Maternal transmission of a rare GABRB3 signal peptide variant is associated with autism

Ryan J. Delahanty, B.S.[1,*], **Jingqiong Kang, M.D., Ph.D.**[2,*], **Camille W. Brune, Ph.D.**[4], **Emily O. Kistner, Ph.D.**[7], **Eric Courchesne, Ph.D.**[6], **Nancy J. Cox, Ph.D.**[5], **Edwin H. Cook Jr., M.D.**[4], **Robert L. Macdonald, M.D., Ph.D.**[2], and **James S. Sutcliffe, Ph.D.**[1,3]

[1]Department of Molecular Physiology & Biophysics, Vanderbilt University, Nashville, TN

[2]Department of Neurology, Vanderbilt University, Nashville, TN

[3]Department of Psychiatry, Vanderbilt University, Nashville, TN

[4]Department of Psychiatry, Institute for Juvenile Research, University of Illinois at Chicago, Chicago, IL

[5]Department of Medicine, The University of Chicago, Chicago, IL

[6]Department of Neurosciences, University of California San Diego, La Jolla, CA

[7]Department of Health Studies, The University of Chicago, Chicago, IL

Abstract

Maternal 15q11-q13 duplication is the most common copy number variant in autism, accounting for ~1-3% of cases. The 15q11-q13 region is subject to epigenetic regulation and genomic copy number losses and gains cause genomic disorders in a parent-of-origin-specific manner. One 15q11-q13 locus encodes the $GABA_A$ receptor $\beta3$ subunit gene *(GABRB3)*, which has been implicated by several studies in both autism and absence epilepsy, and the co-morbidity of epilepsy in autism is well established. We report that maternal transmission of a GABRB3 signal peptide variant (P11S), previously implicated in childhood absence epilepsy, is associated with autism. Analysis of wild-type and mutant $\beta3$ subunit-containing $\alpha1\beta3\gamma2$ $GABA_A$ receptors demonstrates reduced whole cell current and decreased $\beta3$ subunit protein on the cell surface due to impaired intracellular $\beta3$ subunit processing. We thus provide the first evidence for association between a specific $GABA_A$ receptor defect and autism, direct evidence that this defect causes synaptic dysfunction that is autism-relevant, and the first maternal risk effect in the 15q11-q13 autism duplication region linked to a coding variant.

Corresponding Author: James S. Sutcliffe, Ph.D., Center for Molecular Neuroscience, Departments of Molecular Physiology & Biophysics and Psychiatry, Vanderbilt University, Nashville, TN 37232-8548, Tel: 615-936-3626, jim.sutcliffe@vanderbilt.edu.
*Contributed equally to this work

Conflict of Interest Statement: none declared.

Supplemental Information: Supplementary information is available at Molecular Psychiatry's website.

Introduction

Autism spectrum disorders (ASDs) affect approximately 1 in 150 children and are characterized by impaired development of communication and reciprocal social behaviors and the presence of restricted and repetitive behaviors (1, 2). Autism predominantly affects males, with a male:female ratio of 4:1 and is highly genetic, as revealed by concordance rates of 60 to 90% in identical twins and sibling recurrence risk estimates of 25 times or more that of the population prevalence (3, 4). While the underpinnings of autism are predominantly genetic, efforts to identify common susceptibility alleles have been slowed by locus heterogeneity and apparently small effect sizes. In contrast, we now understand that highly penetrant rare variants constitute a major class of genetic risk factors in autism (2). These range from rare point mutations in genes encoding, for example, synaptic proteins (e.g. CNTNAP2, SHANK3, NLGN3), to gains or losses of DNA segments termed copy number variation (CNV) (e.g. 16p11.2, 15q11-q13), to gross chromosomal rearrangements that are estimated to occur in ∼7% of autism cases (1, 2).

The most common chromosomal abnormalities in autism are 15q11-q13 duplications (OMIM: 608636; a.k.a. *AUTS4*) that occur as an interstitial gain or a supernumerary idic(15) chromosome with two additional copies of 15q11-q13 (5). While paternal duplications are observed, autistic phenotypes are almost always associated with duplications of maternal origin. Maternal deletion of this region leads to Angelman syndrome (OMIM: 105830), and paternal deletion to Prader-Willi syndrome (OMIM: 176270), both of which share ASD features (5, 6). As a consequence, the genes in this interval have become strong candidates for investigation of their potential contribution to autism susceptibility. A cluster of GABA$_A$ receptor subunit genes lies within this interval. GABA$_A$ receptors are ligand-gated chloride channels, which mediate the majority of fast synaptic inhibition in the brain. In addition to association studies, multiple lines of evidence have pointed to the potential involvement of GABAergic systems in autism (7-12). Functional GABA$_A$ receptors are composed of five subunits that form a chloride ion channel and are typically composed of two α, two β, and a γ or δ subunit. One gene in the 15q11-q13 cluster encodes the β3 subunit, GABRB3. Several reports have documented association of common alleles at *GABRB3* with autism (13-19), and one report with childhood absence epilepsy (CAE) (20). Data from autism studies (not all of which are positive) show some evidence for replication, but also suggest allelic heterogeneity. Recent epigenetic studies of the 15q GABA$_A$ receptor subunit cluster by Hogart and colleagues indicate biallelic expression of these genes in normal brain samples. A subset of autism samples, however, showed monoallelic or allelic bias in expression (21, 22), suggesting epigenetic dysregulation. To complement ongoing efforts to characterize common allele associations at *GABRB3*, we sought to determine if rare variants in this gene contributed to autism pathogenesis.

Materials and Methods

Subjects

Families included in this study were recruited at Vanderbilt University, the University of Chicago, the University of California at San Diego, or they were obtained from the NIMH Repository (http://nimhgenetics.org). Affected individuals were subject to a research diagnosis based on scoring of assessment using the Autism Diagnostic Interview – Revised and the Autism Diagnostic Observation Schedule. All subjects provided informed consent and this work was conducted under approvals from the Institutional Review Boards at recruiting institutions.

Genetics

Genotyping was conducted using Applied Biosystems (ABI, Foster City, CA, USA) TaqMan Assays-on-Demand (AoD). PCR for the P11S variant (rs25409; ABI assay C__44811455_10) was carried out in 384-well plates in 5μl reactions containing 0.125 μl 20× AoD probe/primer mix, 5ng of genomic DNA and TaqMan Universal PCR Mastermix according to manufacturer's recommendations. Products were scanned on the ABI 7900HT instrument to call genotyped. Genotyping efficiency was 98% and genotyped conformed to expectations under Hardy-Weinberg Equilibrium. Quality control also included inter-plate and intra-plate replicate samples, as well as checks for within-family Mendelian inconsistency using PEDCHECK (23). Family-based association tests and genotype relative risks were calculated using the GenAssoc module for STATA (v9.2) provided by David Clayton (http://www.gene.cimr.cam.ac.uk/clayton/software/) (24). Log-linear models were constructed using SAS version 9.1.

Phenotype

Subjects were classified as affected under a "strict" diagnostic classification if they met criteria for autism on the ADI-R, while "broad" classification also includes individuals who met ASD1 or ASD2 criteria according to Risi et al (25). Items from the ADI-R were the basis for comparing S11 carriers and P11 homozygotes. Subjects with missing data were excluded from analysis. Effect of genotype on seizures was conducted using a Fisher's Exact test comparing numbers of subjects with definite seizures (score of 2) to those with no history of attacks (score of 0) on the "Faints/Fits/Blackouts" item. Individuals with a history of attacks without a diagnosis of epilepsy or suspected attacks (score of 1) or febrile seizures only (score of 7) were treated as missing. For exploratory analyses, core behavior domain and subdomain scores were compared using univariate analysis of variance (ANOVA) with score as the dependent variable, genotype group as the independent variable, and age at ADI-R as a covariate. Algorithm item scores were compared using Mahon's chi-square test to account for the ordinality of the ADI-R scores which were intended to be scored qualitatively (from 0 = "absence of behavior specified" to 2 = "behavior definitely present"). When present, individual item scores of 3 were down-coded to 2 as instructed on the algorithm. The "ever" or most severe scores for the following items were also down-coded and compared when available: "Loss of Skills," "Overall Level of Language," Sensitivity to Noise," "Difficulties with Minor Changes in Subject's Own Routines or Personal Environment," "Resistance to Trivial Changes in the Environment," "Gait," "Coordination,"

"Aggression toward Caregivers or Family Members," "Aggression toward Noncaregivers or Nonfamily Members," "Self-Injury," "Overactivity," and "Special Isolated Skills." Ages for developmental milestones (including 1st steps, 1st words, 1st phrases, and continence) were also compared between groups using ANOVA with age as the dependent variable, genotype group as the independent variable, and age at ADI-R as a covariate; when codes rather than specific ages were given (e.g., 997 for "not known, but apparently delayed") the subject's data was treated as missing.

Ancestry analysis

Classical multidimensional scaling (MDS) was conducted using PLINK (26) for the sample of AGRE parents (founders). MDS dimensions were estimated from genome-wide average proportion of alleles shared identical by state for each possible pair of founders in the sample. Graphical representation of the first two dimensions were used to identify population substructure and ancestry clusters.

Electrophysiology

Expression of recombinant $GABA_A$ receptors and subsequent whole cell recordings from lifted cells were conducted as previously described (27). Human embryonic kidney (HEK) 293-T cells were co-transfected with 2 μg of each subunit-encoding plasmid and 1 μg of the pHook-1 cDNA (Invitrogen, Carlsbad, CA) using a modified calcium phosphate precipitation method and subsequently selected 24 hours after transfection using magnetic hapten-coated beads.

Biotinylation and Western blot analysis

Cell surface receptor biotinylation and western blot procedures were modified from a previous protocol (27). For cell surface receptor biotinylation, live, transfected cells were washed with phosphate buffered saline (PBS) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH 7.4) followed by incubation with sulfo-NHS biotin for 1 hour at 4° C. Sulfo-NHS biotin was quenched with PBS containing 0.1 mM glycine. Cells were lysed in RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1mM EGTA, 0.1% SDS, 1% Triton ×-100, 1% sodium deoxycholate), supplemented with protein inhibitor (Roche) for 1 hour at 4° C. The extracted supernatant was then incubated with immobilized streptavidin overnight at 4° C. Biotinylated proteins were eluted from the streptavidin by incubation with $1\times$ NEB glycoprotein protein denaturing buffer (5% SDS, 0.4 M DTT) at room temperature for 30 minutes. The supernatant was then either undigested or digested with Endo-H or PNGase F prior to fractionation by 10% SDS-PAGE.

35S radiolabeling metabolic pulse-chase assays

^{35}S methionine pulse-chase experiments were conducted with modifications from a previously published protocol (28). Briefly, 48h hours following transfection, cells were replenished with starving medium that lacked methionine and cysteine (Invitrogen), and incubated at 37°C for 30 min. Starving medium was then replaced by 1.5 ml ^{35}S radionuclide methionine (100-250 μCi/ml (1 Ci = 37GBq); PerkinElmer, Wellesley, MA) labeling medium for a series of different time points at 37°C. FLAG-tagged human β3

subunits were then immunoprecipitated from radio-labeled lysates with an anti-FLAG M2-agarose affinity gel by rotating at 4°C overnight. Immunoprecipitated products were then eluted from the beads with FLAG peptide (Sigma-Aldrich)I, and immunopurified subunits were then analyzed by 10 % SDS-PAGE and exposed on a digital PhosphorImager (GE Healthcare, Piscataway, NJ).

Data analysis

Macroscopic currents were low pass filtered at 2 kHz, digitized at 10 kHz, and analyzed using pClamp9 software suite (Axon Instruments). Except for the pulse-chase assays, proteins were quantified by ChemiImager AlphaEaseFC software. Data from pulse-chase experiments were quantified using Quantity One software (Bio-Rad, Hercules, CA). Numerical data were expressed as mean ± SEM. When wild-type data were arbitrarily taken as 1, column statistics were used. Statistical significance, using Student's unpaired t test (GraphPad Prism), was taken as $P < 0.05$.

Results

Association of GABRB3 P11S with Autism

To screen for novel functional variation, *GABRB3* exons were sequenced in a discovery sample of 100 unrelated probands, and from this effort a single nonsynonymous variant (C87T, Pro11Ser) was identified in exon 1a, one of two alternative initiating exons for *GABRB3* (29). This variant, deposited into dbSNP (rs25409), was determined to be inherited and of maternal origin in two unrelated cases in an initial study cohort. We subsequently genotyped this variant in a sample of 1,152 combined simplex and multiplex families to determine its frequency and potential association with autism in a larger population (Supplementary table 1). Seventeen families (1.47%) were found to harbor the rare S11 variant (Figure 1), corresponding to an allele frequency of 0.40%. Given substantial precedent for parent-of-origin effects in the region, parental transmissions were examined separately, and we observed a maternal (but not paternal) S11 over-transmission ($P = 0.045$; Table 1). Indeed, 12 of 16 maternal transmissions resulted in a broad autism phenotype (see Methods), compared with only 3 of 8 paternal transmissions. For the strict autism phenotype the maternal over-transmission was more pronounced ($P = 0.008$), with 12 of 14 transmissions resulting in a strict autism phenotype. The S11 variant confers a genotype relative risk (GRR) of 3.00 (95% CI: 1.26-7.12, $P = 0.013$) for the broad autism phenotype when the transmission is maternal in origin. Similarly, the S11 variant confers a GRR of 6.00 (95% CI: 1.62-22.16, $P = 0.007$) for the strict autism phenotype when transmission is maternal in origin. Genotyping Caucasian controls identified a single S11 carrier from a sample of 584 chromosomes, corresponding to a 0.17% allele frequency, compared with 0.40% observed in the overall autism sample.

Exploratory parent-of-origin tests were also conducted using a log-linear model framework allowing for a maternal genetic effect (30). Application of this approach allowed us to exclude the possibility that a simple effect of maternal genotype, in which offspring of mothers with the S11 allele were at increased risk of developing autism regardless of whether the S11 allele was transmitted to the offspring, accounted for our observations,

since there was clearly preferential transmission of the S11 allele to affected offspring of heterozygous mothers. Using either a broad or a strict autism phenotype, this parent-of-origin effect was statistically significant (p=0.040 and p=0.004 respectively). Although it is theoretically possible that there could be a direct effect of maternal genotype on offspring risk of autism in addition to the parent-of-origin effect that we have established, we have little power to test for a maternal effect in the presence of a parent-of-origin effect. and a fully developed test for such joint effects would require a more fully characterized sample with ascertainment of all affected and unaffected offspring from both mothers and fathers carrying this risk allele.

The *GABRB3* P11S mutation was recently identified in 2 independent families segregating CAE in a Hispanic/Mexican-American cohort of 48 families (31). Self report data for the families genotyped in our study suggested that the S11 variant was on a non-Hispanic Caucasian background. To resolve uncertainty about ancestry, and to ensure that an appropriate control sample was used, we examined genome-wide SNP data available for a subset of subjects from the Autism Genetics Resource Exchange (AGRE) collection, which was previously genotyped using the Affymetrix 5.0 (500k) SNP platform (32). Multidimensional scaling (MDS) of SNP genotype data for all AGRE parents was conducted using PLINK to estimate dimensions of population genetic variation (26). Figure 2 shows a graphical representation of the first two dimensions from this analysis that identified population substructure and ancestral clusters for founders (parents). Our analysis found that the S11 variant was present in Caucasian parents. The rare S11 variant had a frequency of 0.94% (7/743) and 0% (0/105) in Caucasian and Hispanic founders, respectively, though only 75 families screened were determined to have Hispanic ancestry. Given the mixed ancestral history of Hispanic populations with Spanish Caucasians and Native Americans, it is not surprising that the variant was identified previously in a Hispanic sample. Our total sample was 0.48% in the overall sample.

Phenotypic Correlates of P11S in Autism

Given the association of the S11 mutation with CAE, we examined item level data from the Autism Diagnostic Interview-Revised (ADI-R) (33) on seizure history. The rate of confirmed non-febrile seizures was 16.7% (2/12) in affected probands with the maternal S11 compared to 4.9% (55/973) for those homozygous for the P11 allele, but this difference was not significant ($P = 0.10$; Table S2). Seizure rates in autism increase with age, however, age did not significantly differ between the maternal S11 (M = 97 mos ± 45) and homozygous P11 (M = 94 mos ± 52) groups, t(1137) = -0.18, $P = 0.86$.

Supplementary tables 3 and 4 provide characteristics of probands with maternally- and paternally-derived variants, respectively. Inspection of S11 pedigrees indicate that maternal transmission result in a narrowly-defined autism phenotype based on standard diagnostic algorithms including the ADI-R. To further explore P11S phenotypic correlates, we compared ADI-R dimensions between the maternal S11 to the homozygous P11 cases. Individuals with the paternal S11 were considered unknown for these analyses and omitted since the variant was not significantly over-transmitted from fathers to individuals with ASD. Table S5 shows the results for the comparison of groups' domain and subdomain

scores. The maternal S11 and homozygous P11 groups did not significantly differ on any domain score. The maternal S11 group scored more severely than the homozygous P11 group on the subdomain "Stereotyped, repetitive, or idiosyncratic speech" and less severely on "Preoccupations with part of objects or non-functional elements of material." There were no significant differences ($P < 0.01$) in ADI-R item level data between groups considering items for which maternal S11 cases were more severe.

Functional Analysis of *the P11S Variant*

To identify the molecular defect underlying the genetic association, we engineered P11 and S11-encoding human β3 subunit cDNAs for *in vitro* studies in HEK293T cells. β3 subunits are incorporated into hetero-pentameric complexes at the cell surface (e.g. synapses), and the most common combination involves co-assembly of β3 with γ2 and α3 subunits during development and/or α1 subunits in the adult brain. Therefore, we co-expressed both wild-type and mutant β3 subunits with either α1 or α3 and γ2S subunits. The γ2S subunit was used instead of the γ2L subunit since it is much more abundant in the brain. To mimic the heterozygous condition seen in patients, equal amounts of wild-type β3(P11) and mutant β3(S11) subunits were co-transfected with α3 and γ2S subunits (Figure 3a and b). Compared with wild-type α3β3γ2S receptors, mutant receptors displayed reduced peak current amplitudes with either mixed β3(P11)/β3(S11) subunit expression or with only mutant β3(S11) subunit expression, and the amplitude reduction was greater with expression of only mutant β3(S11) subunits than with the mixed condition. Reduced receptor function in the context of the more developmentally relevant α3β3γ2S subunit combination is consistent with recent findings by Tanaka and colleagues (31).

The reduction of mutant receptor channel current could be due to a reduced number of functional receptors. We expressed α1 and γ2S subunits with β3(P11)HA and β3(S11)HA subunits and used surface biotinylation to determine the relative expression of wild-type and mutant β3 subunits. Cell surface membrane proteins were isolated following biotinylation of live cells expressing wild-type α1β3(P11)HAγ2S receptors or mutant α1β3(S11)HAγ2S receptors and treated with PNGase F to remove all carbohydrates attached in the ER and trans-Golgi. Products were then fractionated by SDS-PAGE and immunoblotted with anti-HA antibody. Both mutant β3(S11)HA and wild-type β3(P11)HA subunits on the cell surface appeared to the same size both before and after PNGase treatment, indicating likely signal peptide cleavage for β3(S11) subunits (Figure 3C) as for wild-type subunits Confirmation of signal peptide cleavage, however, will require verification by protein sequencing. While mutant β3(S11) subunits were trafficked to the cell surface, when compared to wild-type β3(P11)HA subunits, mutant β3(S11)[HA] subunit levels were reduced both before (U) (1 vs. 0.4637 ± 0.062; $P < 0.0017$) and after PNGase F treatment (F) (1 vs. 0.3799; $P < 0.0092$) (Figure 3D).

Our data are consistent with a molecular defect produced by the P11S substitution that occurs at a post-translational level, with mutant subunits exhibiting abnormal intracellular processing. Wild-type β3(P11)HA or mutant β3(S11)HA subunits were co-expressed in HEK293T cells with α1 and γ2S subunits. Compared to wild-type β3(P11)HA subunits, mutant β3(S11)HA subunits had a small but significant reduction in protein intensity (1 vs.

0.7316 ± 0.1270; $P = 0.0012$, N = 7). Although surface β3(S11) subunits appeared to have the same molecular mass as wild-type β3(P11) subunits, it was possible that if the signal peptides attached to mutant β3(S11) subunits were not cleaved, the subunits might not fold and oligomerize properly, and thus, would be retained inside cells instead of trafficking to the surface We thus determined the molecular mass of mutant β3(S11) subunit from total cell lysates. Total wild-type (α1β3(P11)HAγ2S) or mutant (α1β3(S11)HAγ2S) receptors were digested with PNGase-F, which removes all carbohydrates attached in both ER and trans-Golgi regions, Without treatment (U), both wild-type β3(P11)HA and the mutant β3(S11)HA subunits migrated in a main band at ∼58-60 kDa, although a faint band with a lower molecular mass representing different glycosylation form was also observed. With PNGaseF (F) treatment, both the wild-type β3(P11)HA and mutant β3(S11)HA subunits migrated in a single main band with the same molecular mass about 52 KDa consistent with previous reports (34, 35), suggesting that the majority of wild-type and mutant β3 subunits had successful signal peptide cleavage (Figure 4C). We also used 35S methionine metabolic labeling to characterize biogenesis of wild-type β3FLAG and mutant β3(S11)FLAG subunits. When expressed alone, both wild-type and mutant subunits migrated in two bands, with the higher molecular mass band representing a more mature form and the lower band representing a less mature form (Figure 4D). Compared to wild-type subunits, the ratio of the higher molecular mass band to the lower band was lower for mutant subunits as soon as 10 min after translation (Figure 4E). The same molecular mass of wild-type β3(P11) and the mutant β3(S11) subunits after PNGase F digestion suggested that the signal peptides were both cleaved. The slightly reduced total amount of mutant β3 (S11) subunit protein suggested that mutant subunits were not as stable as wild-type subunits.

Discussion

We have shown that a rare coding variant of the *GABRB3* gene is associated with autism when transmitted maternally. Statistical association is explained by an intracellular processing defect imparted by the N-terminal P11S substitution in the signal peptide. While the S11-encoded signal peptide is cleaved, molecular evidence indicates that the S11 signal peptide defect results in reduced surface expression and resultant decreased receptor current, probably due to abnormal intracellular processing, intracellular retention and fast degradation of the mutant subunit. We suggest that impaired GABAergic signaling during a critical time window in brain development could lead to both autism and CAE, and possibly other more subtle neurological phenotypes.

To our knowledge, this is the first *rare* coding variant in a GABA receptor gene for which TDT analysis has revealed a significant autism susceptibility effect. In this case, there was an *a priori* expectation that risk would derive from maternal rather than paternal transmissions. Maternal association is indeed significant, although ultimately these findings need to be replicated in an independent cohort. A problem inherent in the study of rare variants is the difficulty in amassing a sufficiently large clinical sample to detect risk effects in the absence of full penetrance. Such is the case for P11S, which is analogous to numerous inherited CNVs that affect loci widely regarded as "autism genes". Loci such as *NRXN1*, *SHANK3* and 16p11.2 have been identified on the basis of *de novo* events in autism probands, however numerous cases at these and other similarly-identified loci show

inherited gene disrupting variants (32, 36-40). Such cases reveal incomplete penetrance of such (e.g.) loss-of-function mutations for a given gene. Indeed families may show unexpected segregation patterns with only one of two affected sibs receiving the variant or both an affected and unaffected sib inheriting the variant. The pattern is much the same for P11S. In fact, even maternal duplication of the *GABRB3*-containing 15q11-q13 region does not always manifest with autism (41).

GABRB3 is known to play a major role in development of the CNS, being the major β isoform in a number of regions in prenatal and neonatal brain (42-44). The result of *Gabrb3* disruption in the mouse is a useful comparison, given the effect of P11S substitution in families segregating autism and epilepsy. In the 10% of null animals that survive to maturity, epilepsy and hypersensitivity and various other behavioral and physiological abnormalities are noted (45). Null mice produce fewer functional GABA$_A$ receptors, and pharmacological evidence indicates that other β subunits do not compensate for the absence of β3 (46). The *Gabrb3* knock-out has been proposed as an autism model because of reduced social and exploratory behaviors and a tendency for diminished nurturing behaviors (47, 48).

In light of the epigenetic regulation of 15q11-q13 genes, and that it is typically maternal, but not paternal duplication of the interval leads to autism, the S11 association is provocative. Maternal bias implicates *UBE3A* in the dup(15) autism phenotype, however, a contiguous gene effect including *GABRB3* is very likely (49). Supporting potential contribution of *GABRB3* in causing dup(15) autism, several genetic and epigenetic studies have independently implicated the *GABRB3* gene in autism in the absence of duplication (13-15, 17, 21, 22, 50-52). While genetic studies of common allele effects are largely positive, the data suggest allelic heterogeneity, with associations seen in two or more regions at the *GABRB3* locus (17). Maternal inheritance has also been noted in genetic epilepsies (53, 54), and given the maternal specificity of dup(15) autism (55, 56), we speculate that epigenetic effects at *GABRB3* may tilt the balance to risk for autism when the origin of the S11 variant is maternal. Studies such as a recent one by Nakatani et al (57) show that duplication of the syntenic region in mouse results in behaviors analogous to some autistic traits. In this instance it is paternal duplication that produces relevant deficits, but the authors recognize that epigenetic regulation may differ between human and mouse. It is not clear at this time if the phenotypic consequence of this murine duplication involves contribution from *Gabrb3*.

We also speculate that the presence of the mutation in both mother and fetus may lead to greater impact at some key developmental stage. To formally test for maternal genotypic effects, analytic approaches must assess any increased association due to the sex of the non-transmitting parent. This would be consistent with maternal genotype-created environment interacting with fetal genotype to increase risk even more in offspring, as opposed to a classic parent-of-origin effect. If S11 effects on maternal environment are important, we would expect to see increased risk in S11-carrier offspring of carrier mothers, relative to offspring of noncarrier mothers (and carrier fathers). Tests of maternal and parent-of-origin effects have been explored in this sample, allowing us to establish a parent-of-origin effect; we were, however, unable to conduct an adequately powered test of whether there are effects of maternal genotype on offspring risk of autism in addition to established parent-of-origin effects.

Epigenetic and expression studies of *GABRB3* provide some context for considering the observed association, although the picture vis-à-vis parent-of-origin effects on gene expression is far from clear From their analysis of cerebral cortex (BA9) brain samples, Samaco and colleagues reported that levels of GABRB3 protein were significantly reduced in five of nine autism samples but not in controls (51). Another study by this group revealed monoallelic expression of *GABRB3* in one of seven autism frontal cortex samples informative for an exon 1a SNP, and evidence from deletion PWS and AS samples suggesting the allelic bias favors paternal expression (22). These reports cannot tell us, however, whether similar effects might occur during fetal development and in other, perhaps more phenotypically-relevant, brain regions. Nevertheless, the possibility that imprinting or allelic bias in gene expression might, like with the serotonin 2A receptor gene (*HTR2A*) (58), be a polymorphic trait, leads us to speculate that incomplete penetrance of S11 relates to inter-individual differences in gene expression and/or epigenetic regulation of the region.

$GABA_A$ receptor activity is critical for neurodevelopment and synaptogenesis in developing brain and for mediating the majority of synaptic inhibition in adult brain. $GABA_A$ receptors switch from excitatory to inhibitory from fetal to postnatal brain. In embryonic and neonatal brains, GABA produces *excitatory* actions and acts as a trophic factor during nervous system development. It plays important roles in proliferation, migration and differentiation of precursor cells, synapse maturation and cell death that orchestrate the development of the embryonic brain.

Spatiotemporal expression of $GABA_A$ receptor subunits is controlled through the use of alternative initiating exons. The P11S variant is in exon 1a, which like exon 1, expresses an alternative signal peptide. In adult, exon 1-containing transcripts are more abundant than those containing exon 1a. However, the relative abundance of exon 1a transcripts is significantly higher in fetal brain than in adult hippocampus (29). Thus, an exon 1a variant is more likely to exert its effects developmentally, prior to the shift in balance of exon 1a to exon1 transcripts. Indeed, age effects are common in epilepsy, with the majority of CAE patients having absence seizures that lessen or abate completely with age (59).

In our phenotype analysis, we asked initially whether or not epilepsy was associated with the S11 variant, given the observation of S11 segregating in families with CAE (31). The rate was higher in P11S carriers (14.3%) compared with P11 homozygotes (5.2%), but not greater than overall estimates of epilepsy in autism (5-38.3%) (60). Aside from ascertainment biases, the broad range of epilepsy estimates may reflect the inherent difficulty in identifying more subtle epilepsy and/or EEG abnormalities in autism. The S11 variant displays incomplete penetrance in both autism and published CAE families, and thus milder phenotypes may be present in S11 carriers (61). As functional data indicate that S11 reduces, but does not eliminate, functional $GABA_A$ receptors, this would not be surprising.

Molecular defects produced by the P11S substitution in *GABRB3* likely include impaired GABAergic signaling and cellular homeostasis. The present study provides a direct link between $GABA_A$ receptor β3 subunit dysfunction and autism, and explains the observed association with epilepsy. Precedent already exists for $GABA_A$ receptor subunit dysfunction leading to epilepsy, with mutations in the γ2 subunit also associated with CAE (27). Given

the nature of its dysfunction, association of this specific, albeit infrequent, allele with autism and epilepsy suggests a potential for individualized treatment in these cases.

In summary, we report (1) the first example of a GABA$_A$ receptor subunit gene coding variant statistically associated with autism; (2) the first signal peptide mutation associated with autism; and (3) the first evidence for maternal over-transmission of a coding variant within this known imprinted, autism-associated region. Since *maternal* duplication of 15q11-q13 is the most frequent chromosomal and copy number abnormality known to cause autism, it is provocative that *maternal* over-transmission of the more discrete *GABRB3* S11 variant may increase risk for autism and epilepsy. These findings complement other common-allele linkage and association studies and provide further support for involvement of *GABRB3* in autism (13-19). It appears then that allelic heterogeneity at *GABRB3*, both common and P11S, act as genetic risk factors. We predict for autism, as found for other complex diseases, that rare variants possessing greater effect sizes will emerge in genes showing association of common alleles conferring more modest effects (62, 63).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Abrahams BS, Geschwind DH. Advances in autism genetics: on the threshold of a new neurobiology. Nat Rev Genet. 2008 May; 9(5):341–355. [PubMed: 18414403]
2. O'Roak BJ, State MW. Autism genetics: strategies, challenges, and opportunities. Autism Research. 2008; 1(1):4–17. [PubMed: 19360646]
3. Chakrabarti S, Fombonne E. Pervasive developmental disorders in preschool children: confirmation of high prevalence. Am J Psychiatry. 2005 Jun; 162(6):1133–1141. [PubMed: 15930062]
4. Jorde LB, Hasstedt SJ, Ritvo ER, Mason-Brothers A, Freeman BJ, Pingree C, et al. Complex segregation analysis of autism. Am J Hum Genet. 1991; 49(5):932–938. [PubMed: 1928098]
5. Schanen NC. Epigenetics of autism spectrum disorders. Hum Mol Genet. 2006 Oct 15;15(Suppl 2):R138–150. [PubMed: 16987877]
6. Sutcliffe J, Nurmi E. Duplication and Inherited Susceptibility of Chromosome 15q11-q13 Genes in Autism. J Am Acad Child Adolesc Psychiatry. 2003; 42:253–256. [PubMed: 12544187]
7. Blatt GJ. GABAergic cerebellar system in autism: a neuropathological and developmental perspective. Int Rev Neurobiol. 2005; 71:167–178. [PubMed: 16512350]
8. Blatt GJ, Fitzgerald CM, Guptill JT, Booker AB, Kemper TL, Bauman ML. Density and distribution of hippocampal neurotransmitter receptors in autism: an autoradiographic study. J Autism Dev Disord. 2001; 31(6):537–543. [PubMed: 11814263]
9. Yip J, Soghomonian JJ, Blatt GJ. Decreased GAD67 mRNA levels in cerebellar Purkinje cells in autism: pathophysiological implications. Acta Neuropathol (Berl). 2007 May; 113(5):559–568. [PubMed: 17235515]
10. Yip J, Soghomonian JJ, Blatt GJ. Increased GAD67 mRNA expression in cerebellar interneurons in autism: implications for Purkinje cell dysfunction. J Neurosci Res. 2008 Feb 15;86(3):525–530. [PubMed: 17918742]

11. Dhossche D, Applegate H, Abraham A, Maertens P, Bland L, Bencsath A, et al. Elevated plasma gamma-aminobutyric acid (GABA) levels in autistic youngsters: stimulus for a GABA hypothesis of autism. Med Sci Monit. 2002 Aug; 8(8):PR1–6. [PubMed: 12165753]

12. Fatemi SH, Halt AR, Stary JM, Kanodia R, Schulz SC, Realmuto GR. Glutamic acid decarboxylase 65 and 67 kDa proteins are reduced in autistic parietal and cerebellar cortices. Biol Psychiatry. 2002 Oct 15; 52(8):805–810. [PubMed: 12372652]

13. Buxbaum JD, Silverman JM, Smith CJ, Greenberg DA, Kilifarski M, Reichert J, et al. Association between a GABRB3 polymorphism and autism. Mol Psychiatry. 2002; 7(3):311–316. [PubMed: 11920158]

14. Cook EH, Courchesne RY, Cox NJ, Lord C, Gonen D, Guter SJ, et al. Linkage-Disequilibrium Mapping of Autistic Disorder, With 15q11-13 Markers. Am J Hum Genet. 1998; 62(5):1077–1083. [PubMed: 9545402]

15. Curran S, Powell J, Neale BM, Dworzynski K, Li T, Murphy D, et al. An association analysis of candidate genes on chromosome 15 q11-13 and autism spectrum disorder. Mol Psychiatry. 2006 Aug; 11(8):709–713. [PubMed: 16868570]

16. Kim SJ, Brune CW, Kistner EO, Christian SL, Courchesne EH, Cox NJ, et al. Transmission disequilibrium testing of the chromosome 15q11-q13 region in autism. Am J Med Genet B Neuropsychiatr Genet. 2008 Oct 5; 147B(7):1116–1125. [PubMed: 18361419]

17. McCauley JL, Olson LM, Delahanty R, Amin T, Nurmi EL, Organ EL, et al. A linkage disequilibrium map of the 1-Mb 15q12 GABA(A) receptor subunit cluster and association to autism. Am J Med Genet B Neuropsychiatr Genet. 2004 Nov 15; 131(1):51–59. [PubMed: 15389768]

18. Salmon B, Hallmayer J, Rogers T, Kalaydjieva L, Petersen PB, Nicholas P, et al. Absence of linkage and linkage disequilibrium to chromosome 15q11-q13 markers in 139 multiplex families with autism. Am J Med Genet. 1999; 88(5):551–556. [PubMed: 10490715]

19. Maestrini E, Lai C, Marlow A, Matthews N, Wallace S, Bailey A, et al. Serotonin transporter (5-HTT) and gamma-aminobutyric acid receptor subunit beta3 (GABRB3) gene polymorphisms are not associated with autism in the IMGSA families. The International Molecular Genetic Study of Autism Consortium. Am J Med Genet. 1999; 88(5):492–496. [PubMed: 10490705]

20. Urak L, Feucht M, Fathi N, Hornik K, Fuchs K. A GABRB3 promoter haplotype associated with childhood absence epilepsy impairs transcriptional activity. Hum Mol Genet. 2006 Aug 15; 15(16):2533–2541. [PubMed: 16835263]

21. Hogart A, Leung KN, Wang NJ, Wu DJ, Driscoll J, Vallero RO, et al. Chromosome 15q11-13 duplication syndrome brain reveals epigenetic alterations in gene expression not predicted from copy number. J Med Genet. 2008 Oct 7.

22. Hogart A, Nagarajan RP, Patzel KA, Yasui DH, Lasalle JM. 15q11-13 GABAA receptor genes are normally biallelically expressed in brain yet are subject to epigenetic dysregulation in autism-spectrum disorders. Hum Mol Genet. 2007 Mar 15; 16(6):691–703. [PubMed: 17339270]

23. O'Connell JR, Weeks DE. PedCheck: a program for identification of genotype incompatibilities in linkage analysis. American journal of human genetics. 1998 Jul; 63(1):259–266. [PubMed: 9634505]

24. Cordell HJ, Clayton DG. Genetic association studies. Lancet. 2005 Sep 24-30; 366(9491):1121–1131. [PubMed: 16182901]

25. Risi S, Lord C, Gotham K, Corsello C, Chrysler C, Szatmari P, et al. Combining information from multiple sources in the diagnosis of autism spectrum disorders. J Am Acad Child Adolesc Psychiatry. 2006 Sep; 45(9):1094–1103. [PubMed: 16926617]

26. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet. 2007 Sep; 81(3):559–575. [PubMed: 17701901]

27. Kang JQ, Macdonald RL. The GABAA receptor gamma2 subunit R43Q mutation linked to childhood absence epilepsy and febrile seizures causes retention of alpha1beta2gamma2S receptors in the endoplasmic reticulum. J Neurosci. 2004 Oct 6; 24(40):8672–8677. [PubMed: 15470132]

28. Gallagher MJ, Ding L, Maheshwari A, Macdonald RL. The GABAA receptor alpha1 subunit epilepsy mutation A322D inhibits transmembrane helix formation and causes proteasomal degradation. Proc Natl Acad Sci U S A. 2007 Aug 7; 104(32):12999–13004. [PubMed: 17670950]

29. Kirkness EF, Fraser CM. A strong promoter element is located between alternative exons of a gene encoding the human gamma-aminobutyric acid-type A receptor beta 3 subunit (GABRB3). J Biol Chem. 1993; 268(6):4420–4428. [PubMed: 8382702]

30. Weinberg CR. Methods for detection of parent-of-origin effects in genetic studies of case-parents triads. American journal of human genetics. 1999 Jul; 65(1):229–235. [PubMed: 10364536]

31. Tanaka M, Olsen RW, Medina MT, Schwartz E, Alonso ME, Duron RM, et al. Hyperglycosylation and reduced GABA currents of mutated GABRB3 polypeptide in remitting childhood absence epilepsy. Am J Hum Genet. 2008 Jun; 82(6):1249–1261. [PubMed: 18514161]

32. Weiss LA, Shen Y, Korn JM, Arking DE, Miller DT, Fossdal R, et al. Association between microdeletion and microduplication at 16p11.2 and autism. N Engl J Med. 2008 Feb 14; 358(7):667–675. [PubMed: 18184952]

33. Lord C, Rutter M, Le Couteur A. Autism Diagnostic Interview-Revised: a revised version of a diagnostic interview for caregivers of individuals with possible pervasive developmental disorders. J Autism Dev Disord. 1994; 24(5):659–685. [PubMed: 7814313]

34. Taylor PM, Thomas P, Gorrie GH, Connolly CN, Smart TG, Moss SJ. Identification of amino acid residues within GABA(A) receptor beta subunits that mediate both homomeric and heteromeric receptor expression. J Neurosci. 1999 Aug 1; 19(15):6360–6371. [PubMed: 10414965]

35. McDonald BJ, Amato A, Connolly CN, Benke D, Moss SJ, Smart TG. Adjacent phosphorylation sites on GABAA receptor beta subunits determine regulation by cAMP-dependent protein kinase. Nat Neurosci. 1998 May; 1(1):23–28. [PubMed: 10195104]

36. Moessner R, Marshall CR, Sutcliffe JS, Skaug J, Pinto D, Vincent J, et al. Contribution of SHANK3 mutations to autism spectrum disorder. American journal of human genetics. 2007 Dec; 81(6):1289–1297. [PubMed: 17999366]

37. Szatmari P, Paterson AD, Zwaigenbaum L, Roberts W, Brian J, Liu XQ, et al. Mapping autism risk loci using genetic linkage and chromosomal rearrangements. Nature genetics. 2007 Mar; 39(3):319–328. [PubMed: 17322880]

38. Marshall CR, Noor A, Vincent JB, Lionel AC, Feuk L, Skaug J, et al. Structural variation of chromosomes in autism spectrum disorder. American journal of human genetics. 2008 Feb; 82(2):477–488. [PubMed: 18252227]

39. Feng J, Schroer R, Yan J, Song W, Yang C, Bockholt A, et al. High frequency of neurexin 1beta signal peptide structural variants in patients with autism. Neuroscience letters. 2006 Nov 27; 409(1):10–13. [PubMed: 17034946]

40. Yan J, Oliveira G, Coutinho A, Yang C, Feng J, Katz C, et al. Analysis of the neuroligin 3 and 4 genes in autism and other neuropsychiatric patients. Molecular psychiatry. 2005 Apr; 10(4):329–332. [PubMed: 15622415]

41. Bolton PF, Dennis NR, Browne CE, Thomas NS, Veltman MW, Thompson RJ, et al. The phenotypic manifestations of interstitial duplications of proximal 15q with special reference to the autistic spectrum disorders. American journal of medical genetics. 2001 Dec 8; 105(8):675–685. [PubMed: 11803514]

42. Laurie DJ, Wisden W, Seeburg PH. The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J Neurosci. 1992 Nov; 12(11):4151–4172. [PubMed: 1331359]

43. Wisden W, Laurie DJ, Monyer H, Seeburg PH. The distribution of 13 GABAA receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J Neurosci. 1992 Mar; 12(3):1040–1062. [PubMed: 1312131]

44. Ma W, Saunders PA, Somogyi R, Poulter MO, Barker JL. Ontogeny of GABAA receptor subunit mRNAs in rat spinal cord and dorsal root ganglia. The Journal of comparative neurology. 1993 Dec 15; 338(3):337–359. [PubMed: 7509352]

45. Homanics GE, DeLorey TM, Firestone LL, Quinlan JJ, Handforth A, Harrison NL, et al. Mice devoid of gamma-aminobutyrate type A receptor beta3 subunit have epilepsy, cleft palate, and

hypersensitive behavior. Proceedings of the National Academy of Sciences of the United States of America. 1997 Apr 15; 94(8):4143–4148. [PubMed: 9108119]

46. Krasowski MD, Rick CE, Harrison NL, Firestone LL, Homanics GE. A deficit of functional GABA(A) receptors in neurons of beta 3 subunit knockout mice. Neuroscience letters. 1998 Jan 9; 240(2):81–84. [PubMed: 9486477]

47. DeLorey TM, Sahbaie P, Hashemi E, Homanics GE, Clark JD. Gabrb3 gene deficient mice exhibit impaired social and exploratory behaviors, deficits in non-selective attention and hypoplasia of cerebellar vermal lobules: a potential model of autism spectrum disorder. Behavioural brain research. 2008 Mar 5; 187(2):207–220. [PubMed: 17983671]

48. DeLorey TM. GABRB3 gene deficient mice: a potential model of autism spectrum disorder. International review of neurobiology. 2005; 71:359–382. [PubMed: 16512358]

49. Sutcliffe JS, Nurmi EL, Lombroso PJ. Genetics of childhood disorders: XLVII. Autism, part 6: duplication and inherited susceptibility of chromosome 15q11-q13 genes in autism. Journal of the American Academy of Child and Adolescent Psychiatry. 2003 Feb; 42(2):253–256. [PubMed: 12544187]

50. Meguro M, Mitsuya K, Sui H, Shigenami K, Kugoh H, Nakao M, et al. Evidence for uniparental, paternal expression of the human GABAA receptor subunit genes, using microcell-mediated chromosome transfer. Hum Mol Genet. 1997; 6(12):2127–2133. [PubMed: 9328477]

51. Samaco RC, Hogart A, Lasalle JM. Epigenetic overlap in autism-spectrum neurodevelopmental disorders: MECP2 deficiency causes reduced expression of UBE3A and GABRB3. Hum Mol Genet. 2005 Feb 15; 14(4):483–492. [PubMed: 15615769]

52. Weiss LA, Gonen D, Kim SJ, Yang Z, Cox NJ, Cook EH. Association of variation between GABRB3 exon 3 and 155CA-2 with Autistic Disorder. Am J Hum Genet. 2002; 71:A448.

53. Tsuboi T, Endo S. Incidence of seizures and EEG abnormalities among offspring of epileptic patients. Human genetics. 1977 Apr 15; 36(2):173–189. [PubMed: 404230]

54. Ottman R, Annegers JF, Hauser WA, Kurland LT. Higher risk of seizures in offspring of mothers than of fathers with epilepsy. American journal of human genetics. 1988 Sep; 43(3):257–264. [PubMed: 3414683]

55. Cook EH Jr, Lindgren V, Leventhal BL, Courchesne R, Lincoln A, Shulman C, et al. Autism or atypical autism in maternally but not paternally derived proximal 15q duplication. American journal of human genetics. 1997 Apr; 60(4):928–934. [PubMed: 9106540]

56. Schanen NC. Epigenetics of autism spectrum disorders. Human molecular genetics. 2006 Oct 15; 15(Spec No 2):R138–150. [PubMed: 16987877]

57. Nakatani J, Tamada K, Hatanaka F, Ise S, Ohta H, Inoue K, et al. Abnormal behavior in a chromosome-engineered mouse model for human 15q11-13 duplication seen in autism. Cell. 2009 Jun 26; 137(7):1235–1246. [PubMed: 19563756]

58. Bunzel R, Blumcke I, Cichon S, Normann S, Schramm J, Propping P, et al. Polymorphic imprinting of the serotonin-2A (5-HT2A) receptor gene in human adult brain. Brain Res Mol Brain Res. 1998 Aug 15; 59(1):90–92. [PubMed: 9729300]

59. Loiseau P, Duche B, Pedespan JM. Absence epilepsies. Epilepsia. 1995 Dec; 36(12):1182–1186. [PubMed: 7489694]

60. Tuchman R, Rapin I. Epilepsy in autism. Lancet neurology. 2002 Oct; 1(6):352–358. [PubMed: 12849396]

61. Levisohn PM. The autism-epilepsy connection. Epilepsia. 2007; 48(Suppl 9):33–35. [PubMed: 18047599]

62. Sutcliffe JS, Delahanty RJ, Prasad HC, McCauley JL, Han Q, Jiang L, et al. Allelic heterogeneity at the serotonin transporter locus (SLC6A4) confers susceptibility to autism and rigid-compulsive behaviors. American journal of human genetics. 2005 Aug; 77(2):265–279. [PubMed: 15995945]

63. Nejentsev S, Walker N, Riches D, Egholm M, Todd JA. Rare Variants of IFIH1, a Gene Implicated in Antiviral Responses, Protect Against Type 1 Diabetes. Science. 2009 Mar 5.
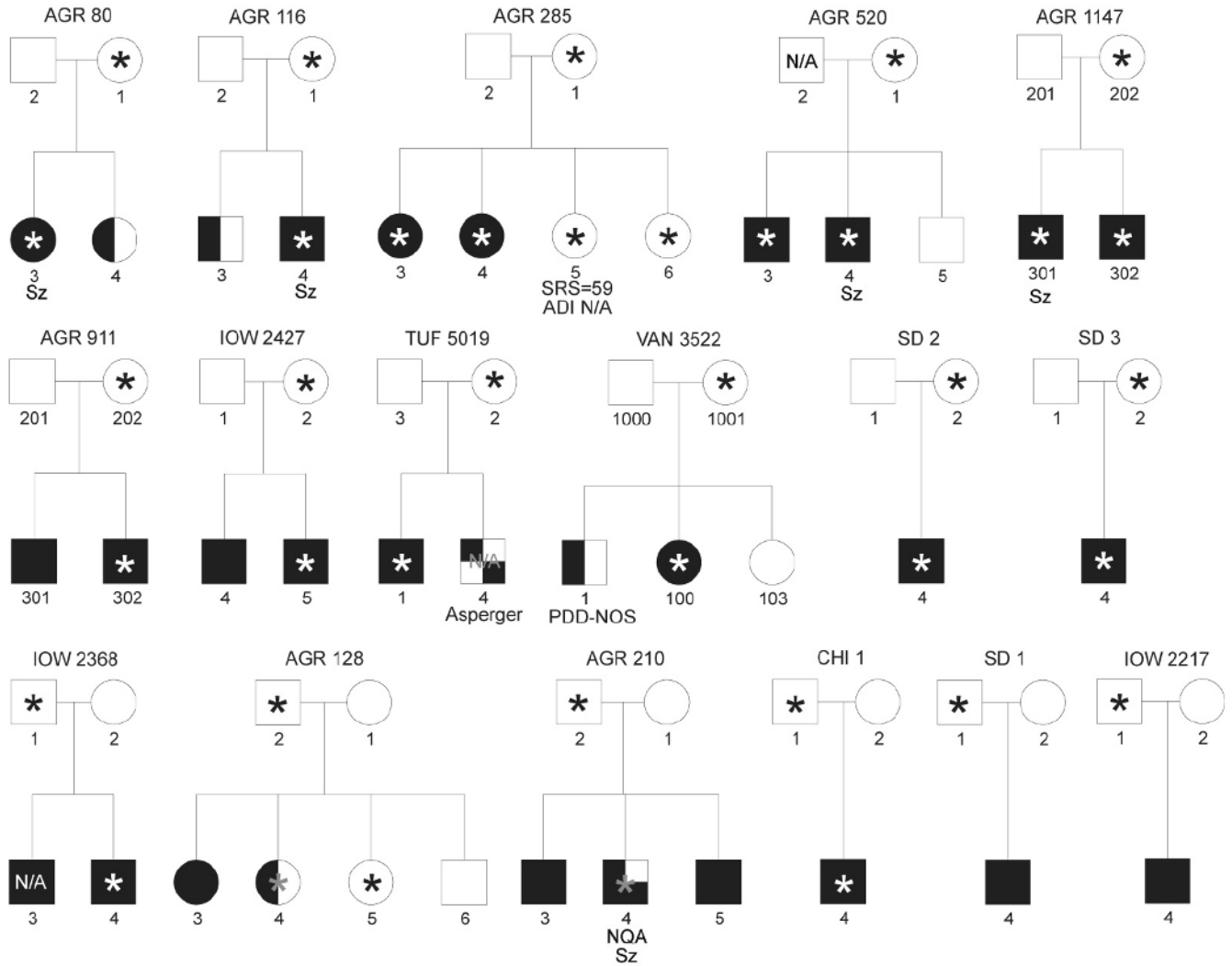
**Figure 1. Pedigree structure of ASD families bearing the P11S variant**

17 ASD families in total were identified to harbor the variant. Individuals carrying the variant are marked with asterisks. Individuals for whom DNA was not available are marked N/A. Our strict analyses considered individuals affected if they met criteria for autism on the ADI-R diagnostic algorithm (completely filled black). Our broad analyses considered these individuals and individuals who met the AGP criteria ASD1 or ASD2 as affected (half filled black) (see Risi et al. 2006 (25) for details); note no individuals met ASD2. Unfilled individuals are considered unknown. For individuals who did not meet broad criteria Social Responsiveness Scale (SRS) t-scores from teacher (/parent) report are provided when available. SRS scores ranging from 60-75 are considered mild to moderate range for ASD; children with high functioning autism may score in the t-score range of 55-59. AGR 80-4 had significant language delay and impairment per the ADI-R, but did not meet our broad criteria or have a SRS. Individuals with definite seizures per the ADI-R are marked SZ = 2; those with suspected seizures per the ADI-R are marked SZ = 1.
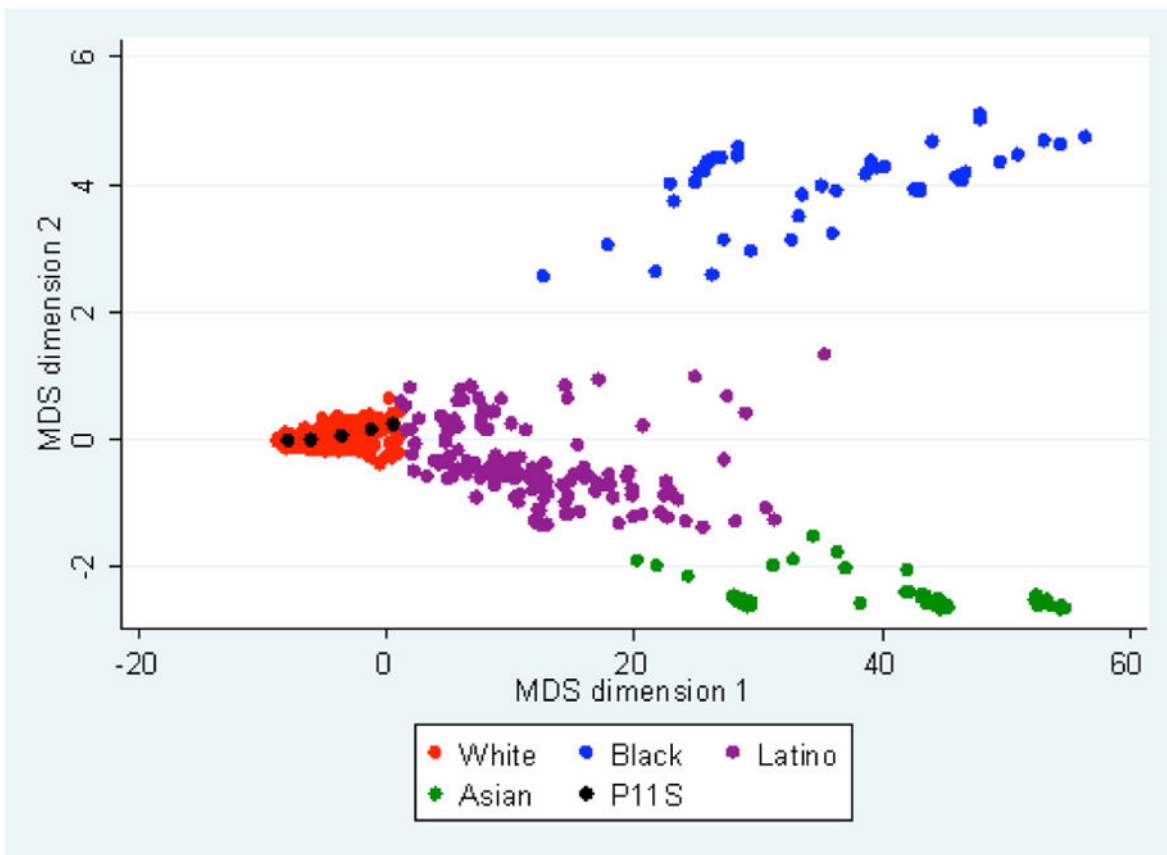
Figure 2. MDS plot of all AGRE parents carrying the S11 risk allele
Black dots represent parents with p11s mutation. Out of 22 AGRE samples with rare
variation, 7 are founders with AGRE Affy 5.0 genome-wide data available. Classical
multidimensional scaling (MDS) was conducted in PLINK using the total sample of AGRE
parents in order to estimate dimensions of population genetic variation. These dimensions
are estimated from genome-wide average proportion of alleles shared by state for each
possible pair of individuals in the sample. Graphical representation of the first two
dimensions is used to identify population substructure and ancestry clusters. Figure 3 shows
the variant to be present in Caucasian parents. Here colors represent best race and ethnicity
approximations while black represents parents with the P11S variant.
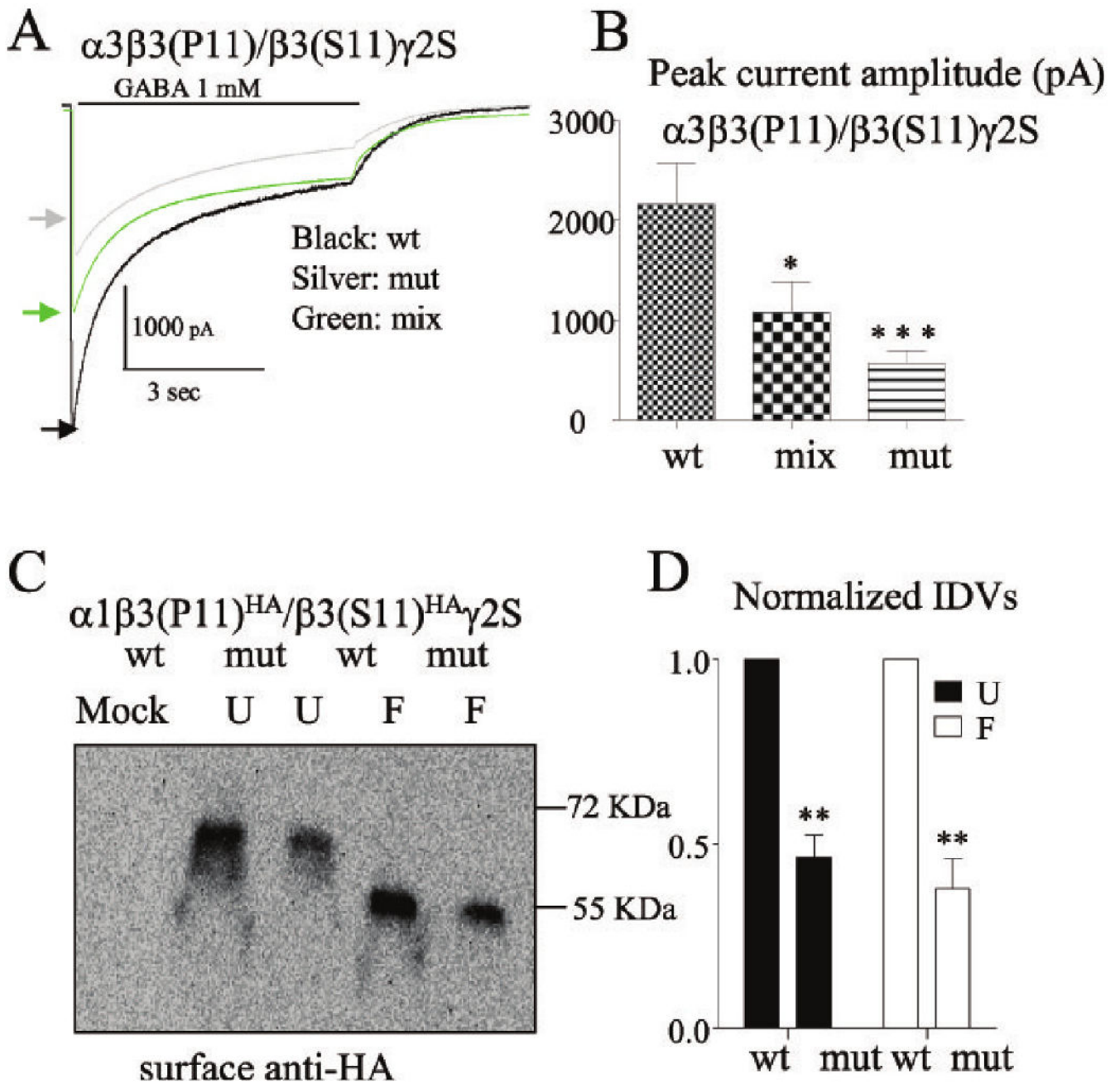
Figure 3. Mutant β3(S11) subunit harboring receptors had reduced current and subunit surface expression

(A) Human GABA$_A$ receptor currents were obtained from HEK 293T cells co-transfected with α3 and γ2S subunit cDNAs and wild-type β3(P11) and the mutant β3(S11) subunit for wild-type (wt α3β3γ2S 1:1:1 cDNA ratio, black), mixed of the wild-type β3(P11) and mutant β3(S11) (1:0.5:0.5:1, mix, green) or for mutant (mut α3β3(S11)γ2S) and evoked with 1 mM GABA for 6 sec (A). In A arrows indicates the peak of each actual trace. (B) The mean peak amplitude of each group was plotted (n = 10 for wt, n = 15 for mix, n = 13 for mutant from three different transfections). (C) HEK 293T cells co-transfected with α1, β3^{HA}

(wt) or β3(P11S) ^{HA} (mut) and γ2S subunit cDNAs. Equal amounts of membrane-bound protein from live cells cell biotinylation, were pulled down with immobilized streptavidin, eluted with 1× NEB glycoprotein protein denaturing buffer (5% SDS, 0.4 M DTT) at room temperature for 30 min. The eluted products were then incubated in absence (U) or presence of PNGAse F (F) for 1hr at 37°C before fractionated by 10% SDS-PAGE and probed with monoclonal anti-HA antibody. (D) The relative amount of surface β3 ^{HA} subunit protein of wild-type and mutant receptors from C was plotted (n = 4). In B and D, the data ere plotted as mean ± SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. wild-type).
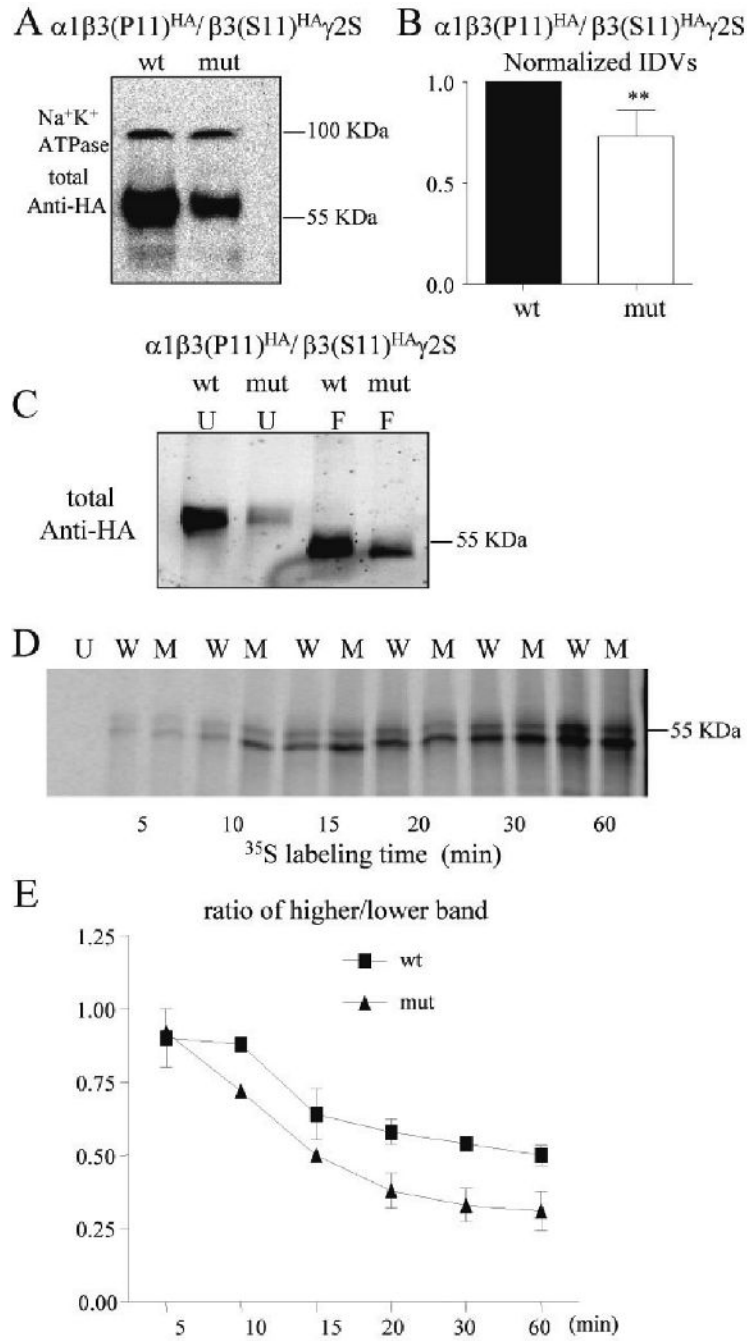
Figure 4. Mutant β3(P11S) subunit protein had impaired intracellular processing
(A) HEK 293T cells co-transfected with α1 and γ2S subunits with HA tagged β3(P11)[HA]
(Wt) or β3(S11) [HA] (mut) subunit cDNAs. Equal amounts of total lysates protein were
analyzed by 10% SDS-PAGE and probed with monoclonal anti-HA and with monoclonal
anti-Na^{+} K^{+} ATPase antibody as internal loading control. **(B)** The relative amount of surface
β3[HA] subunit protein versus loading control of wild-type and mutant receptors from A was
plotted (n = 7) **(C)** Equal amount of total cell lysates from A were undigested (U) or
PNGase-F **(F)** at 37°C for 3 hr. **(DE)** HEK 293T cells containing pulse-chase [35]S

methionine radio-labeled wild-type $\beta 3^{\text{FLAG}}$ (W) and mutant $\beta 3(\text{S11})^{\text{FLAG}}$ (M) subunits were pulse-labeled for a series of time points. The cells were lysed and the same amount of protein for each sample was used for immunopurification and SDS-PAGE (C). The relative ratio of radioactivity of the upper versus lower band is plotted at each time point for either the wild-type or mutant subunits (D, n = 4).

Table 1

Transmission statistics, parental origin and genotype relative risk estimates are provided for the Pro11Ser (C87T from NM_021912) variant rs25409

Affection	Parental origin	Fams.	Informative Trans.	P11 (C) Observed Trans.	P11 (C) Expected Trans.	S11 (T) Observed Trans.	S11 (T) Expected Trans.	χ^2	P	Genotype Relative Risk
Spectrum	Both parents	17	24	9	12	15	12	1.5	0.220	1.67(0.73-3.81)
	Maternal	11	16	4	8	12	8	4.0	**0.046**	**3.00 (0.97-9.30)**
	Paternal	6	8	5	4	3	4	0.5	1.000	0.60(0.14-2.51)
Strict	Both parents	17	21	7	10.5	14	10.5	2.3	0.130	2.57(1.01-5.86)
	Maternal	11	14	2	7	12	7	7.1	**0.008**	**6.00(1.34-26.81)**
	Paternal	6	7	5	3.5	2	3.5	1.3	0.250	0.40 (0.08-2.06)