Phenotypic consequences of deletion of the γ_3, α_5, or β_3 subunit of the type A γ-aminobutyric acid receptor in mice

(p-locus deletions/neurotransmitter receptor/null mutation/Angelman syndrome)

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ABSTRACT   Three genes (Gabrg3, Gabra5, and Gabrb3) encoding the γ_3, α_5, and β_3 subunits of the type A γ-aminobutyric acid receptor, respectively, are known to map near the pink-eyed dilution (p) locus in mouse chromosome 7. This region shares homology with a segment of human chromosome 15 that is implicated in Angelman syndrome, an inherited neurobehavioral disorder. By mapping Gabrg3 on a panel of p-locus deletions, we have determined that the order of genes within this cluster is centromere–p (D15S12h)–Gabrg3–Gabra5–Gabrb3–telomere. Like Gabrb3, neither the Gabra5 nor Gabrg3 gene is functionally imprinted in adult mouse brain. Mice deleted for all three subunits die at birth with a cleft palate, although there are rare survivors ($\approx$5%) that do not have a cleft palate but do exhibit a neurological abnormality characterized by tremor, jerky gait, and runtiness. We have previously suggested that deficiency of the β_3 subunit may be responsible for the clefting defect. Most notably, however, in this report we describe mice carrying two overlapping, complementing p deletions that fail to express the γ_3 transcript, as well as mice from another line that express neither the γ_3 nor α_5 transcripts. Surprisingly, mice from both of these lines are phenotypically normal and do not exhibit any of the neurological symptoms characteristic of the rare survivors that are deleted for all three (γ_3, α_5, and β_3) subunits. These mice therefore provide a whole-organism type A γ-aminobutyric-acid receptor background that is devoid of any receptor subtypes that normally contain the γ_3 and/or α_5 subunits. The absence of an overt neurological phenotype in mice lacking the γ_3 and/or α_5 subunits also suggests that mutations in these genes are unlikely to provide useful animal models for Angelman syndrome in humans.

In adult vertebrate brain, binding of the neurotransmitter γ-aminobutyric acid to type A (GABA$_A$) receptors results in the opening of the receptor's integral membrane Cl$^-$ channel, which leads to an inhibition of neuronal activity (1, 2). Other studies have suggested that during early postnatal development this receptor–ligand interaction has an excitatory function (3). The clinical importance of understanding the biochemistry and the regulation of expression of GABA$_A$ receptors is realized when one considers that these receptors are also targets for a wide variety of pharmaceutically important drugs, such as antiepileptic agents, anxiolytics, muscle relaxants, sedatives, and hypnotics (2, 4).

Comparison of amino acid sequences predicted from cloned cDNAs reveals that GABA$_A$ receptors belong to a superfamily of ligand-gated ion channels, which also includes the glycine and nicotinic acetylcholine receptors (1, 2, 5). Current data strongly suggest an extraordinary diversity of

GABA$_A$ receptors (referred to as subtypes) formed by combinations of four to five subunit polypeptides, although the exact subunit composition is not yet known for any native receptor. For example, at least five GABA$_A$-receptor subtypes have been detected in neurons by in situ immunofluorescence staining with subunit-specific antibodies (6). The 16 subunits so far identified are grouped into five classes: α_{1-6}, β_{1-4}, γ_{1-3}, δ, and ρ_{1-2} (4, 36). Members of the same class exhibit 70–80% amino acid-sequence homology as compared with 30–40% between different classes.

The genes encoding subunits of the GABA$_A$ receptor are differentially expressed depending on the developmental age and tissue type (6–9). In some brain tissues of the rat, there is a developmental switch from embryonic to adult combinations of subunits, but in others the same subunits are expressed throughout life (7). Moreover, although they are primarily distributed in vertebrate brain, certain subunits are also expressed in non-central-nervous-system tissues (7, 8). These observations have raised the possibilities that various modes of GABAergic inhibition are brought about by changes in receptor composition during development and/or in different tissues, which may be a way of fine-tuning neuronal activity (6, 7) and that, in nonneuronal tissues, certain GABA$_A$-receptor subtypes may be involved in functions other than neuronal inhibition (10).

Mapping studies have shown that genes encoding GABA$_A$-receptor subunits occur in gene clusters distributed into different human chromosomes: α_5–β_3 in chromosome 15 (11, 12), γ_1–α_2–β_1 in chromosome 4 (13), and γ_2–α_1 in chromosome 5 (13). Comparative mapping in mice has localized two of these clusters: α_5–β_3 in chromosome 7 (10, 14) and γ_1–α_2–β_1 in chromosome 11 (15). We recently mapped the genes encoding the β_3 and α_5 subunits (Gabrb3 and Gabra5, respectively) on a panel of deletions of the mouse pink-eyed-dilution (p) locus and showed a complete concordance between genomic or transcriptional alterations at the Gabrb3 gene and a usually neonatal-lethal defect in facial development (isolated cleft palate) (10). Recently, the γ_3 subunit (Gabrg3) was also mapped to a p-locus deletion (16).

We report here the mapping of Gabrg3 to a site between p (D15S12h) and Gabra5 by the analysis of the Oak Ridge series of p-locus deletions. Importantly, we describe a phenotypically normal balanced-lethal line of mice carrying overlapping, complementing p deletions that fails to express the γ_3 transcript, as well as another phenotypically normal line that expresses neither the γ_3 nor α_5 transcripts. Thus, the absence of the γ_3 subunit of the GABA$_A$ receptor alone or the absence of both the γ_3 and α_5 subunits does not result in any overt neurological phenotype in mice.

MATERIALS AND METHODS

Mice. The origin, maintenance, and analysis of mouse *p*-locus deletions, as well as construction of *p*-deletion compound heterozygotes, have been described (10, 17).

DNA and RNA Analysis. DNA and RNA extraction and Southern/Northern blot analysis were done as described (17), except that washing of blots was done at a stringency of 0.2× standard saline/citrate/0.2% SDS at 68°C for 30 min. The derivation of the *Gabra5* probe (300/301) is described elsewhere (10). The dilute-gene probe, designated C23, corresponds to nt 1549–3928 of the dilute cDNA sequence and detects three high-molecular-weight transcripts (12, 8, and 7 kb) in adult mouse-brain RNA (18). This probe was included as a control for loading as well as for degradation of high-molecular-mass RNAs (such as the 9.5-kb *Gabrg3* transcript). The probe for *Gabrg3* (designated 304/305) was derived by PCR amplification with primers 5'-TCAAGCTGTC-GAAAGCCAAC and 5'-GTCCAGCTCAGAGACATCAA from first-strand cDNA templates synthesized from mouse brain RNA using a cDNA synthesis kit (Amersham). These primers amplify a 306-bp fragment extending from nt 1046–1352 in the mouse cDNA sequence, a region that codes for the putative intracytoplasmic domain (19). This region displays little homology with other subunits of the same class, thus minimizing cross-hybridization. The PCR was carried out with reagents from Perkin–Elmer and the conditions used were 95°C, 4 min; (94°C, 30 sec; 55°C, 1 min; 65°C, 3 min) for 36 cycles; 72°C, 4 min. All PCR products were purified by affinity chromatography with PrimeErase Quik kit (Stratagene).

RESULTS

Deletion Mapping of *Gabrg3*. Based on analysis of deletion mutations at the *p* locus in mouse chromosome 7, we recently reported that the genes encoding the α_5 and β_3 subunits of the GABA$_A$ receptor (*Gabra5* and *Gabrb3*, respectively) map distal to *p* in the order *p–Gabra5–Gabrb3* (10, 17). By hybridizing a PCR-derived Gabrg3 cDNA subclone to Southern blots of DNAs obtained from a variety of genotypes involving numerous *p* deletions, we have found that the gene (*Gabrg3*) encoding the γ_3 subunit of this receptor likewise maps to this chromosomal segment. Fig. 1*a* shows that a *Gabrg3* probe, which recognizes 23.0-kb and 6.6-kb *Pvu* II fragments in wild-type DNA, fails to hybridize to $p^{4THO-II}/p^{4THO-II}$ DNA, which is also deleted for the *Gabra5* and *Gabrb3* genes (10). Each of 10 other mutations that delete *Gabrb3* and *Gabra5* (p^{8R250M}, p^{47DTD}, p^{2HATh}, p^{45DTD}, p^{116G}, p^{132G}, p^{30PUb}, $p^{23DFiOD}$, p^{55PB}, and $p^{7FR60Lb}$) (10), when tested as compound heterozygotes with $p^{4THO-II}$, also remove *Gabrg3* (data not shown). p^{83FBFo}, the only *p* deletion that removes *Gabra5* but not *Gabrb3* (10), also deletes *Gabrg3* (Fig. 1*a*). Of the six *p* deletions tested that do not delete *Gabra5* and *Gabrb3*, four ($p^{46DFiOD}$, p^{6H}, p^{80K}, p^{4R250H}) delete *Gabrg3*, whereas two (p^{25DVT}, $p^{12R30Lb}$) do not (Fig. 1*a*). These deletion-mapping data, summarized in Fig. 1*b*, are consistent with the order *p–Gabrg3–Gabra5–Gabrb3* and extend recent results that provided evidence that the *Gabrg3* locus maps to the p^{cp} deletion (16).

Mice Homozygously Deleted for *Gabrg3* Alone or for Both *Gabrg3* and *Gabra5*. The deletion map presented in Fig. 1*b* shows that certain compound heterozygotes carrying *p* deletions that complement for prenatal-lethal factors mapping proximal to *p* (10, 17, 21) will be deleted for one or more of the GABA$_A$ subunit genes. For example, the genotype $p^{46DFiOD}/p^{4THO-II}$ would be homozygously deleted for *Gabrg3*, whereas the genotype $p^{83FBFo}/p^{4THO-II}$ would be homozygously deleted for both *Gabrg3* and *Gabra5*. Surprisingly, mice of either of these genotypes are completely viable
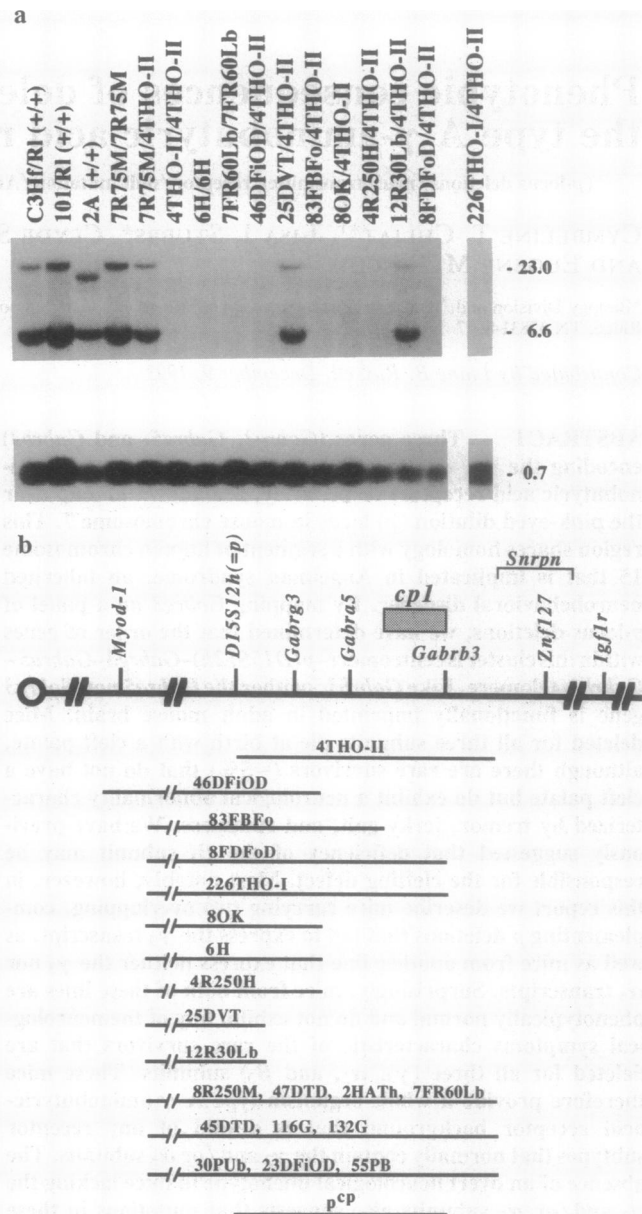

FIG. 1. Mapping *Gabrg3* to the *p–Gabra5* interval. (*a*) A Southern blot of 10 μg of *Pvu* II-digested DNA from animals of the indicated genotypes was hybridized to the Gabrg3 cDNA subclone 304/305, which detects wild-type fragments 23 kb and 6.6 kb in size. Each genotype is given in abbreviated form; for example, "4THO-II/4THO-II" represents $p^{4THO-II}/p^{4THO-II}$. Note that *Gabrg3* is not deleted in p^{25DVT}, which is deleted for the entire coding region of the *p* (*D15S12h*) gene (20), but that *Gabrg3* is missing in the p^{83FBFo} chromosome, which is also deleted for *Gabra5* (10), and in four mutations ($p^{46DFiOD}$, p^{6H}, p^{80K}, and p^{4R250H}) that do not delete *Gabra5*. At bottom is shown hybridization of the same blot to control probe 34-1-111 from the *Znf127* (*D15S9h-1*) locus (17). This control probe detects 4.1-kb and 0.7-kb *Pvu* II fragments, but only the 0.7-kb fragment is shown. A polymorphic fragment of ≈17.0 kb detected by the Gabrg3 probe in stock 2A DNA is irrelevant to this analysis. (*b*) A map of mouse chromosome 7 positioning the GABA$_A$-receptor cluster relative to markers around the *p* locus and to the endpoints of *p* deletions. The centromere is designated by the open circle at left. The abbreviated name of each deletion is written above the line that represents its extent. The deletion mapping of *Gabra5* and *Gabrb3* is presented elsewhere (10, 17). Endpoints of independently isolated deletions that cannot yet be differentiated by available molecular probes are shown as terminating at the same point. Endpoints of the p^{cp} deletion are positioned within the *p* (*D15S12h*) gene proximally and the *Gabrb3* gene distally, according to the data of Nakatsu *et al.* (16).
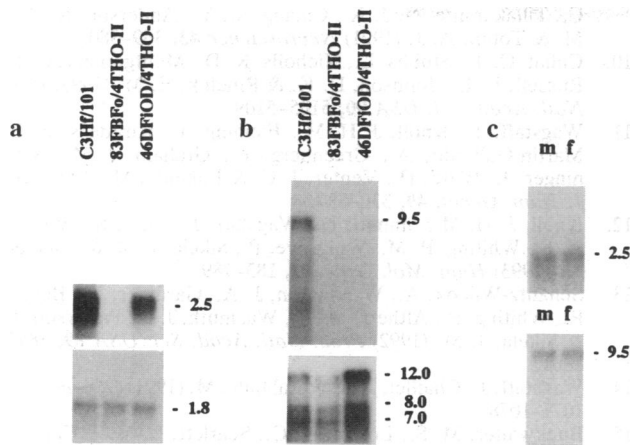
FIG. 2. Expression analysis of Gabrg3 and Gabra5 transcripts. (*a* and *b*) A blot of poly(A)$^+$ RNAs extracted from adult brains of (101 × C3Hf)F$_1$ (C3H/101); $p^{83FBFo}/p^{4THO-II}$ (83FBFo/4THO-II); and $p^{46DFiOD}/p^{4THO-II}$ (46DFiOD/4THO-II) mice was hybridized with a probe for Gabra5 (*a* upper blots) or Gabrg3 (*b* upper blots). Control probes chicken tubulin (*a*) or the chromosome 9 dilute gene (*b*) are shown beneath the respective blots. The sizes of transcripts (Gabra5, 2.5 kb; Gabrg3, 9.5 kb; dilute, 12, 8, and 7 kb; chicken tubulin, 1.8 kb) were determined by comparison to an RNA ladder (Life Technologies, Grand Island, NY) and are indicated at right of *a–c*. The decrease in hybridization intensity of the 2.5-kb Gabra5 transcript in $p^{46DFiOD}/p^{4THO-II}$ brain is due to hemizygous expression of the gene from the $p^{46DFiOD}$ chromosome [Gabra5 is deleted in $p^{4THO-II}$ (10)]. (*c*) A blot containing poly(A)$^+$ RNAs from adult brains of heterozygotes inheriting the $p^{4THO-II}$ deletion from either the male (m) or female (f) parent was hybridized to the Gabra5 (upper) or Gabrg3 probes (lower) as described above. Note that both genes are expressed equally well from the wild-type chromosome inherited from either parent.

and fertile, and, other than manifesting the pink-eyed dilution hypopigmentation phenotype, these animals appear normal. Indeed, there are no obvious neurological abnormalities evident either in gross brain morphology or in behavior. Because Gabrg3 expression has been observed in the developing intestine (7), we also examined mice homozygously deleted for Gabrg3 for defects in this organ. The stomach and all sections of the large and small intestines of adult $p^{46DFiOD}/p^{4THO-II}$ animals appeared grossly normal, and they all had histologically normal submucosal and myenteric ganglia (data not shown).

To confirm that there were indeed no Gabrg3 or Gabra5 transcripts in the apparently neurologically normal animals of the $p^{83FBFo}/p^{4THO-II}$ and $p^{46DFiOD}/p^{4THO-II}$ genotypes, cDNA probes for Gabra5 and Gabrg3 were hybridized to Northern blots containing poly(A)$^+$ RNAs from adult brain. Fig. 2*a* shows that the wild-type 2.5-kb Gabra5 message is absent in $p^{83FBFo}/p^{4THO-II}$ brain but, as expected, is present in $p^{46DFiOD}/p^{4THO-II}$ brain. Also as expected, the 9.5-kb wild-type Gabrg3 transcript is absent in mice of both genotypes (Fig. 2*b*).

Neither *Gabrg3* nor *Gabra5* Is Imprinted in Adult Brain RNA in Mice. The lack of a detectable phenotype in mice heterozygous for the $p^{4THO-II}$ deletion (i.e., $+/p^{4THO-II}$) inherited from either parent suggests that neither Gabrg3 nor Gabra5 is functionally imprinted, similar to the situation found for Gabrb3 (17, 22). The RNA blots of adult mouse brain poly(A)$^+$ RNA presented in Fig. 2*c* confirm this prediction for both genes by showing that the corresponding transcript is expressed equally well from the maternal chromosome (where the $p^{4THO-II}$ deletion is inherited from the sire) or from the paternal chromosome (where the deletion is inherited from the dam).

DISCUSSION

Analysis of mice that carry overlapping, complementing deletions of the *p* locus in chromosome 7 has demonstrated

that the γ$_3$ and α$_5$ subunits of the GABA$_A$ receptor are dispensable for survival and that their absence does not contribute to any overt neurological or other phenotype. Clearly, additional studies will be needed to evaluate whether there are any subtle behavioral or pharmacological consequences of the loss of the γ$_3$ subunit in $p^{46DFiOD}/p^{4THO-II}$ mice and the loss of both the γ$_3$ and α$_5$ subunits in $p^{83FBFo}/p^{4THO-II}$ mice. For example, it is known that a point mutation in the gene encoding the α$_6$-subunit polypeptide is associated with alcohol intolerance in rats, in which animals are highly susceptible to the impairment of postural reflexes by alcohol and benzodiazepines (23).

Disruptions in Gabrb3 expression, however, are completely concordant with neonatally lethal cleft palate (10), and we have hypothesized, based on these findings and on earlier teratological studies (24, 25), that deficiency of the β$_3$ subunit during facial development may be responsible for the clefting defect (10). It is possible that failure to express a normal β$_3$ transcript may also contribute to the variable neurological phenotype (i.e., tremor and jerky gait) exhibited by those rare $p^{4THO-II}$ (10) or p^{cp} (16, 21, 26, 27) homozygotes that escape neonatally lethal cleft palate. On the other hand, the compound heterozygotes reported here demonstrate that failure to express the α$_5$ and γ$_3$ subunits does not contribute to this neurological phenotype, a result in conflict with a recent suggestion that these subunits may play such a role (16).

Intercrossing of $p^{83FBFo}/p^{4THO-II}$ compound heterozygotes creates a balanced-lethal line in which both homozygous classes die [p^{83FBFo}/p^{83FBFo} embryos die prenatally owing to deletion of a lethal factor mapping proximal to *p* (L.B.R. and C.S.M., unpublished data), and ≥ 95% of $p^{4THO-II}/p^{4THO-II}$ neonates die at birth from cleft palate (10)]. Thus, such a cross leaves only the next generation of $p^{83FBFo}/p^{4THO-II}$ compound heterozygotes that are deleted for the γ$_3$ and α$_5$ genes. The availability of completely viable and fertile mutant mouse strains that are deleted for both the γ$_3$ and α$_5$ subunits of the GABA$_A$ receptor (or for the γ$_3$ subunit alone, as with $p^{46DFiOD}/p^{4THO-II}$ heterozygotes) obviates the need for creating null mutations at these loci by gene targeting in embryonic stem cells. These mutant lines thus provide whole-animal models useful for dissecting the role(s) of specific receptor subtypes in the mammalian nervous system, as well as in other tissues of the developing fetus. Such lines should be particularly useful in the analysis of the organismal function(s) of the γ$_3$ and α$_5$ subunits by making available large numbers of animals for a wide range of behavioral or pharmacological studies. In comparison, we estimate that only 1 in 80 to 1 in 100 mice obtained from an intercross of heterozygotes carrying the p^{cp} deletion (16, 21, 26, 27) or the $p^{4THO-II}$ deletion (10) will survive past birth and be homozygously deleted for the γ$_3$ and α$_5$ subunits (as well as for the β$_3$ subunit); moreover, these rare survivors are not very fit, and they often die by weaning age (10, 21, 26, 27).

Angelman syndrome (AS) in humans is a severe neurological disorder that is characterized by seizures, ataxia, absence of speech, stiff jerky movements, and severe mental retardation (28, 29). AS is thought to arise by mutations in an imprinted gene and has been associated with maternal deletion, or uniparental paternal disomy, of a segment of chromosome 15q11–q13 designated the AS critical region (ANCR) (12, 29–31). Because of a potential relationship between defects in GABA$_A$-receptor genes and AS in humans (11, 32), we tested whether Gabra5 or Gabrg3 is functionally imprinted in mouse and now show that neither gene is imprinted in mouse brain, as has been observed for Gabrb3 (17, 22). It has been demonstrated that the Igf2r gene is imprinted in mice but not in humans (33), suggesting that imprinting of specific genes need not be conserved between species; further, it is known that imprinting of expression is not necessarily uniform from tissue to tissue (34). Such considerations

are, however, overshadowed by the observation that no obvious neurological phenotype is detectable in mouse compound-deletion heterozygotes not expressing the γ_3 and α_5 subunits. This finding thus makes it highly unlikely that the *GABRG3* or *GABRA5* genes will contribute to AS in humans or to an AS-like syndrome in mice that could be useful as a model for the human disease. This latter possibility was previously suggested (16) on the basis of the neurological phenotype of the rare p^{cp}/p^{cp} mice that escape cleft-palate-associated neonatal death but are deleted for the γ_3, α_5, and β_3 subunits.

Genetic- and physical-mapping studies have suggested that the order of loci in human 15q11-q13 is centromere–*ZNF127 (D15S9)–[ANCR, GABRB3]–GABRA5–D15S12* (P)–telomere (12, 30, 31). The existence of an AS patient (35) who carries an unbalanced translocation and is hemizygous for loci proximal to *GABRB3* but is heterozygous for a polymorphism in the 3' (proximal) end of the *GABRB3* gene suggests that *GABRB3* itself may not be involved in AS, although it is still not certain whether the β_3 subunit is indeed expressed in this patient. The concordance between genomic or transcriptional alterations at the *Gabrb3* locus and cleft palate in mice (10) provides additional, but not yet conclusive, evidence that this gene is involved in facial development but not in AS. Interestingly, Cattanach *et al.* (22) have suggested that there may not be a paternal-duplication imprinting phenotype in mouse chromosome 7 that is reflective of the human AS phenotype. Comparison of the gene order in mouse of centromere–*D15S12h* (p)–*Gabrg3–Gabra5–Gabrb3–Znf127 (D15S9h-1)*–telomere with that in humans suggests that if mouse models for AS can be identified, they will probably arise from mutations in genes (whether imprinted or not) that map distal to *Gabrb3* (Fig. 1*b*).

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