or not such types of activity-dependent mechanisms could have contributed to downregulated cortical *GAD1* expression in some of the SCZ cases remains unclear given that the clinical studies are confined to postmortem brain. However, this is a plausible hypothesis given that there is ample evidence for functional hypoactivity affecting multiple cortical areas in SCZ (Cieslik et al., 2013; Diwadkar et al., 2011; Eack et al., 2013; Fujiki et al., 2013; Lee et al., 2014; Ota et al., 2014; Thoresen et al., 2014; Wolwer et al., 2012; Yoon et al., 2013)(and earlier references cited therein).

Prefrontal GAD1 expression steadily increases during childhood and adolescence, and is sensitive to developmental perturbations

Maturation of the cerebral cortex, including its prefrontal areas, extends beyond the second decade of life (Kolb et al., 2012), and such type of prolonged developmental periods may play a key role in the neurobiology of SCZ as a psychiatric disorder with a typical onset of clinical symptoms around adolescence and young adulthood (Weinberger, 1987). Studies monitoring the expression and developmental trajectory of key molecules at inhibitory synapses in primate prefrontal cortex during the transitions from the early postnatal period to adulthood have emphasized the temporal association with a range of environmental risk factors for SCZ, including cannabis abuse (Hoftman and Lewis, 2011). This developmental window of vulnerability is likely to include much earlier time points too, because studies in rodents have linked early postnatal stressors (Zhang et al., 2010), and even prenatal exposure to toxins (Mackowiak et al., 2014) and maternal immune activation (Richetto et al., 2014) to deficits in *Gad1/Gad67* expression in adult cortex and hippocampus. Therefore, it is interesting to note that *GAD1* expression in human prefrontal cortex is subject to a developmentally regulated steady increase from the late prenatal period to early adolescence and adulthood (Huang et al., 2007), together with a progressive switch from an early *GAD1* transcript encoding a 25kDa protein lacking enzymatic activity to full length (67kDa) *GAD1* (Hyde et al., 2011). Such broad temporal window of vulnerability would make it appear plausible that developmental defects or environmental insults impacting the immature brain indeed play a role for the observed alterations of cortical *GAD1* expression later in life.

Developmental and disease-associated changes in epigenetic signatures at the GAD1 promoter

The regulatory networks governing the molecular architectures of cortical inhibitory circuitry are exceedingly complex and include a diverse array of transcriptional and post-transcriptional mechanisms. To mention just one recent example from the SCZ literature, prefrontal deficits in the expression of a subset of GABA neuron-specific mRNAs, including Neuropeptide (NPY), were found to be dependent on the regional supply of Brain-derived Neurotrophic Factor (BDNF), which in turn was subject to post-transcriptional control by a microRNA-dependent mechanism (Mellios et al., 2009).

In contrast, as will be further discussed below, defective *GAD1* expression in SCZ has been primarily linked to epigenetic abnormalities in chromatin surrounding the *GAD1* promoter and transcription start site (note that while the more traditional definition of *epi*-(greek for 'over', 'above') *genetics* is often equated with heritable changes in gene expression and function in the absence of DNA sequence alterations, the term is nowadays much more broadly applied to describe chromatin structure and function in the context of transcription, splicing, genome organization and maintenance and many other mechanisms). It should be mentioned that the process of gene expression and the gene (sequence)-specific occupancies of activated RNA polymerase II complex and subunits associated with transcriptional initiation or elongation cannot be measured easily in postmortem specimens. However, some

of the covalent modifications of the nucleosomal core histones that are differentially enriched at sites of actively expressed genes versus repressed and condensed chromatin are sufficiently stable after death. Thus, a number of postmortem brain studies have successfully employed chromatin immunoprecipitation assays, a standard approach to quantify levels of specific histone modifications, at specific genes and loci (Huang et al., 2006; Kurita et al., 2012; Matevossian and Akbarian, 2008; Stadler et al., 2005; Tang et al., 2011), and even embarked on genome-scale epigenome mappings (Cheung et al., 2010; Zhu et al., 2013).

These techniques have now also been applied to *GAD1* promoter sequences which are located within a few hundred base pairs from the transcription start site (Chen et al., 2011). Alterations observed include a shift from facilitative towards repressive chromatin-associated histone modifications, and changes in DNA methylation signatures, often in conjunction with altered *GAD1* expression in the prefrontal cortex (PFC) of the affected SCZ cases (Grayson and Guidotti, 2012; Huang and Akbarian, 2007; Huang et al., 2007; Tang et al., 2011). It is also noteworthy that common polymorphisms in the proximal *GAD1* promoter confer genetic risk for SCZ, impaired working memory performance and accelerated loss of gray matter (Addington et al., 2005; Straub et al., 2007), possibly in conjunction with altered expression of the cation chloride co-transporters NKCC1/KCC2, two key regulators of postsynaptic GABA_A receptor-mediated currents (Hyde et al., 2011). These findings, taken together, would suggest that the genetic and epigenetic architecture of the *GAD1* promoter is a potential factor for the gene's dysregulated expression in at least some cases with SCZ. Furthermore, at least one of the histone modifications found at reduced levels at *GAD1* regulatory sequences in SCZ, histone H3 trimethylated at lysine 4 (H3K4me3), shows a progressive upregulation at human and mouse *GAD1/Gad1* during the extended period of postnatal cortical development until reaching mature levels, in parallel to the corresponding increase of the RNA (Huang et al., 2007). Strikingly, the same epigenetic mark was significantly upregulated in *GAD1* chromatin from PFC of SCZ subjects treated with the atypical antipsychotic clozapine before death, and in cerebral cortex of mice subjected to a subchronic (21 days) regimen of daily clozapine injections (Huang et al., 2007). Therefore, the *GAD1*-bound H3K4me3 could be viewed as a molecular link that interconnects three important factors in the neurobiology of SCZ – GABA neuron dysfunction, developmental mechanisms and the molecular response after exposure to one of the most effective antipsychotic drugs currently available.

Spatial architecture of the *GAD1* locus in normal and diseased PFC

The preceding paragraph summarized evidence for localized epigenetic dysregulation of sequences surrounding the *GAD1* transcription start site. However, the regulation of gene expression in a vertebrate cell goes far beyond the genetic and epigenetic architectures of proximal promoters and transcription start sites. Instead, chromosomes and gene expression units inside the cell nucleus are organized as highly complex dynamic 3-dimensional structures that includes chromosomal loopings and physically interactions of enhancer and promoter elements with transcription start sites (Deng and Blobel, 2010; Ribeiro de Almeida et al., 2011; Singh et al., 2012). To date, however, the regulation of supranucleosomal higher order chromatin, beyond the level of DNA methylation and posttranslational modifications of the core histones, largely remains unexplored in normal or diseased human brain.

However, this may soon change because it was recently reported that chromosome conformation capture, commonly referred to as 3C (Dekker et al., 2013; van Berkum and Dekker, 2009) and widely viewed as the standard approach to map chromosomal loopings, is applicable to brain tissue collected postmortem (Mitchell et al., 2014). The 3C technique explores physical interactions between DNA fragments separated by Kb or Mb of interspersed sequence; crosslinked chromatin is digested with a specific restriction enzyme, religated and amplified using primer pairs for which forward and reverse primers match to different portions of the genomic locus-of-interest (Mitchell et al., 2014). Chromosomal conformations have been explored at the *GAD1* locus (Bharadwaj et al., 2013). One of these loop formations, initially detected in PFC and then confirmed in neuronal cultures derived from pluripotent skin cells, interconnected promoter-proximal sequences, positioned within 1–2kb from the *GAD1* transcription start site, with non-coding sequences positioned 50 kb further upstream. These loopings showed a significant weakening in PFC of SCZ subjects affected by decreased *GAD1* expression (Bharadwaj et al., 2013). Remarkably, DNA sequences at the *GAD1* TSS and 50kb upstream show conservation from rodents to primates, and the *GAD1* TSS – 50kb chromatin loop is strikingly similar in both mouse and human brain (Bharadwaj et al., 2013). Furthermore, reported that 3C studies in reporter mice expressing green fluorescent protein in GABAergic neuronal nuclei showed that the *Gad1* TSS-50kb loop was much stronger in cortical GABAergic interneurons—which express *Gad1*—compared to other cortical cells that do not express *Gad1*. This study (Bharadwaj et al., 2013) therefore could be viewed as ‘proof of principle’, showing that higher order chromatin is specific for cell-type and potentially altered in SCZ, and amenable for follow-up work in animal models and human cell culture systems.

Synopsis and implications for future treatments of SCZ

If dysregulated *GAD1* expression in PFC and other brain regions is indeed critical in the pathophysiology of at least some cases with SCZ, then one could speculate whether these mechanisms would offer novel therapeutic avenues for a subset of patients carrying this diagnosis. Of note, antipsychotic medications targeting dopaminergic, serotonergic and monoaminergic receptor systems are still the mainstay in SCZ treatment (Kim and Stahl, 2010; Taly, 2013) and broadly applied to a large majority of patients. However, the majority of subjects with SCZ experience an incomplete response to currently prescribed antipsychotic drugs, resulting in significant disability and reduced quality of life (Lieberman et al., 2005; Swartz et al., 2007). Rationale for the development of *GAD1* and other GABAergic gene-targeted therapeutic interventions could be built on the following five lines of evidence and starting points: First, prefrontal *GAD1* expression is dysregulated in a significant subset, or approximately 30%, of subjects with SCZ (Volk et al., 2012). Second, common polymorphisms in 5′ regulatory sequences of *GAD1* that affects its expression also confer a statistical risk for childhood-onset SCZ and cortical volume loss (Addington et al., 2005), and are in epistasis, or co-regulated, with other SCZ-relevant genes (Straub et al., 2007; Tao et al., 2012). Third, conditional deletion of one of the two *Gad1* alleles in parvalbumin-positive interneurons in the juvenile cortex elicited transient synaptic deficits and increased pyramidal cell excitability (Lazarus et al., 2013). Furthermore, *Gad1/Gad67* deficiencies in select interneuron populations of the adolescent cortex result in cell-

autonomous defects in connectivity (Chattopadhyaya et al., 2007). Decreased expression of GAD1/GAD67 in conjunction with other SCZ-related deficits and behavioral alterations has also been reported in conditional mutant mice with interneuron-specific ablation of NMDA glutamate receptor subunits (Belforte et al., 2010). These elegant mouse models further emphasize the potential importance of even moderately decreased *GAD1* expression in disease states, and warrants additional examination of the homeostatic and compensatory mechanisms that normalized synaptic inhibition in the adult mouse mutant cortex. Fourth, cortical *Gad1* expression and levels of transcription-associated histone methylation at the *Gad1* promoter showed in some rodent studies a subtle but significant up-regulation after treatment with commonly prescribed antipsychotic drugs (Huang et al., 2007; Lipska et al., 2003). Fifth, bulk GABA tissue levels in the cerebral cortex show robust associations with gamma band oscillations and working memory performance, even under conditions of system and cognitive impairments as seen in SCZ (Chen et al., 2014; Yoon et al., 2010). While cell-type specific prefrontal deficits in GAD1 expression probably are not well represented in measurements of bulk tissue GABA levels, the five independent lines of preclinical and clinical evidence, as summarized above, taken together, provide very reasonable rationale to pursue targeted therapeutic interventions aimed at cortical GAD1 expression in SCZ.

Looking forward: Targeted Epigenetic Interventions at the GAD1 locus for the treatment of SCZ

Genetic engineering-induced GAD1/GAD67 deficiency in cortical interneurons is detrimental for neuronal signaling and cognition and social interactions (Belforte et al., 2010; Chattopadhyaya et al., 2007; Lazarus et al., 2013; Schmidt et al., 2014). Whether or not an experimentally induced increase in *Gad1*/*Gad67* would elicit therapeutic effects in preclinical SCZ models remains to be determined. Among the various options available in the present-day molecular toolbox for altering the expression of a gene-of-interest, we would favor *de novo* engineered designer transcription factors targeted against specific sequences in the gene-proximal promoter near the TSS, or at distal cis-regulatory elements, including long-range enhancers (Figure 1A). Such type of chromatin-based therapy would bear important advantages over transgene-derived overexpression that relies on some artificial or endogenous promoter that is constitutively active. Instead, promoter- or enhancer-based interventions would modulate, or boost physiologically driven *Gad1* expression. For example, a good candidate would be targeting the sequences that engage in a chromosomal loop formation interconnecting the gene-proximal GAD1 promoter with regulatory elements positioned 50kb further upstream to edit looping interactions (Bharadwaj et al., 2013).

A number of different genome editing approaches, including zinc finger nucleases, transcription activator-like effector nucleases (TALEN), clustered regularly interspaced short palindromic repeat (CRISPR)/CAS9 RNA-guided nucleases—all of which were recently introduced to the field (Gaj et al., 2013; Li et al., 2014)—allow for design of sequence-specific DNA binding factors, which when fused to well described transcriptional activation domains such as the VP64 protein, could function as sequence-specific transcription factors (Figure 1C and 1D). Each method targets the sequence differently. Zinc

finger proteins are generated in 3 bp selectivity modules. Each module can then be linked together to target a specific region of interest. TALEs are composed of 33–35 amino acid repeat domains that recognize a single base pair with specificity determined by two hypervariable amino acids. Distinct from zinc finger proteins and TALEs CRISPR/Cas operates via RNA-guides. The CRISPR/Cas system can be guided to specific regions via short guide RNA (sgRNA). These transcription factors and editors can be combined with different effector domains (nucleases, transcriptional activators and repressors, recombinases, transposases, DNA and histone methyltransferases, and histone acetyltransferases) to strengthen the *Gad1*-TSS/50-kb loop and upregulate *Gad1* gene expression in cell culture, mouse GABAergic PFC neurons, and possibly eventually in humans (Cho et al., 2013; Cong et al., 2013). A TALE-based novel engineered transcription factor already has contributed to better understanding of the regulatory mechanisms governing *Gad1*/*Gad67* expression in culture mouse neurons. In cell culture, a TALE-based transcription factor positioned at gene-proximal *Gad1* promoter sequences was used with good success to upregulate *Gad1* gene expression in cultured neurons (Konermann et al., 2013). It is likely that these approaches will become the essential parts in the molecular toolbox to up-regulate cortical GAD1 expression in the animal model, or at some point in the future, also in human subjects.

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Figure 1. GAD1/Gad67 TALE and CRISPR Engineering

(A) A chromatin loop exists between the *Gad1*-TSS and a region upstream by 50kb marked by H3K4me3 sites in mouse cortex. It is marked by H3K4me3 sites in cortex and AP1 (c-Jun) sites in CH12 cell lines (ENCODE data) (Bernstein et al., 2012). (B) This 50-kb loop also exists in humans and is characterized by RNA polymerase II binding and H3K4me3 sites. The loop is weakened in the prefrontal cortex (PFC) of subjects with schizophrenia along with decreased *GAD1* gene expression, loss of the H3K4me3 mark, and probably also RNA polymerase II binding. (C and D) Designer transcription factors can be targeted to the human or mouse *GAD1*(*Gad1*)-TSS or to the upstream 50-kb loop to effectively increase gene expression. (C) We propose to test these mechanisms in mice *in vivo*, using (C) a TALE or (D) a guide RNA ('sg-RNA 1') for the CRISPR-Cas9 protein, each fused to the VP64 enhancer to increase gene expression and EGFP as a fluorescent mark. 'NG' (pink) box in (C) marks the hypervariable region of the TALE repeat region.