Mapping the Genomic Pathways That Dysregulate Brain Inhibition in Disease

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Introduction: The Role of GABA in the CNS

GABA is the major inhibitory neurotransmitter in the CNS. It activates three different classes of receptors: the ionotropic type A receptor ($GABA_AR$) and type C receptor ($GABA_C$) and the G protein-coupled type B receptor ($GABA_B$). In the retina, the $GABA_C$ receptor regulates fast synaptic inhibition, while in the brain, this function is specific to the $GABA_AR$ (MacDonald and Olsen, 1994; Bormann and Feigenspan, 1995; Rabow et al., 1995; Sieghart and Sperk, 2002). $GABA_B$ receptors are involved in slower, more prolonged inhibitory signaling (Jacob, et al., 2008).

$GABA_A$ receptors

Similar to other members of the ligand-gated ionotropic receptor family, such as the nicotinic acetylcholine receptor, the $GABA_AR$ is defined by the assembly of five subunits, as well as the presence of GABA and benzodiazepine (BZ) binding sites (Choi et al., 1981; MacDonald and Olsen, 1994; Chebib and Johnston, 1999). $GABA_ARs$ mediate fast synaptic inhibition by regulating the flow of Cl^- ions down their concentration gradient into the cell to hyperpolarize the postsynaptic neuronal membrane, hindering the spread of excitability (Costa, 1998). While GABA is an inhibitory neurotransmitter in the adult brain, in embryonic and early postnatal mammalian hippocampal neurons, synaptically released or exogenously applied GABA depolarizes and excites postsynaptic membranes via $GABA_AR$ activation (Cherubini et al., 1991). This excitatory response has been attributed to the presence of an embryonic chloride transporter (NKCC1 [sodium–potassium–chloride cotransporter 1]) that increases intracellular chloride concentration opposed to KCC2, which is expressed in adult neurons and extrudes Cl^- (Ben-Ari, 2002).

Role in normal development and disease

Dynamic changes in NKCC1 expression during brain development have recently been associated with the critical migration of neuroblasts to their targets (Mejia-Gervacio et al., 2011), while misexpressed NKCC1 has been implicated in multiple disorders, including epilepsy (Palma et al., 2006). Moreover, a role for the excitatory function of GABA has been proposed for the development of synaptic connections, as well as the subsequent plasticity and establishment of key neuronal networks dysregulated in developmental disorders such as autism (Kriegstein and Owens, 2001).

GABA plays a highly significant role in both the developing nervous system, the adult brain, and the compromised brain (as reflected in multiple disease states). As a result, our laboratory has had a longstanding interest in identifying specific protein–DNA interactions that regulate the transcription of unique $GABA_AR$ subunit genes (*GABRs*) and critical $GABA_AR$-associated proteins. Our investigations are meant to shed light on the genome response that shapes both present and future affective and cognitive behavior.

The $GABA_AR$ Genome and Its Gene Products

$GABA_AR$ subunits

In mammals, $GABA_AR$ subunits are divided into seven subunit classes based on sequence homology; including α_1-6, β1-3, γ1-3, δ, ε, θ, and π (Rabow et al., 1995). Although the majority of genes coding for multi-subunit receptor families lie scattered in the genome, evolution has preserved the organization of the $GABA_AR$ subunit genes, challenging us to understand the forces behind cluster preservation and expansion. Conservation of gene order and orientation on human chromosomes is shown in Figures 1 and 2. Taken together with the conservation of intron position in the β genes (Russek and Farb, 1994), it demonstrates that the diversity of $GABA_A$ receptor subunit genes originated from the duplication of an ancestral gene and the subsequent translocation of an ancestral gene cluster (Russek, 1999). Head-to-head orientation of the α and β subunit genes also suggests that they may be positively or negatively regulated by the proximity of regulatory elements.

Additional $GABA_AR$ subunit variants are observed from alternative splicing of individual subunit transcripts (Barnard et al., 1998; Jacob et al., 2008). Subunits within groups share 60–80% homology, whereas between different subunit families, homology is only 30% (Costa, 1998). $GABA_ARs$ are assembled from subunits in the endoplasmic reticulum (ER) (Jacob et al., 2008). Their departure from the ER depends on proteins reaching conformation maturity, contributing to a diverse population of $GABA_ARs$ at the cell surface. While many subunit conformations are possible, only a limited number of $GABA_ARs$ actually exit the ER, as less than 25% of translated subunits assemble into $GABA_ARs$ (Gorrie et al., 1997). Misfolded or nonassembled subunits are degraded through the ubiquitin–proteasome pathway (Bedford et al., 2001; Jacob et al., 2008). Once assembled, $GABA_ARs$ are trafficked to the Golgi to be packaged into vesicles for transport to and insertion into cellular membranes (Jacob et al., 2008).

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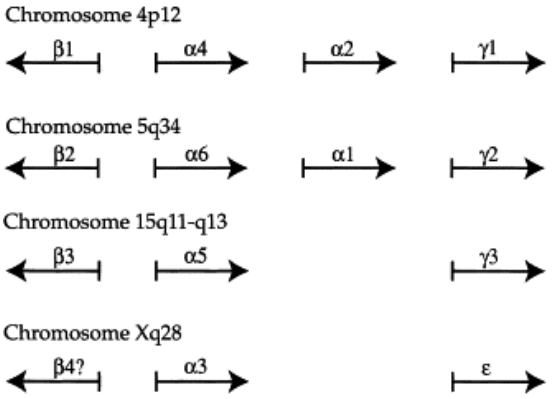

Figure 1. Gene organization is conserved for the $GABA_A$ receptor gene clusters on human chromosomes 4, 5, 15, and X. Orientation of subunit genes are indicated by arrow direction. (The schematic is not drawn to scale.) The most current cytogenetic localization of the gene cluster is indicated next to the chromosome number. Information for chromosomes 4 and 5 is presented in Russek, 1999. Information for chromosomes 15 and X is from Greger et al., 1995; Levin et al., 1996; and Wilke et al., 1997. Note that no β4 (avian) was ever reported in mammals and that sequencing of the genome revealed presence of θ in that position for rodents and humans.) Russek, 1999, Figure 5, reprinted with permission.

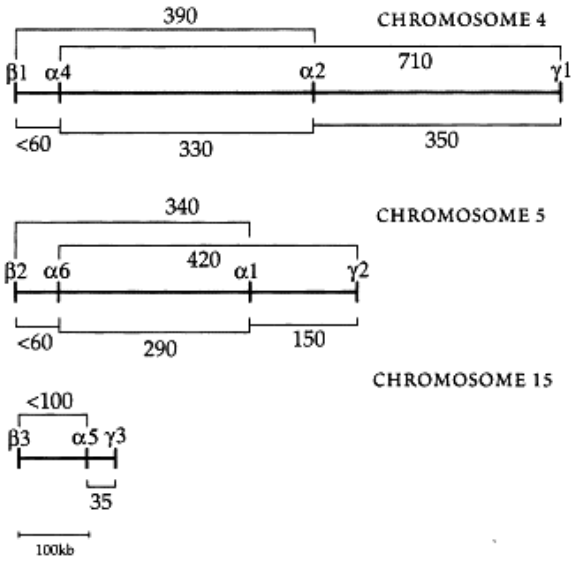

Figure 2. Schematic representation of the genomic organization of $GABA_A$ receptor gene clusters on chromosomes 4, 5, and 15 in the human genome. Estimates of genomic distance between genes on chromosomes 4 and 5 were obtained from interphase mapping. Distance measurements for the genes on chromosome 15 were obtained by restriction fragment-length fingerprinting and interphase fluorescence *in situ* hybridization (FISH) mapping (Greger et al., 1995) and by restriction fragment length analysis with field-inversion gel electrophoresis (Sinnet et al., 1993). Measurements are close to the distance verified by sequencing of the human genome. The diagram has been drawn to scale. Scale bar: 100 kb. Russek, 1999, Figure 3, reprinted with permission.

Pharmacological properties of $GABA_A$Rs

Different $GABA_A$R subtypes, a product of differential subunit composition, confer distinct receptor localization and function. Fully functional $GABA_A$Rs require at least one α, one β, and one other subunit type, allowing for GABA-gated Cl^- flux (Pritchett et al., 1989; Johnston, 1996; Chebib and Johnston, 1999). The most common receptor subtype contains 2α, 2β, and 1γ (or δ) subunit (MacDonald and Olsen, 1994; Jacob et al., 2008).

$GABA_A$Rs are the site of action for many therapeutics, including barbiturates, benzodiazepines (BZs), ethanol, and anesthetic steroids (Vicini, 1991; MacDonald and Olsen, 1994; Brooks-Kayal et al., 1998a). Research has demonstrated that different subunits confer distinct pharmacological properties to $GABA_A$Rs. For example, BZs act as allosteric modulators of $GABA_A$Rs, amplifying GABA signaling with varying levels of efficacy depending on the α and γ subunits present in the complex (Pritchett et al., 1989). Neurosteroids and barbiturates can also amplify GABA-gated current in most $GABA_A$R subtypes, by increasing chloride channel open time (Costa, 1998; Puia et al., 1990). In the adult brain, α_1 is the most abundant $GABA_A$R subunit and is found in 50% of $GABA_A$Rs (Duggan and Stephenson, 1990; McKernan et al., 1991). In general, receptors containing α_1 are mostly synaptic, sensitive to BZ, insensitive to zinc inhibition, and mediate most phasic inhibition in the brain (Pritchett et al., 1989; Puia et al., 1991; MacDonald and Kapur, 1999).

Levels of α_1 subunit expression can be altered by treatment with different mediators of synaptic signaling, suggesting that its expression may be activity-dependent. Treatment with NMDA stimulates α_1 expression in cultured cerebellar granule cells (Harris et al., 1994; Zhu et al., 1995). In contrast, prolonged treatment with GABA or BZ decreases α_1 expression in cortical and hippocampal neurons, respectively (Tietz et al., 1993; Lyons et al., 2000). Additional experiments using immunoprecipitation with subunit-specific antibodies followed by radiolabeled muscimol binding (a ligand that binds to the GABA binding site between α and β subunits) found that α_1 precipitated 70–90% of radiolabeled muscimol binding sites from rat or mouse brain membrane extracts (Sieghart and Sperk, 2002).

Alpha4 $GABA_A$ receptors

$GABA_A$Rs containing α_4 are less abundant, detected mainly in the hippocampus and thalamus (Rabow et al., 1995; Whiting et al., 1995; Benke et al., 1997; Sur et al., 1999). Furthermore, $GABA_A$Rs containing α_4 subunits are predominately extrasynaptic, insensitive

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to BZs, sensitive to zinc inhibition, and mediate tonic inhibition (Knoflach et al., 1996; Benke et al., 1997; Fisher and MacDonald, 1998; Lagrange et al., 2007). Immunoprecipitation experiments with α_4 subunit–specific antibodies detected α_4 in only 6% of $GABA_A$Rs in the brain (Sieghart and Sperk, 2002).

Altered expression of $GABA_A$Rs

Differences in the number and subunit composition of $GABA_A$Rs contribute to their unique function in discrete brain regions. Any alteration in such expression has been observed in multiple disease states, including alcoholism, Alzheimer's disease, autism, drug abuse, epilepsy, and schizophrenia. Changes in subunit expression are also observed in many of the comorbidities associated with epilepsy, such as anxiety disorders, cognitive deficits, and depression (Jacob et al., 2008).

With GABA and its type A receptors playing such critical roles in brain development and in brain inhibition more generally, they present a unique opportunity for the research community. The goal is to test the power of modern transcriptomics as a means of uncovering basic principles of brain design and function, which may be represented in the structure of the genome.

Gene Regulatory Networks That Control $GABA_A$R Subunit Genes

As discussed above, altered GABAergic function has been associated with multiple brain disorders. An additional feature of these disorders is a marked change in neurotrophic signaling, especially as orchestrated by brain-derived neurotrophic factor (BDNF). Work from our laboratory (in collaboration with Amy Brooks-Kayal and her group, who model temporal lobe epilepsy *in vivo*) has uncovered a unique relationship between these two receptor systems: $GABA_A$Rs and BDNF receptors (trkB and p75 neurotrophin receptor [p75NTR]). These findings suggest the two systems are part of an important gene regulatory network that is active in normal and diseased brain (Brooks-Kayal et al., 2009). Briefly, by activating the trkB receptor and downstream mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) intracellular cascades, BDNF increases levels of early growth response factor 3 (Egr3). Egr3, in turn, is an activator of the *GABRA4* promoter that drives the expression of $GABA_A$R α_4 subunits (Roberts et al., 2005, 2006).

In parallel, working via a novel pathway we have recently shown links p75NTR to the JAK/STAT cascade, BDNF increases levels of inducible cAMP

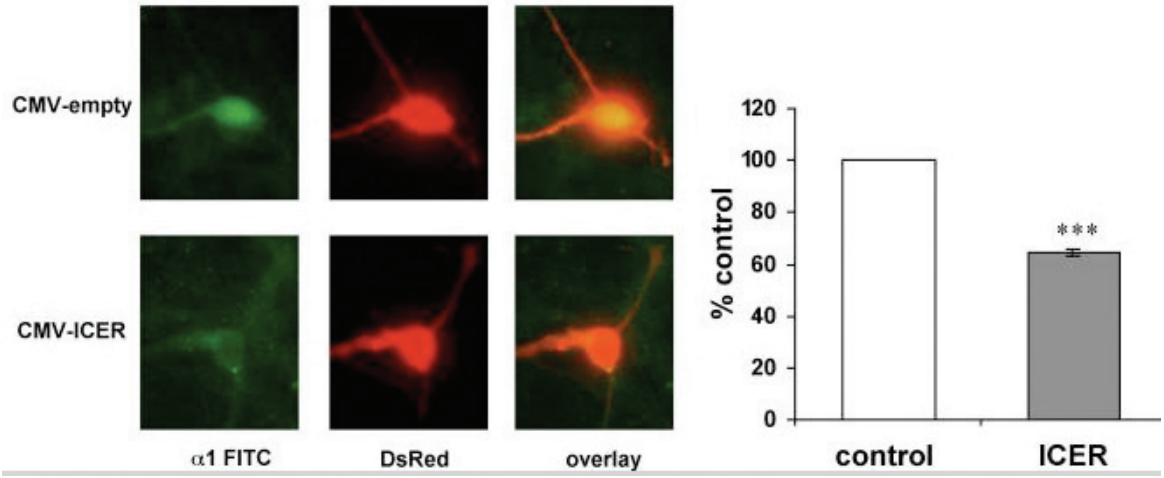

Figure 3. Effects of ICER induction on cell-surface α_1 expression. Overexpression of ICER decreases the endogenous levels of α_1 subunit detected at the cell membrane. Primary cultured neocortical neurons were cotransfected with pDsRed2-Monomer and ICER expression (*CMV-ICER*) or control vectors (*CMV-empty*). At 48 h after transfection, unpermeabilized cells were fixed and stained with an α_1-specific antibody using a standard protocol. The DsRed-transfected cells were viewed by using an Olympus IX71 inverted fluorescence microscope (Olympus America, Center Valley, PA), and the images were analyzed by using IPLab software (Becton Dickinson, Franklin Lakes, NJ). Representative images are shown (empty vector, top panel; ICER construct, bottom panel). Quantitation data are presented in the *histogram* (***, $p < 0.01$; mean ± S.E.; $n = 3$). FITC, fluorescein isothiocyanate. Hu et al., 2008, their Figure 9B, reprinted with permission.

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early repressor (ICER): a repressor of the core *GABRA1* promoter (Lund et al., 2008). Figure 3 depicts the overexpression of ICER in primary neurons, a process that alters the number of α_1 subunits at the cell surface (Hu et al., 2008). For the first time, the presence of ICER has been demonstrated to be directly relevant to the disappearance of the subunit from a functional compartment. Multiple intracellular signaling pathways regulate *GABRA1* transcription. Figure 4 depicts the process in which activation of PKC enhances transcription while activation of protein kinase A (PKA), like BDNF, represses transcription, dependent on the presence or absence of ICER.

Dynamics of $GABA_AR$ Transcription as Revealed by High-Density ChIP Sequencing

Increased access to new opportunities to probe genome activity at a global level holds great promise of open-ended discovery in GABA biology in the years to come. Recent evidence suggests that paracrine GABA, released from emerging neuroblasts, may participate in a negative feedback mechanism that causes cells to exit the cell cycle, producing fewer progenitors and supporting cellular differentiation (LoTurco et al., 1995, Kreigstein and Owens, 2001; Andäng et al., 2008) . The identification of early GABA as a switch that may control the potential pool of neural progenitors in the developing brain has shed light on the importance of studying this receptor system in the embryo, in addition to the adult brain, where most researchers have concentrated their efforts.

As a first step in this direction, we examined the chromatin state of *GABR* gene clusters in mouse embryonic stem cells (ESs) as compared with ES-derived neural progenitors (NPs), mouse embryonic fibroblasts (MEFs), and whole brain tissue (WB). We used the UCSC Genome Browser (http://genome.ucsc.edu) to analyze the results of chromatin immunoprecipitation (ChIP). ChIP was performed at the Broad Institute, using antibodies to three markers: H3K4me3 (a histone marker of transcriptional activation found at or close to active transcriptional start sites); H3K27me3 (a histone marker associated with genes that are silenced); and H3K36me3 (a histone marker usually found immediately after transcriptional start sites associated with active transcription) (Mikkelsen et al, 2007; Meissner et al., 2008). Genes displaying a sharp peak at both H3K4me3 and H3K27me3 contain a bivalent chromatin mark that has been associated with marks that play key roles in lineage-specific activation or repression.

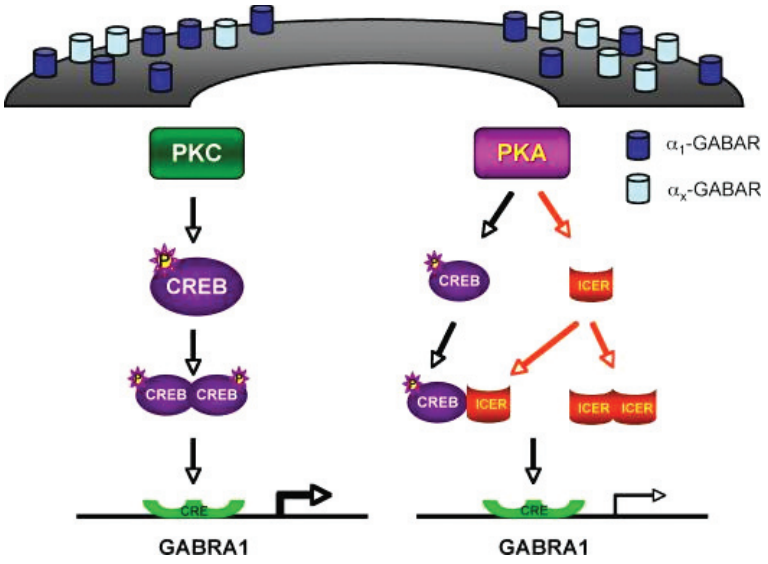

Figure 4. A model for the role of CREB and ICER in the regulation of *GABRA1* transcription. Activation of the PKC pathway leads to phosphorylation of CREB without induction of ICER. Phosphorylated CREB at Ser-133 forms homodimers to increase *GABRA1* expression (left). Activation of the PKA pathway induces synthesis of ICER and phosphorylation of CREB. Homodimers of ICER or ICER and CREB heterodimers repress transcription of *GABRA1* (right) and alter the number of α1-containing $GABA_AR$s (α1-GABAR) at the cell surface. None α_1 subunit–containing receptors are as indicated by "αx" key. CREB, cAMP response element-binding protein. Hu et al., 2008, their Figure 10, reprinted with permission.

Figures 5–7 display the results of ChIP sequencing through three *GABR* clusters in the mouse genome. Proposed *GABRs* with the highest probability of being expressed, either at the ES-cell level or upon commitment to NP or MEF, are indicated by red lettering. Analysis of histone marks in these different cell populations suggests that the early $GABA_AR$ is composed of *GABRA2*, *GABRB3*, and *GABRG1*. Results of RNA sequencing will confirm or refute this hypothesis and provide the necessary feedback to determine whether unique histone marks can predict the expression of $GABA_AR$ gene clusters in normal and diseased brains.

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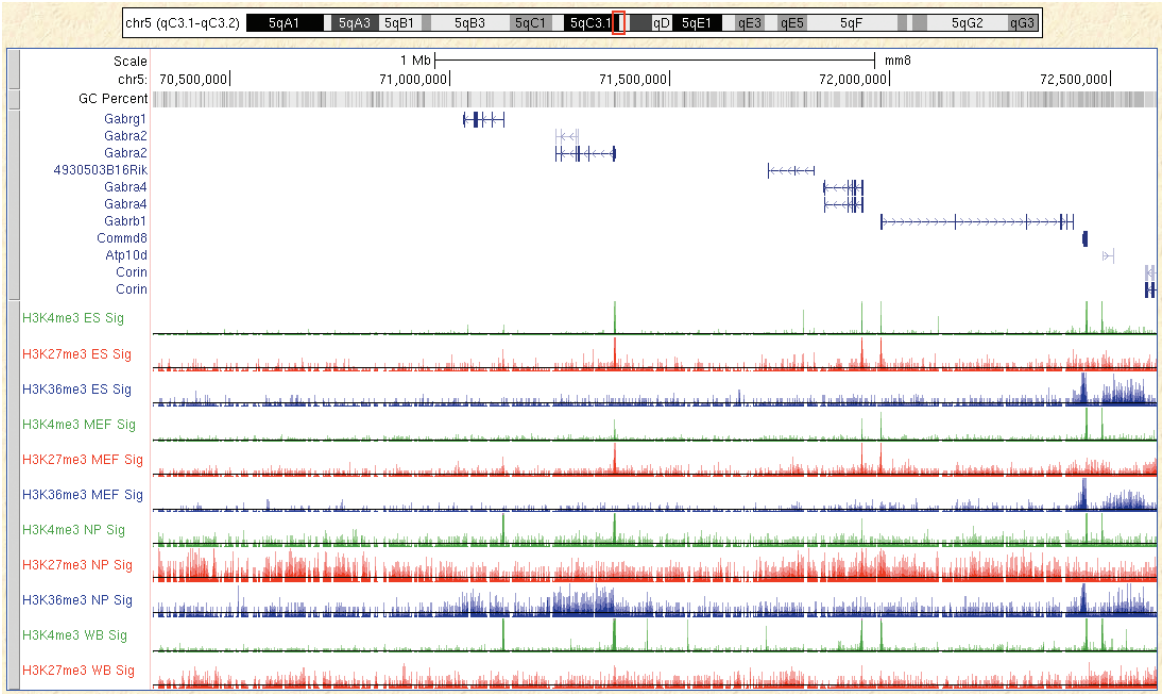

Figure 5. *GABRG1*, *GABRA2*, *GABRA4*, *GABRB1* gene cluster. Note that *GABRG1* is not univalent but is marked for transcription in NP, while *GABRA2* is univalent with high levels of expression in NP and H3K36me3 close to the start site.

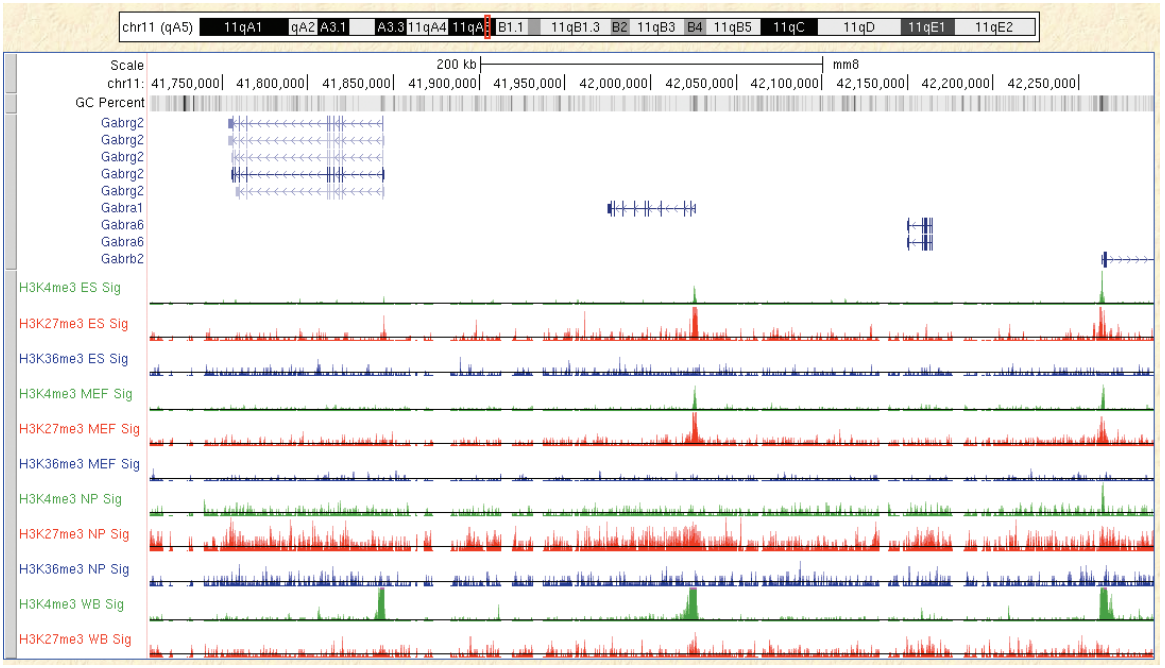

Figure 6. *GABRG2, GABRA1, GABRA6, GABRB2* gene cluster. Note that there are no peaks associated with *GABRG2* or *GABRA6*. However, there are univalent marks for ES and MEF with their loss in NP for *GABRA1*.

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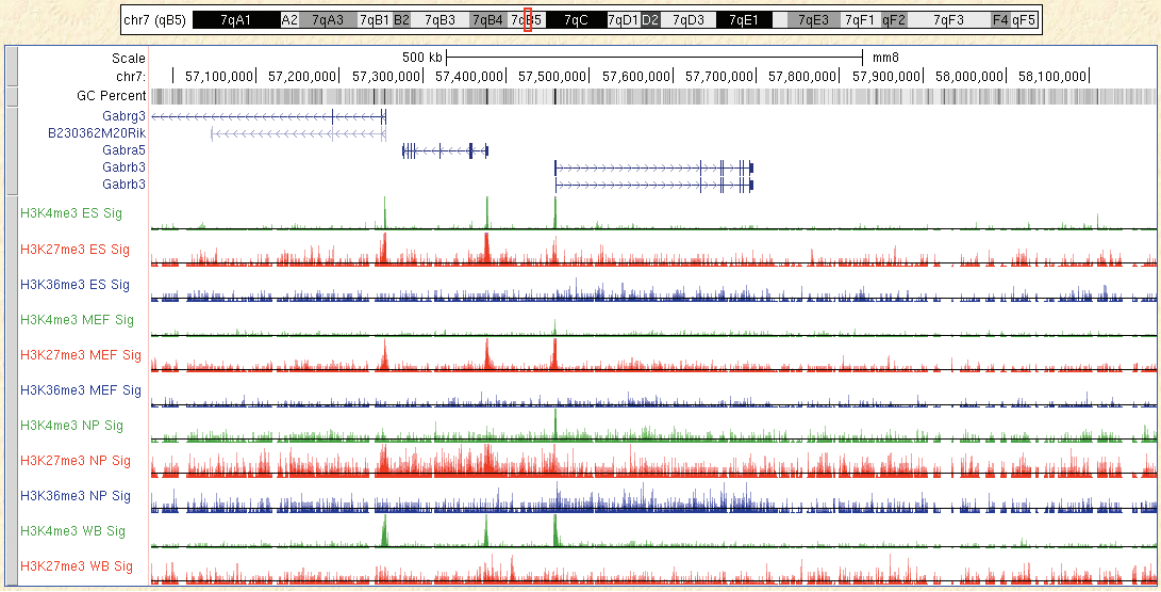

Figure 7. *GABRG3, GABRA5, GABRB3* gene cluster. Note that all genes in this cluster are univalent with evidence for some *GABRB3* in stem cells. There is a loss of a *GABRB3* univalent mark in MEF and strong H3K36me3 in NP, suggestive of a transcribed gene.

Breaking New Ground in a Familiar Landscape

Our lab and others across the country have identified a handful of gene regulatory proteins that are critical to the altered expression of certain *GABRs* in disease models. We have done so through a combination of traditional candidate gene regulatory assays and investigator-driven bioinformatic analysis. These discoveries have opened up new avenues for whole-genome investigations, using the power of ChIP-Seq and RNA-Seq analysis, to determine the transcriptome that is regulated in a coordinate or independent manner. Little is still known about how *GABRs* are coordinately regulated and why they have remained in clusters throughout our evolution. New techniques such as chromosome conformation capture—as used first to describe the beta-globin locus (Tolhuis et al, 2011)—may be powerful tools for exploring this new and complex territory.

Future discoveries in the field of GABA subunit gene regulation will take place in the background of an extensive history of GABA receptor biology that parallels the development of the larger field of neuroscience. Identifying gene duplications and inversions within *GABR* clusters that associate with human diseases will also provide a window onto the relationship between $GABA_AR$ number and kind that is key to maintaining a healthy balance of GABAergic neurotransmission in the young and old. These important questions have perplexed neuroscientists for over two decades; finally, the techniques are powerful enough to provide some answers.

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