Published in final edited form as:

Alcohol Clin Exp Res. 2015 September ; 39(9): 1654–1664. doi:10.1111/acer.12807.

GABRA2 alcohol dependence risk allele is associated with reduced expression of chromosome 4p12 GABA_A subunit genes in human neural cultures

Richard Lieberman^{1,2}, Henry R. Kranzler^{4,5}, Pujan Joshi⁶, Dong-Guk Shin^{3,6}, and Jonathan Covault^{1,3,*}

¹Alcohol Research Center, Department of Psychiatry, University of Connecticut School of Medicine, Farmington, CT 06030-1410

²Department of Neuroscience, University of Connecticut Health Center, University of Connecticut School of Medicine, Farmington, CT 06030

³Institute for Systems Genomics, University of Connecticut, Storrs, CT 06269

⁴Center for Studies of Addiction, Department of Psychiatry, Perelman School of Medicine of the University of Pennsylvania, Philadelphia, PA 19104

⁵V ISN4 MIRECC, Philadelphia VAMC, Philadelphia, PA 19104

⁶Department of Computer Science and Engineering, University of Connecticut, Storrs, CT 06269

Abstract

Background—Genetic variation in a region of chromosome 4p12 that includes the GABA_A-subunit gene *GABRA2* has been reproducibly associated with alcohol dependence (AD). However, the molecular mechanisms underlying the association are unknown. This study examined correlates of *in vitro* gene expression of the AD-associated *GABRA2* rs279858*C-allele in human neural cells using an induced pluripotent stem cell (iPSC) model system.

Methods—We examined mRNA expression of chromosome 4p12 GABA_A subunit genes (*GABRG1*, *GABRA2*, *GABRA4*, and *GABRB1*) in 36 human neural cell lines differentiated from iPSCs using quantitative PCR and Next Generation RNA Sequencing. mRNA expression in adult human brain was examined using the BrainCloud and Braineac datasets.

Results—We found significantly lower levels of *GABRA2* mRNA in neural cell cultures derived from rs279858*C-allele carriers. Levels of *GABRA2* RNA were correlated with those of the other three chromosome 4p12 GABA_A genes, but not other neural genes. Cluster analysis based on the relative RNA levels of the four chromosome 4p12 GABA_A genes identified two distinct clusters of cell lines, a low-expression cluster associated with rs279858*C-allele carriers and a high-

*Correspondence: Department of Psychiatry, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030-1410, Telephone: 860-679-7560, Fax: 860-679-1296, jocovault@uchc.edu.

Author Contribution

RL, HK and JC were responsible for the conceptualization and design of the study. RL was responsible for the acquisition of data. PJ and DGS were responsible for alignment and quantification of RNA sequencing data. RL was responsible for drafting the manuscript. JC and HK provided critical revision of the manuscript. All authors critically reviewed the content and approved the final version for publication.

expression cluster enriched for the rs279858*T/T genotype. In contrast, there was no association of genotype with chromosome 4p12 GABA_A gene expression in post-mortem adult cortex in either the BrainCloud or Braineac datasets.

Conclusions—AD-associated variation in *GABRA2* is associated with differential expression of the entire cluster of GABA_A subunit genes on chromosome 4p12 in human iPSC-derived neural cell cultures. The absence of a parallel effect in post-mortem human adult brain samples suggests that AD-associated genotype effects on GABA_A expression, although not present in mature cortex, could have effects on regulation of the chromosome 4p12 GABA_A cluster during neural development.

Keywords

Alcohol Dependence; GABA_A Receptor; *GABRA2*; Gene Expression; Induced Pluripotent Stem Cells

Introduction

GABA (γ -aminobutyric acid) is the major inhibitory neurotransmitter in the human brain (Olsen and Sieghart, 2009). The fast inhibitory effect of GABA in the mature nervous system is mediated through GABA type-A (GABA_A) receptors, which are heteropentameric ligand-gated ion channels permeable to chloride (Hevers and Luddens, 1998). GABA_A receptors are distributed throughout the brain and composed of a diverse set of 19 subunits (α 1–6, β 1–3, γ 1–3, δ , ϵ , θ , π , and ρ 1–3) that vary in expression across brain regions (for a review, see (Olsen and Sieghart, 2009). The various GABA_A receptor subtypes differ in subunit stoichiometry, a major determinant of their diverse pharmacological and electrophysiological properties (Barnard et al., 1998, Olsen and Sieghart, 2009). The majority of GABA_A receptors contain two α , two β , and 1 γ subunit, with the α 1 β 2 γ 2 and α 2 β 3 γ 2 combinations comprising 75–85% of GABA_A receptors in adult cortex (Rudolph et al., 2001).

In the human genome, the majority of GABA_A subunit genes are clustered on four chromosomes: 4p12 (β 1, α 4, α 2, γ 1), 5q34 (β 2, α 6, α 1, γ 2), 15q11 (β 3, α 5, γ 3) and Xq28 (θ , α 3, ϵ) (Steiger and Russek, 2004). Homologous clusters of genes are found on mouse chromosomes 5, 11, 7 and $\times$ and are thought to have derived from a single ancestral $\alpha\beta\gamma$ cluster by gene duplication (Russek, 1999). Positioning of GABA_A genes in tandem is believed to facilitate coordinated and tissue-specific co-regulation of gene expression by allowing clustered genes to share regulatory elements (Steiger and Russek, 2004, Barnard et al., 1998). Co-regulation of genes encoding the GABA_A receptor subunits is evidenced by variation in RNA levels in post-mortem human brain samples over a range of developmental ages. At earlier stages of development, chr4p12 GABA_A genes are expressed at higher levels than chromosome 5q34 genes. However, during development, chr4p12 genes are down regulated while chromosome 5q34 genes are up regulated (Fillman et al., 2010). Similarly, in rhesus monkeys, there is a developmental up regulation of the α 1 subunit and a down regulation of the α 2 subunit in the dorsolateral prefrontal cortex, which coincide with decreases in the decay time of miniature inhibitory post-synaptic potentials (Hashimoto et al., 2009).

Alcohol use disorders are prevalent, affecting more than 8% of the U.S. population (Grant et al., 2004). They have multiple and complex etiologies including a strong genetic component, with heritability estimates ranging from 50–60% (Gelernter and Kranzler, 2009). Among the most widely studied genetic associations with alcohol dependence (AD) are those involving single nucleotide polymorphisms (SNPs) contained in a ~140 kb haplotype block spanning the 3'-region of *GABRA2* and the adjacent intergenic *GABRA2-GABRG1* region on chr4p12 (Covault et al., 2008, Edenberg et al., 2004, Covault et al., 2004). The association of SNPs in this region with AD has been replicated in multiple studies and across different populations (Ittiwut et al., 2012, Enoch et al., 2009, Lappalainen et al., 2005, Bauer et al., 2007, Fehr et al., 2006, Soyka et al., 2008, Li et al., 2014), but lack of replication has also been reported (Matthews et al., 2007, Lydall et al., 2011). SNPs in this haplotype block have also been associated with drug dependence (Agrawal et al., 2006), suggesting that variability in replication may relate in part to differing co-morbidities among samples. The most commonly examined tag-SNP in this region, rs279858, a synonymous SNP in exon 5 of *GABRA2*, was the only candidate marker associated with AD in a genome-wide analysis of candidate genes (Olfson and Bierut, 2012). The molecular mechanism by which *GABRA2* polymorphisms influence risk for AD is unknown. The absence of a linked coding variant suggests that AD-associated variation in this region is in linkage disequilibrium with an as-yet-unidentified functional variant that influences the developmentally regulated expression of *GABRA2* or adjacent chr4p12 GABA_A subunit genes. This could result in subtle differences in neural connectivity and subsequent behavioral effects. Support for this hypothesis comes from reports that SNPs in *GABRA2* are associated with intermediate neural phenotypes, including fast beta frequency electroencephalographic (EEG) activity (Edenberg et al., 2004, Lydall et al., 2011), increased activation of the insular cortex during reward anticipation (Villafuerte et al., 2012), differences in activation of the medial frontal cortex and ventral tegmental area by alcohol cues (Kareken et al., 2010) and increased activation of the nucleus accumbens in reward anticipation paradigms (Heitzeg et al., 2014).

To explore molecular effects of AD-associated variants in the *GABRA2* haplotype block, we used an induced pluripotent stem cell (iPSC) model system (Takahashi and Yamanaka, 2006, Takahashi et al., 2007). Generation of iPSCs from individuals with known genotypes and subsequent differentiation into neural cells provides an opportunity to explore molecular phenotypes of AD-associated genetic variants *in vitro*. We examined neural cell cultures differentiated from 36 iPSC lines from 21 donor subjects with different *GABRA2* rs279858 genotypes and RNA expression of genes encoding GABA_A receptor subunits was examined. Our results indicate that chr4p12 AD risk alleles are associated with differences in RNA expression of all four GABA_A genes located in this region in this *in vitro* model system.

Materials and Methods

iPSC Generation

Fibroblast cell lines were generated from skin punch biopsies donated by participants enrolled in a study of the effects of alcohol in social drinkers (9 males) (Milivojevic et al., 2014) or in a clinical trial for heavy drinkers (10 males and 2 females) (Kranzler et al., 2014) at the University of Connecticut Health Center (UCHC). All fibroblast samples were from

Caucasian donors. Fibroblasts were reprogrammed to pluripotency by the UCHC Stem Cell Core using retrovirus constructs expressing five factors (*OCT4*, *SOX2*, *KLF4*, *c-MYC*, and *LIN28*) for 34 lines or sendai virus constructs expressing four factors (*OCT4*, *SOX2*, *KLF4*, *c-MYC*) for 2 lines. 2 to 4 weeks after viral transduction, individual iPSC colonies were identified by morphological characteristics and selected clones were expanded and cultured as separate cell lines in human embryonic stem cell media on a feeder layer of irradiated mouse embryonic fibroblasts.

Neural Differentiation

Neural cells generated from 36 iPSC lines were examined, including two independent lines from each of 15 donors and one line each from an additional 6 donors. iPSCs were differentiated into forebrain lineage neural cultures using an embryoid body-based protocol developed by the WiCell Institute (#SOP-CH-207 Rev A, www.wicell.org, Madison, WI). For details on the neural differentiation methods used see (Lieberman et al., 2012). Mature cultures (12 weeks post-neural plating) generated using this protocol contain a mixture of glial cells and neurons with spontaneous electrical activity, functional ionotropic GABA_A and glutamate receptors, and the ability to generate action potentials (Lieberman et al., 2012). A comparison of transcriptome profiles for human iPSC-derived forebrain lineage neural cultures and human post-mortem brain tissue suggests that 6–12 week iPSC neural cultures have a gene expression profile most similar to first trimester forebrain tissue (Brennand et al., 2014, Mariani et al., 2012).

Reverse Transcription- Quantitative Polymerase Chain Reaction (RT-qPCR)

Neural cultures 12–17 weeks post-plating were mechanically harvested and RNA extracted using TRIzol (Life Technologies, Carlsbad, CA). RNA concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Pittsburgh, PA) and cDNA was synthesized using a High Capacity cDNA Reverse Transcription kit (Life Technologies) using 2 µg of total RNA. Relative transcript levels represented in the resulting cDNA were measured by real-time qPCR using an Applied Biosystems 7500 instrument and FAM-labeled TaqMAN Assays (Life Technologies) for: *GABRA1* (Hs00971228_m1), *GABRA2* (Hs00168069_m1), *GABRA4* (Hs00608034_m1), *GABRB1* (Hs00181306_m1), *GABRG1* (Hs00381554_m1), *GABRG2* (Hs00381554_m1), *GABRD* (Hs00181309_m1), *RBFOX3* (Hs01370653_m1), *GAD2* (Hs00609534_m1), and *SLC17A7* (Hs00220404_m1). VIC-labeled *GUSB* (β-glucuronidase, 4326320E) was used as a within-well reference gene for normalization. Each sample was assayed in triplicate and a standard curve using cDNA generated from mature iPSC-derived neural cells from one subject was included on each qPCR assay plate to allow pooling of relative RNA expression levels among all samples. C_T values for target genes and the internal reference gene were converted to relative expression levels using the standard curve method, which compares each experimental sample with a calibrator reference RNA sample whose RNA level for each probe is defined as 1 (Wong and Medrano, 2005). PCR cycles were: 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds.

Allelic Expression

TaqMan (Life Technologies) FAM- and VIC-labeled genotyping probe and primer sets targeting exonic SNPs in *GABRG1* (rs976156, C__8723970_1_), *GABRA2* (rs279858, C__2073557_1_), *GABRA4* (rs7660336, C__11278069_10), *GABRB1* (rs10028945, C__2119811_30), and *AKR1C3* (rs12529, C__8723970_1_) were used to examine allelic expression bias of the chr4p12 GABA_A cluster. 20 μ L TaqMan reactions containing 2 μ L of cDNA from iPSC-derived neural cells or genomic DNA from human fibroblasts were carried out in triplicate using Universal Master Mix II (Life Technologies) and an Applied Biosystems 7500 instrument. C_T values were generated as the difference between FAM- and VIC-probes for the iPSC-derived neural cell cDNA samples or heterozygote fibroblast gDNA for each SNP. Consistent with other studies (Lin et al., 2012, Serre et al., 2008), we used a threshold for allelic imbalance as a 50% increase in RNA from one of the two alleles (i.e. $\pm 0.5 C_T$).

Post-mortem human brain datasets

To compare our results from *in vitro* neural cultures with adult human brain GABA_A gene expression *in vivo*, we examined data from two publically available post-mortem datasets: Brain Cloud and Braineac. For the Brain Cloud sample the microarray gene expression dataset (<http://braincloud.jhmi.edu>) (Colantuoni et al., 2011) was merged with SNP genotype data for each sample (dbGap dataset phs000417.v2.c1). We selected all adult Caucasian cases age 18–80 yo (mean=43.1) with an RNA integrity number ≥ 7.0 (mean RIN=8.25), resulting in a sample of n=60. The SNP dataset did not include rs279858, but rather a nearby marker, rs279856, tightly linked to rs279858 in Caucasians ($r^2=0.91$; http://www.broadinstitute.org/mammals/haploreg/haploreg_v3.php). The Braineac dataset [<http://braineac.org>; UK Brain Expression Consortium, (Trabzuni et al., 2011)] consists of microarray gene expression datasets for each of 134 Caucasian subjects together with whole genome SNP data. Donors ranged in age from 16–102 yo (mean=59.0) and RNA samples had an average RIN of 3.85 (range=1–8.5). The Braineac public dataset does not allow the selection of a subset of subjects based on age or RIN. Both rs279858 and rs279856 were reported in this dataset and were in complete LD ($r^2=1.0$).

RNA Sequencing

RNA from 4 wells per neural culture was pooled as input for Next Generation Sequencing (NGS). RINs ranged from 7–9.7, with an average of 8.63. Poly-A selected (n=5) or ribosomal-RNA depleted (n=11) RNA was used to generate randomly primed libraries (200–500 bp inserts). NGS was conducted at the Genomics Core of the Yale Stem Cell Center using Illumina TruSeq Chemistry for library preparation and the Illumina HiSeq 2000 platform to generate 100-bp reads (average 44 million reads per sample). Sequence reads were aligned using Bowtie and TopHat (Trapnell et al., 2009) and gene expression levels quantified using Cufflinks (Trapnell et al., 2010) using a computational pipeline in the Department of Computer Science and Engineering, UConn-Storrs. Sequence tag reads were aligned to reference human genome hg19 and results normalized for exon length and the total sequence read number for each sample to generate Reads Per Kilobase of exon per Million mapped reads (RPKM).

Statistical Analysis

Statistical analysis was conducted using SPSS software v22 (IBM, Armonk, NY). Linear mixed-effects models were used to compare gene expression as a function of genotype or cluster membership to account for biological replicates for a subset of lines (biological replicates were available for 15 lines for qPCR data and 6 lines for RNA sequence data). Analysis was repeated using a single value per donor subject by averaging biological replicates and using t-tests for comparison of group means. Statistically significant contrasts identified using mixed-effects models were also seen using t-tests. Pearson correlations and two-step cluster analysis were used to examine the correlation in expression of GABA_A genes and to group samples based on patterns of chromosome 4 GABA_A gene expression. One-way ANOVA was used to compare adult brain RNA expression as a function of genotype. P values of 0.05 were considered significant.

Results

GABRA2 RNA Expression Differs by rs279858 Genotype

Mixed-effects models examining RT-qPCR *GABRA2* RNA levels revealed a significant effect of *GABRA2* rs279858 genotype ($F=3.7$, $df=2,15.8$, $p=0.046$). Neural cell cultures derived from rs279858*C-allele donor subjects had lower levels of *GABRA2* RNA than T-allele homozygotes, Figure 1A (T/T vs. C/T $F=4.9$, $df=1,20$, $p=0.039$, T/T vs. C/C $F=6.1$, $df=1,12.1$, $p=0.03$, C/C vs. C/T $F=0.10$, $df=1,10.8$, $p=0.76$). In contrast, there was no significant effect of rs279858 genotype on RNA expression of *RBFOX3* (Figure 1B, $F=0.562$, $df=2,19.3$, $p=0.58$), which encodes the neuron-specific transcription factor NeuN, suggesting that the between-sample differences in *GABRA2* expression were not due to variability in the capacity of iPSC lines to differentiate into neurons.

RNA levels for Chromosome 4p12 GABA_A Genes are Correlated and Identify Two Clusters of iPSC Lines

RT-qPCR analysis revealed a significant correlation of RNA expression among the four consecutive chr4p12 GABA_A subunit genes (*GABRG1*, *GABRA2*, *GABRA4*, and *GABRB1*) in iPSC-derived neural cultures (Table 1). This suggests that AD-associated genetic variation in *GABRA2* may have regional effects on the expression of all four GABA_A subunit genes. There were no correlations between RNA levels for any of the chr4p12 GABA_A genes and *GABRD* (chr1p36, encoding the GABA_A δ subunit), or *RBFOX3* (chr17q25, encoding NeuN).

Cluster analysis identified two groups among the 36 neural cell lines: 17 in a low-expression Cluster 1 and 19 in a high-expression Cluster 2. There was a significant association between cluster membership and *GABRA2* rs279858 genotype ($\chi^2=9.9$, $df=2$, $p=0.007$): Cluster 1 cell lines had a greater frequency of C alleles than those in Cluster 2 (0.76 vs. 0.34; $\chi^2=12.9$, $df=1$, $p=0.003$) (Table 2). Scatter plots comparing RNA levels for *GABRA2* to those for *GABRG1*, *GABRA4*, or *GABRB1* RNA, Figure 2, illustrate group differences in chr4p12 GABA_A subunit gene expression levels between the two clusters (Figure 2 A–C). Mixed-effects models identified significant differences in RNA expression between Cluster 1 and Cluster 2 for each of the chr4p12 GABA_A genes (Figure 3 A–D) (*GABRG1* $F=44.6$,

df=1,34, $p<0.001$, *GABRA2* $F=44.5$, df=1,34, $p<0.001$, *GABRA4* $F=45.9$, df=1,34, $p<0.001$, *GABRB1* $F=82.7$, df=1,34, $p<0.001$). There was no between-cluster difference in RNA levels for *GABRD* (Figure 3E; $F=0.034$, df=1,30, $p=0.86$) or *RBFOX3* (Figure 3F; $F=0.41$, df=1,29, $p=0.53$).

RNA Sequencing

RNA-Seq data were available from other projects for 10 of the iPSC-derived neural cell lines. Sequence data for biological replicates from 6 of these lines were also available (i.e. a subset of iPSC lines were differentiated into neural cells on 2 distinct occasions). Including biological replicates, RNA-Seq data were available for 16 mature neural cultures derived from 10 iPSC lines.

RNA-Seq results confirmed the qPCR findings of lower expression of chr4p12 GABA_A genes in neural cell cultures from Cluster 1 vs. Cluster 2 (Figure 4B 2–5) (*GABRG1* $F=22.2$, df=1,7.6, $p=0.002$; *GABRA2* $F=7.7$, df=1,8.2, $p=0.024$; *GABRA4* $F=15.7$, df=1,7.8, $p=0.004$; *GABRB1* $F=6.0$, df=1,8.5, $p=0.039$). There was no significant difference ($p>0.50$) in the expression of genes encoding GABA_A subunits located on chromosomes 5q34 (*GABRG2*, *GABRB2*), 15q12 (*GABRB3*), or 1p36 (*GABRD*) (Figure 4C 1–4), or in the expression of the cation-chloride transporters NKCC1 (encoded by *SLC12A2*) and KCC2 (encoded by *SLC12A5*) (Figure 4C 5–6), which regulate the intracellular chloride concentration during development and determine whether GABA is an inhibitory or excitatory neurotransmitter (Stein et al., 2004). Similarly, with the exception of *GABRA2* and *GABRB1* genes, there were no significant differences between Cluster 1 and Cluster 2 neural cultures in the level of RNA for a panel of 74 neural patterning genes used by other investigators (Brennand et al., 2014) to compare developmental characteristics of iPSC-derived neural cultures (Supplemental Table 1). These results suggest that the two clusters had a similar degree of neuronal maturation and forebrain lineage features. Interestingly, there was no difference in expression of the two genes directly adjacent to the chr4p12 GABA_A cluster, *GNPDA2* and *COMMD8* (Figure 4B 1 and 6), located ~1.3 Mb and 25 kb from *GABRG1* and *GABRB1*, respectively (Figure 4A). Taken together, these findings strongly suggest that genetic variation in the *GABRA2* AD-associated haplotype block influences developmental regulation of the entire cluster of chr4p12 GABA_A subunit genes in this iPSC-derived neural culture model. Further, our findings were replicable across multiple neural differentiations of individual iPSC lines, as there were no differences observed in GABA_A expression patterns from the iPSC-derived neural cells differentiated on different dates.

Lack of Association of Genotype and GABA_A Expression in Adult Post-Mortem Brain

We examined the expression of chr4p12 GABA_A genes in a set of 60 adult post-mortem Caucasian brain samples using a whole genome microarray gene expression dataset [<http://braincloud.jhmi.edu>; (Colantuoni et al., 2011)], merged with SNP array data for each sample (dbGap dataset phs000417.v2.c1). Because rs279858 was not available in the SNP dataset for these samples, we examined 4p12 GABA_A gene expression as a function of the *GABRA2* intron 4 SNP, rs279856 located 3.3 kb from rs279858 and in near-complete linkage disequilibrium with it ($r^2=0.91$) in Caucasians. There was no significant relationship of rs279856 genotype with *GABRA2* RNA expression ($F=0.67$, df=2,57, $p=0.52$). Similar

results were obtained examining expression data for a collection of post-mortem adult brain samples from 134 subjects of European descent [<http://braineac.org/>; UK Brain Expression Consortium, (Trabzuni et al., 2011)]. There was no association of rs279858 genotype with *GABRA2* RNA expression in either frontal cortex ($F=0.43$, $df=2,124$, $p=0.65$) or hippocampus ($F=1.25$, $df=2,119$, $p=0.29$).

Complex Genetic Regulation of the Chromosome 4p12 GABA_A Cluster

When we examined the within-cluster expression of *GABRA2* as a function of rs279858 genotype in the set of 36 iPSC-derived neural cultures, we found that expression did not differ by genotype among cell lines within Cluster 1 or 2 (Figure 5). The lack of a gene-dose effect was not unexpected in Cluster 1, given the very low expression phenotype and small variability between lines (Figure 2). In contrast, we expected that an autosomal Mendelian cis- or trans-acting effect of a rs279858-linked functional variant on RNA levels would be observable in Cluster 2 cultures because *GABRA2* expression was readily detectable with significant between-sample variation in Cluster 2 lines.

A potential alternative genetic model more consistent with these results would include a cis-acting genetic locus influencing expression of the chr4p12 GABA_A gene cluster paired with a random, mitotically-stable, allelic expression bias. In this model, a stochastic clonal allelic expression bias for the chromosome carrying a cis-acting repressive functional variant linked with the rs279858*C-allele would determine Cluster 1 vs. 2 membership for a given iPSC line. Support for a model that includes allelic expression bias in this region was obtained using RT-qPCR and TaqMan allele-specific probes to examine neural cell RNA from 16 Cluster 2 cell lines heterozygous for exonic SNPs in at least one of the chr4p12 GABA_A genes (*GABRG1*, *GABRA2*, *GABRA4* and *GABRB1*). We observed allelic imbalance [a 50% or greater increase in RNA from one of the two alleles, i.e. $>\pm 0.5$ C_T (Serre et al., 2008, Jeffries et al., 2012)] in chr4p12 GABA_A RNA expression compared to fibroblast genomic DNA in 7 of 16 lines (44%). In contrast, 0 of 11 lines (0%) heterozygous for an exonic SNP in the *AKRIC3* gene (chr10p15) showed an imbalance in expression of the two alleles (Fisher's exact test, $p=0.02$). Of the lines that were heterozygous at two or more chr4p12 GABA_A genes, 6 of 8 lines displayed concordant allelic expression across markers (i.e., either a greater than ± 0.5 C_T difference for each gene marker or no bias for each gene). Both of the lines that were discordant over the interval displayed allelic bias in 3 of 4 GABA_A genes, but with the most distal gene (*GABRG1* in one line, *GABRB1* in the other line) showing less than a 0.5 C_T difference. In the case of donor 727 (rs279858*T/T genotype), neural cells differentiated from two independent iPSC clones (727-6 and 727-8) had allelic bias for opposite chromosomes over this region (Figure 6). This is consistent with a random, mitotically-stable clonal event occurring at the time of iPSC generation producing complementary allelic bias in the chr4p12 GABA_A genomic interval for the 2 independent iPSC lines from this subject.

Discussion

iPSC technologies provide novel model systems to examine the molecular and cellular effects of disease-associated genetic variation using disease-relevant cell types not otherwise

readily available. In the current study, we generated neural cultures from 36 iPSC lines derived from 21 donor subjects characterized by rs279858, a synonymous T-to-C polymorphism in exon 5 of *GABRA2*. Previous research has shown associations between the rs279858*C allele and increased risk for AD (Ittiwut et al., 2012, Enoch et al., 2009, Lappalainen et al., 2005, Bauer et al., 2007, Fehr et al., 2006, Soyka et al., 2008, Li et al., 2014), marijuana and illicit drug dependence (Agrawal et al., 2006), anxiety (Enoch et al., 2006), and differences in the risk of relapse or likelihood of drinking following treatment for substance abuse (Bauer et al., 2012, Bauer et al., 2007). Despite these multiple behavioral associations of *GABRA2* variation in humans, the cellular and molecular components underlying the associations are not understood.

We found that relative levels of RNA for the chr4p12 GABA_A subunit genes *GABRG1*, *GABRA2*, *GABRA4*, and *GABRB1* were significantly inter-correlated in neural cultures derived from iPSC lines. RNA levels for these GABA_A subunits identified distinct low-expression and high-expression clusters of cell lines. The low-expression cluster had a higher frequency (0.76 vs. 0.34) of AD-associated *GABRA2* rs279858*C alleles than the high-expression cluster. Examination of next-generation RNA-Seq data available for 10 neural cell lines allowed us to extend our analysis to include the expression of GABA_A genes located on multiple chromosomes. RNA-Seq data confirmed our qPCR results showing low expression of chr4p12 GABA_A genes in cell lines that comprise Cluster 1, and extended our findings by showing that GABA_A subunit genes located on other chromosomes showed no difference in expression as a function of expression cluster. Furthermore, transcripts of the *GNPDA2* (glucosamine-6-phosphate deaminase 2) and *COMMD8* (COMM domain-containing protein 8) genes that flank the chromosome 4 GABA_A cluster were readily detected in samples in which the four GABA_A genes were minimally expressed. Together, these RNA-Seq and qPCR results suggest that the effects of the AD-associated *GABRA2* polymorphism on gene regulation are specific for the ~1.4 Mb region containing the chr4p12 GABA_A gene cluster.

Our results suggest that the *GABRA2* haplotype block containing rs279858 harbors a functional polymorphism with regulatory effects that influence the expression of all four GABA_A-subunit genes on chr4p12 in parallel. These results are consistent with other work suggesting the presence of multi-gene locus control elements within the 4p12 and 5q34 GABA_A subunit gene clusters. In two independently derived mouse *GABRA6* knockout lines, in which neomycin insertion cassettes were used to target exon 8 of *GABRA6*, expression of the two adjacent genes, *GABRA1* and *GABRB2*, was reduced in the forebrain, suggesting the presence of a locus control element in the chromosome 5q34 GABA_A gene cluster (Uusi-Oukari et al., 2000). In addition, human and primate studies provide evidence of a coordinated developmental switch in the relative expression of GABA_A subunits (Fillman et al., 2010, Hashimoto et al., 2009).

The lack of association of rs279858 with the expression of chr4p12 GABA_A genes in adult post-mortem brain suggests that genetic variation in this region may increase risk for substance-related disease via developmental mechanisms. However, more research is needed to show a developmental influence of chr4p12 variation *in vivo*. Evidence of genetic variation influencing development but contributing to disease risk in adulthood has been

described for the serotonin transporter-linked polymorphic region, 5-HTTLPR of *SLC6A4*. Carriers of the 5-HTTLPR S-allele are more susceptible to anxiety and depression (Caspi et al., 2010), have reduced functional and white matter connectivity of the amygdala and anterior cingulate cortex (Pacheco et al., 2009, Pezawas et al., 2005) and show enhanced amygdala activation to fear stimuli (reviewed in (Munafò et al., 2008). In rodents, anxiety- and depression-like behavior in adult mice were only seen when the serotonin transporter was disrupted early in development (Ansorge et al., 2004), suggesting a developmental effect of *SLC6A4* genotype on susceptibility to anxiety, depression, and regional brain connectivity in adulthood.

GABA_A receptors play key roles in cortical development. Early in development, GABA_A receptors are excitatory due to a high intracellular chloride concentration and provide the main excitatory drive onto immature neural cells (Lee et al., 2005). This early excitatory function is important for normal corticogenesis, cell proliferation, and synaptic formation (Wang and Kriegstein, 2009). Because GABA_A subunit stoichiometry affects channel properties (Olsen and Sieghart, 2009), we speculate that the genotype-associated differences in GABA_A subunit expression may impact GABA signaling effects on cellular connectivity during development. Reported associations of *GABRA2* genetic variation with EEG beta frequency oscillations (Edenberg et al., 2004, Lydall et al., 2011), increased activation of the insula (Villafuerte et al., 2012) and nucleus accumbens (Heitzeg et al., 2014) during reward anticipation, and differences in the activation of reward pathways by alcohol cues (Kareken et al., 2010) are consistent with a developmental model of the effects of the AD-associated *GABRA2* genetic variant rather than an alteration in ligand binding.

Analysis of RNA expression by RT-qPCR produced two unexpected observations. First, although the rs279858*C-allele was enriched in low expression Cluster 1 cell lines (Table 1), the within-cluster expression of chr4p12 GABA_A genes did not differ by *GABRA2* genotype (Figure 5). Therefore, the genotypic association with expression level illustrated in Figure 1A reflects the greater frequency of rs279858*C-allele carriers in Cluster 1 vs. Cluster 2 (0.94 vs. 0.47), suggesting that the genetic effect linked to rs279858 strongly determines Cluster membership. The absence of an rs279858 genotype effect within Cluster 2 cell lines suggests that a simple Mendelian genetic model is not sufficient. One model consistent with our results includes a cis-acting genetic locus (in linkage disequilibrium with rs279858) that influences the expression of the chr4p12 GABA_A gene cluster together with a recently discovered phenomenon of random clonal allelic-biased expression of a subset of autosomal non-imprinted genes (Chess, 2012). Specifically, we hypothesize that a random stochastic process occurring during the reprogramming of individual fibroblasts to generate iPSC clones results in random allelic bias for the expression of chr4p12 GABA_A genes. In this model, iPSC allelic expression bias for the chromosome carrying a cis-acting repressive functional variant linked with the rs279858*C-allele would determine Cluster membership for a given iPSC line.

Autosomal random stochastic inactivation was initially described for a group of immune and nervous system gene families (see Chess, 2012 for review) including immunoglobulins, interleukin receptors, odorant receptors, and neural protocadherin genes. More recent genomic studies have revealed that random mono-allelic expression is widespread and

involves a much larger number of autosomal genes (5–20% in mammalian cells) (Gimelbrant et al., 2007, Serre et al., 2008). Mitotically stable, random mono-allelic expression has been demonstrated in clonal lymphoblastoid cell lines (Gimelbrant et al., 2007, Serre et al., 2008), in clonal groups of cells in placental tissue (Gimelbrant et al., 2007), in iPSCs and iPSC-derived neural cultures (Lin et al., 2012), and in human clonal neural stem cells derived from fetal brain and spinal cord (Jeffries et al., 2012). This process typically does not result in exclusively mono-allelic expression but rather clonal lines expressing either one or both alleles at loci showing random clonal allelic bias potential (Gimelbrant et al., 2007, Jeffries et al., 2012). Functional outcomes of allelic bias in the expression of cell surface proteins include the potential for increasing the cellular diversity of immune and neural cells.

A second unexpected observation was the discordance in cluster assignment for pairs of neural cell lines derived from 4 individual donors. The 36 iPSC lines examined included 2 independent iPSC clones from each of 15 donor subjects. Paired lines from 11 donors (9 rs279858 homozygotes and 2 heterozygotes) were concordant (i.e., both lines from a given donor were grouped in the same Cluster), while paired lines from 4 subjects (1 rs279858*T/T, 1 C/C, and 2 T/C) were discordant. We speculate that the AD-associated rs279858*C-allele is in moderate but not complete linkage disequilibrium with an as-yet-unidentified repressive functional variant, and that iPSC-derived rs279858 homozygote neural cells that are discordant in chr4p12 GABA_A gene expression are in fact heterozygous for this unidentified functional variant. Allelic bias could then reveal the cis-effect of the functional repressive C-allele linked variant in heterozygotes. Our results demonstrating a modest allelic bias in expression of the entire chr4p12 GABA_A gene region (e.g. Figure 6) suggests that allelic bias may partially explain the complex genetic regulatory features in this region.

In summary, we found correlated gene expression of four genes encoding GABA_A receptor subunits located in a 1.4 Mb region of chr4p12, with GABA_A gene expression levels significantly associated with a tag-SNP for this region that has been linked to AD. The endophenotype of low mRNA expression for the chr4p12 GABA_A gene cluster in this iPSC *in vitro* model may be a more useful marker than the diagnosis of AD to identify functional polymorphisms in this region. Our results suggest that such functional variants may produce behavioral effects relevant to AD via effects on GABA_A gene expression in the developing nervous system. This work supports the utility of iPSCs as a model system to explore previously unknown molecular effects of genetic variation associated with risk for neuropsychiatric disease. Limitations of such models include the need to examine multiple donor and iPSC lines to address inter- and intra-cell line variability and the limited ability of cultures to replicate the prolonged and complex development of the human nervous system *in vivo*. Finally, our finding that nearly half of iPSC lines produce neural cultures with strikingly low levels of expression of the chr4p12 GABA_A gene cluster may be of interest to other investigators using this model system to examine neuropsychiatric conditions, particularly if only a limited number of disease and control donor lines are compared.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

We thank Chris Abreu at the UConn Clinical Research Center Core Lab for assistance in developing subject-specific fibroblast lines. We thank Leann Crandall at the UConn Stem Cell Core for her valued assistance in generating iPSC lines and Mei Zhong at the Genomics Core of the Yale University Stem Cell Center for RNA library preparation and sequencing. Supported by NIH grants P60 AA03510 (Alcohol Research Center), K24 AA13736 (HRK), M01 RR06192 (University of Connecticut General Clinical Research Center) and by a grant from the CT Department of Public Health (JC).

Dr. Kranzler has served as a consultant or advisory board member for the following companies: Alkermes, Lilly, Lundbeck, Otsuka, Pfizer, and Roche. He is a member of the Alcohol Clinical Trials Group of the American Society of Clinical Psychopharmacology, which is supported by AbbVie, Alkermes, Ethypharm, Lilly, Lundbeck, and Pfizer.

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Figure 1. *GABRA2* RNA Expression by rs279858 Genotype

(A) qPCR data from 36 iPS-derived neural cell lines reveals a significant effect of *GABRA2* rs279858 genotype on *GABRA2* RNA levels. Expression was significantly lower in rs279858 C-allele carriers than in lines from T/T individuals. (B) There was no difference in RNA expression of *RBFOX3* (NeuN) as a function of rs279858 genotype. (* $p < 0.05$)

Figure 2. Clustering of Cell Lines by Chromosome 4p12 GABA_A Expression in Differentiated Neural Cells

Scatterplots of qPCR RNA levels contrasting (A) *GABRA2* vs. *GABRG1*, (B) *GABRA2* vs. *GABRA4*, and (C) *GABRA2* vs. *GABRB1* RNAs for 36 iPS-derived neural cell lines. RNA levels for Cluster 1 cultures (x symbol) were lower for all 4 genes than those for Cluster 2 cultures (gray circles).

Figure 3. RNA Levels for Chromosome 4p12 GABA_A Genes by Cluster Group

RNA levels for chr4p12 GABA_A genes measured by qPCR were significantly lower in Cluster 1 cell lines (n=17) than in Cluster 2 cell lines (n=19): (A) *GABRG1*, (B) *GABRA2*, (C) *GABRA4*, and (D) *GABRB1*. There were no significant differences in RNA levels between clusters for (E) *GABRD* or (F) *RBFOX3* (NeuN). (***) $p < 0.001$

Figure 4. RNA-Seq Confirms qPCR Results and Delineates Differences in Expression of Chromosome 4p12 GABA_A Genes

RNA-seq data from 16 iPSC-derived neural cell lines generated from 10 donor subjects. (A) Schematic depicting the orientation of the chr4p12 GABA_A cluster and adjacent genes. Arrows indicate the direction of transcription, with the approximate distance shown between each gene. (B) There were significant differences in expression between neural cell lines in Cluster 1 and Cluster 2 for the chr4p12 GABA_A genes *GABRG1*; *GABRA2*; *GABRA4*; *GABRB1*, but not for the genes directly adjacent to the chr4p12 GABA_A cluster *GNPDA2*, and *COMMD8*. (C) GABA_A genes located on other chromosomes did not differ in expression between neural cell lines in Cluster 1 and Cluster 2 (*GABRG2*, *GABRB2*, *GABRB3*, *GABRD*). Genes encoding cation-chloride transporter proteins also did not differ between Cluster 1 and Cluster 2 (*SLC12A2* and *SLC12A5*). (* $p < 0.05$; ** $p < 0.005$)

GABRA2 mRNA Expression by Genotype and Cluster

Figure 5. Expression of *GABRA2* RNA by rs279858 Genotype in Cluster 1 vs. Cluster 2 Neural Cell Lines

Cluster 1 iPSC-derived neural cell lines had lower levels of *GABRA2* RNA and a greater number of C-allele carriers than Cluster 2 lines, but levels of *GABRA2* RNA did not show a rs279858 allele dose effect between cell lines within each cluster.

Figure 6. Random Allelic Expression Bias of Chromosome 4p12 GABA_A Genes in independently derived iPSC-neural cultures from a Cluster 2 Subject

Allele biased gene expression in mature neural cultures generated from two independently derived iPSC lines (727-6 and 727-8) from a Cluster 2 donor homozygous for the T-allele at *GABRA2* rs279858 but heterozygous at exonic SNPs in the other three chr4p12 GABA_A genes (rs976156 *GABRG1* exon 3 Thr88Thr, rs7660336 *GABRA4* 3UTR, and rs10028945 *GABRB1* 3UTR). An opposite pattern of allelic bias (C_T between FAM and VIC probes) was observed comparing lines 727-6 and 727-8 for each of the heterozygous SNPs in (A) *GABRG1*, (B) *GABRA4*, and (C) *GABRB1*. Expression of opposite alleles for each gene in the two lines spanning the 4p12 GABA_A gene region indicates the presence of a random process during iPSC induction that can produce bias in expression from either the maternal or paternal chromosome.

Table 1
Correlations Among Chromosome 4p12 GABA_A Subunit mRNA Expression Levels

	<i>GABRG1</i> Chr 4p12	<i>GABRA2</i> Chr 4p12	<i>GABRA4</i> Chr 4p12	<i>GABRB1</i> Chr 4p12	<i>GABRD</i> Chr 1p36	<i>RBFOX3</i> Chr 17q25
<i>GABRG1</i>	1	.865**	.727**	.781**	.137	-.165
<i>GABRA2</i>	.865**	1	.699**	.713**	.109	-.098
<i>GABRA4</i>	.727**	.699**	1	.748**	.105	-.061
<i>GABRB1</i>	.781**	.713**	.748**	1	.118	.033
<i>GABRD</i>	.137	.109	.105	.118	1	.132
<i>RBFOX3</i>	-.165	-.098	-.061	.033	.132	1

**
p<0.01 (Pearson Correlation, 2-tailed)

Table 2
GABRA2 rs279858 Genotype by Chromosome 4p12 *GABRA* Gene Expression Levels

Cluster #	<i>GABRA2</i> rs279858				
	Genotype Count (Frequency) ^a		Allele Count (Frequency) ^b		
	C/C	C/T	T/T	C	T
1 (low expression)	10 (0.59)	6 (0.35)	1 (0.06)	26 (0.76)	8 (0.24)
2 (high expression)	4 (0.21)	5 (0.26)	10 (0.53)	13 (0.34)	25 (0.66)

^a cluster × genotype $\chi^2 = 9.9$, df=2, p=0.007

^b cluster × allele $\chi^2 = 12.9$, df=1, p=0.003