# Respective roles of isoforms of glutamic acid decarboxylase in GABA synthesis and neuronal function

*Fengyun Ji*

*Doctor of Philosophy*

**Department of Physiological Science,**
**School of Life Science,**
**The Graduate University for Advanced Studies**
**and**
**Laboratory of Neurochemistry,**
**National Institute for Physiological Science,**
**Myodaiji, Okazaki, Aichi 444-8585,**
**Japan**

**1999**

## CONTENTS

# Figures

## *Summary*

γ-Aminobutyric acid (GABA) is believed to play important roles in nervous system development and control of behavior. To elucidate the developmental and functional roles of GABA and to evaluate the GABA synthesis by two isoforms (GAD67 and GAD67) of glutamic acid decarboxylase (GAD) in normal and GAD-deficient tissue, mice carrying null mutations for either GAD65 or GAD67 and the both were analyzed.

Adult GAD65 null mutant mice showed 50% reduction of GABA content in the amygdala and hypothalamus and changes in emotional behavior including aggressive, anxiety etc. GABA contents of GAD67 null mutant mice hippocampus and cerebellum were less than 5% on the first day after birth and increased markedly in organotypic culture. GABA-containing fiber networks also developed well during culture. GABA was barely detectable in GAD65/GAD67-deficient mice but these mice did not show any discernible disorders of histogenesis.

These results indicate that the two isoforms of GAD may have different roles in GABA synthesis and GABA-mediated neural function: GABA is mainly generated by GAD67 during prenatal development while GAD65 is not required in the young mice but appears to be crucial control of behavior in the adult. Although trophic functions of GABA have been suggested, our results indicate that deficits in GABA synthesis have no influence on neurogenesis in GAD mutant mice.

# 1. Introduction:

γ-Aminobutyric acid (GABA) is a principal inhibitory neurotransmitter both in the mammalian central nervous system (Obata, 1977) and in invertebrates including crustacea and nematoda (Kuffler and Nicholls, 1976; McIntire et al., 1993). Its various effects are exerted through two ligand-gated channels, $GABA_A$ and GABAc receptors, and a third receptor, $GABA_B$, which acts through G proteins to regulate potassium and calcium channels (Bowery, 1993). In addition to its inhibitory function, roles as a trophic factor have also been suggested (Barker et al., 1998; Redburn & Schousboe., 1987; Redburn et al., 1986). At developmental stages, GABA is expressed transiently in various non-GABAergic cells in addition to GABAergic neurons (Ma et al., 1992; Van Eden et al., 1989). GABA can enhances neuronal differentiation of both primary culture and neuroblastoma cells in culture (Eins et al, 1983; Spoerri et al., 1981), and induces upregulation of low-affinity GABA receptors (Meier et al., 1983). On freshly dissociated cells or cultured cells, GABA and related substances can affects cell proliferation (LoTurco et al., 1995), cell migration (Behar et al., 1994, 1996, 1998), cell survival (Ikeda et al., 1997 ), neurite extension (Barbin et al., 1993) and synapse formation (Spoerri, 1988). Such effects, however, have so far not been demonstrated during *in vivo* neurogenesis.

GABA is also present in non-neural tissues such as β-cells in the pancreas, in testis and ovary as well as in plants and bacteria. While GABA has been shown to be involved in the formation of the palate in mice (Wee et al., 1983; Asada et al., 1997) and influences

metamorphosis in abalone (Morse et al., 1980), the function of GABA in plants and bacteria remains to be clarified (Satyanarayan et al., 1990; Padmanabhan et al., 1969).

A growing body of evidence suggests a role for altered GABAergic function in neurological and psychiatric disorders of humans, including Huntington's chorea, epilepsy, tardive dyskinesia, alcoholism, sleep disorders, depression, anxiety, panic disorders, Stiffman syndrom and Angelman syndrom (Reberts et al., 1976; Enna et al., 1987; Tanaka et al., 1996). Pharmacological manipulation of GABAergic transmission is an effective approach for the treatment of anxiety (Enna et al., 1987).

GABA is almost exclusively synthesized by α-decarboxylation of L-glutamic acid through glutamic acid decarboxylase (GAD),which is expressed in GABAergic neurons. Possible alternate sources of GABA include putrescence, spermine, spermidine and ornithine, which may be converted to GABA via deamination and decarboxylation reactions, while L-glutamine is a major sources of glutamic acid via deamination (Niranjala et al., 1995). However, the physiological roles of GABA possibly produced by such alternate routes in nervous system are yet unknown. Although GAD appears as a single molecule in C.elegans (Bargmann, 1998), mammalian GAD has two isoforms, GAD65 and GAD67, named after their molecular masses of 65 and 67 kDa and which are encoded by two independent genes respectively (Erlander et al., 1991). The mouse GAD65 and GAD67 genome have been mapped to chromosome 2A2-B and 2D, respectively, and in human, there are at least two homologous genes, located on chromosome 2 and 10, respectively (Edelhoff et al., 1993; Szabo et

al., 1994). The amino acid sequences of rat GAD65 and GAD67 are 65% identical and 80% similar (counting conserved changes), with the N-terminal 100 amino acids being the most divergent (Erlander et al., 1991).

The two isoforms of GAD are coexpressed in GABAergic neurons (Feldblum et al.,1993., Erlander, et al.,1991). Whereas GAD67 is a cytosolic enzyme and distributed throughout the cell body, GAD65 is preferentially located in nerve termini, and is reversibly anchored to the membrane of synaptic vesicles (Kaufman et al.,1991,. Christgau et al.,1991, 1992., Reetz et al.,1991). Such different distribution might derive from the difference in N-terminal structure. Some studies also demonstrated that the two proteins are associated and that through its interaction with GAD65, GAD67 may also be targeted to intracellular membrane compartments, speculating that GAD67 is transported to nerve terminals only after association with GAD65 in the neuronal soma (Dirkx et al., 1995). However, our investigations on the GAD65-deficient mice showed that in despite of the absence of GAD65, GAD67 was transported in nerve terminals (Obata et al., unpublished observations). Studying the roles of either GAD isoform may promote our understanding of GABA function.

In order to disclose any different functions of GAD65 and GAD67 and to elucidate the developmental roles of GABA, we have produced GAD65- and GAD67-deficient mice by gene targeting techniques (Asada et al.,1996; 1997). In previous studies, we demonstrated that mice lacking GAD65 show increased susceptibility to seizures and GABA content are not much different among

GAD65-deficient and wild-type brain until 2 month old. The mice lacking GAD67 are born with greatly reduced levels of GABA but their neural development and functions cannot be investigated since they die of cleft palate soon after birth (Asada et al., 1996, 1997). The present investigations were carried out to clarify the following issues. 1. Although GAD65 is considered to be more involved in neurotransmitter synthesis (Martin and Barke, 1998). Our previous studies (Asada et al., 1996) did not find severe phenotypes in GAD65-deficient mice. Therefore, I investigated the change in GABA content with ages and region and emotional behaviors. 2. GAD67-deficient newborn mice contains GABA less than 5% of wild-type, suggesting that GAD65 is not much active, but have no remarkable defect in neurogenesis. To assess the possible postnatal development of GABA system in GAD67-deficient newborn tissue, I kept it in organotypic culture and analyzed biochemically and immunohistologically. 3. It might be still possible that a small amount of GABA is sufficient for neurogenesis in GAD67-deficient mice, in order to study development of the neural tissue lacking most part of GABA and possible participation of any GAD-independent pathway in GABA synthesis, I produced the mice lacking both GAD isoforms. The results indicated:

1) During prenatal development, GABA was mainly generated by GAD67. In contrast, the production of GABA by GAD65 increased after birth and played an important role in modulation of emotional behavior in adult mice.

2) Morphological abnormality of the nervous system could not be found in the GAD67 mutant mice in spite of the fact that GABA

contents were decreased approximately 15-fold at the first day of life. Although GABA was barely detectable, discernible disruption of histogenesis was not revealed histologically or immunochemically.

3) The alternative pathway for synthesis of GABA by ornithine decarboxylase was only minimally active in fetal mouse brain.

# 2. Materials and Methods:

## 2.1. Production of mutant mice.

GAD65/GAD67-doubly deficient mice were produced by repeated mating of GAD65- and GAD67-mutant mice. Production of GAD65- and GAD67-deficient mice was reported previously (Asada et al., 1996;1997). As GAD67 null mutant mice die of cleft palate immediately after birth (Asada et al, 1997) and most GAD65-/-/GAD67+/- died probably of spontaneous convulsion by the age of 15 weeks especially during pregnancy, GAD65+/-/GAD67+/- double mutant mice were used as breeders in the present study. Analysis was done at the embryonic day14 (E14) and postnatal day 0.5 (P0.5). Genotypes were determined by PCR using genomic DNA from the body or the tail with the probes specific to GAD65 and GAD67 genes (Asada et al., 1996, 1997).

## 2.2. Tissue preparation.

GAD65 mutant mice aged 1-4 month were anethesized with Nembutal and decapitated, their brains were removed, rapidly frozen in liquid nitrogen and stored at -80°C. The cerebral cortex, amygdala, hypothalamus and cerebellum were dissected out from cryosections of frozen brains at -20°C according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997). The tissue samples were homogenized in 300-600 µl of ice-cold phosphate-buffered saline (PBS) (pH7.4) ( Asada et al., 1996), and used to determine amino acid

levels (GABA, glutamic acid and aspartic acid) and for immunoblotting.

### 2.3. Measurement of the contents of GABA and other amino acids.

The GABA contents were measured by high performance liquid chromatography (HPLC) with fluorescence detection (CMA 280, BAS) (Ding et al 1998). The OPA/MCE reagent was prepared by adding 14 μl of 2-mercaptoethanol (1:10 diluted in MeOH) (Sigma) to 1ml of O-phthal aldehyde (OPA) solution (Sigma incomplete, Cat. No. P7914). The samples were mixed with 3.5 μl of OPA/MCE reagent and allowed to react for 1 min. After the reaction was completed, 12 μl of the mixture was applied to HPLC with a capillary column (c-18.5 μm, monomeric 1.0×150 mm, BAS). The mobile phase was delivered at a flow rate of 0.8 ml/min. 0.1M acetate buffer (pH 5.4) and 20% acetonitrile was used for GABA, and 0.1M acetate buffer (pH 6.0) and 10% acetonitrile was used for glutamic acid/aspartic acid, respectively. Chromatograms were stored and analyzed using an on-line computerized data acquisition system (BAS). For the tissue preparation, the samples were homogenized in phosphate-buffered saline (pH 7.4) (see above) and the particulate fraction was removed by centrifugation (5000 rpm, 5 min). Protein concentration of supernatant fraction was determined with BCA Protein Assay Reagent (Pierce, Rockford, IL). After high-molecular weight substances were removed by filtering through a membrane (Ultrafine-MC, 5,000 NMWL Filter Unit; Millipore, Bedford, MA),10 μl of the sample was analyzed

using HPLC. The values of GABA levels were represented as nmol/mg protein.

### 2.4. *In vivo* microdialysis of cerebellum.

Mice (20-30g) aged~16 weeks were anesthetized with Nembutal (50 mg/kg, i. p.) and mounted on a sterotaxic frame (Narishige, Tyokyo, JAPAN). A guide cannula (CUP11, BAS, Tyokyo, JAPAN) was implanted by cementing to the skull following the the coordinated given in the atlas of Franklin and Paxinos (Franklin and Paxinos,1997). The tips were 2 mm posterior to the bregma, -2 mm ventral to the aura and 0 mm from the midline. After 3-4 days, each mouse was transferred to a Plexiglas bowl (40 cm in diameter) allowing free movement and a probe with a 1 mm-long tubular membrane (o. d. 240 μm; molecular cut-off 20 kDa; CUP11, BAS, Tyokyo) was inserted through the guide cannula and perfused with modified Ringer's solution containing (mM): NaCl: 147, KCl: 4, $CaCl_2$: 2.3 , buffered at pH7.2 with 2 mM phosphate buffer at a rate of 1.0 μl/min, by a microinjecter pump (3channels, CMA/200, BAS, Sweden). For high potassium stimulation, the concentration of KCl was increased to 100 mM by replacing NaCl. Two hours after the probe was inserted, dialysate samples were automatically collected every 10 min by a refrigerated microsampler (CMA/200, microinjection pump, CMA Microdialysis, Sweden) and were stored at 4°C throughout the whole experiment. The duration of high potassium stimulation was 10 min (Ding et al., 1998).

## 2.5. Immunoblotting.

Tissue samples (amygdala from GAD65 mutant mice and cultured hippocampus from GAD67 mutant fetuses) were homogenized in PBS containing 0.2% protease inhibitor cocktail solution (Boehringer Mannheim), then 2 volumes of SDS sample buffer were added. Protein levels were quantified (Pierce, BCA protein assay kit) and equal amounts were loaded in each lane. Molecular weight standards (rainbow molecular weight markers from 10,000, to 250,000, Amersham) were loaded along with the samples. Homogenates were separated by electrophoresis on10% or12.5% acrylamide gels. Proteins were transferred to Immunobilon membrane (Millipore). Blots were incubated overnight in Tris-buffered saline (TBS) containing 4% skim milk to block non-specific binding of protein. Proteins were immunostained using anti-GAD antibodies (Asada et al., 1996) and alkaline phosphatase-conjugated second anti-rabbit IgG (Vector). Incubation with antibodies was performed at 37℃ (primary antiserum for 1.5 hr and secondary antibody for 1.5 hr). Blots were incubated in NBT/BCIP solution (Boehringer, Mannheim, GMBH, Germany) to reveal the presence of immunoreaction.

## 2.6. Behavioral Method

***Fear conditioning***

Mice were tested for their response to a conditioned fear stimulus (experimental group). Both contextual and tone-dependent fear conditioning were studied with a four-day test protocol. After

two adaptation sessions (context 1: a plastic cage, 12 x 20 cm, in a sound-proof test box, with dim light and a fan providing background noise), animals were trained in an altered context (context 2: 25 x 25 cm, grid floor, strong light, peppermint smell, fan off) on day two. Animals received three CS/ US pairings in one-minute intervals (CS: 4 Khz, 75 dB for 10 sec; US: 0.7 mA footschock, delivered during the last second of the CS). A control group received the same training stimuli without temporal coincidence. On day three, tone-specific memory of both experimental and control mice was tested in context 1. As behavioral indicator of fear, occurrence of freezing was determined by the experimenter every ten seconds for two minutes without the CS, then for two minutes with the CS (twice 30 seconds duration with 30 seconds pause). At the same time suppression of locomotor activity was measured using a photobeam system (SCANET, Toyo Sangyo). On day four, experimental animals were tested for their context-specific memory over a period of two minutes in the context. Since initial measurements indicated the occurance of an altered expression of fear in GAD mutants, exploratory ambulation, risk assesment behavior(forward-strechíng, observing in crouched position), as well as flight and jumping were also recorded by the experimenter during the retrieval sessions.

***Aggression test***

Intruder-aggression tests were performed as described previously (Stork et al., 1997). In brief, mice were separated at twelve weeks of age and housed singly in transparent cages ("residents"). After four weeks of single housing they were confronted with a

twelve-week-old unfamiliar C57B6/J male ("intruder") for fifteen minutes. Aggressive and other social behavior was evaluated visually in a blind test, measuring the latency for tail-rattling and first attack as well as the total number of attacks and intruder-grooming during the test.

***Forced swimming***

Mice were tested for their swimming activity in a forced swimming model of depression (Porsolt et al., 1978). To measure swimming activity and immobility, mice were placed in a glass cylinder (12 cm in diameter, 15 cm high) filled with water (24 ± 1℃) at a height of 10 centimeters. During the last four minutes of a six minute test, immobility time was determined visually by the experimenter, who was blind for the animals genotype. Swimming activity was measured at the same time using a photobeam system (SCANET).

***Light/dark avoidance test***

Mice were tested for anxiety-like behavior in a light / dark avoidance test (modified from Crawley, 1981) in a single trial of five minutes. After adaptation to the observation room for one hour, animals were placed into the center of the light compartment in a light / dark avoidance apparatus. The total time spent in the dark (L/D time), transitions between dark compartment and light compartment (L/D transitions) and activity in the light and dark compartment were recorded in a blind test using a photobeam system

(SCANET). For pharmacological experiments, animals were injected intraperitoneally with the anxiolytic benzodiazepine diazepam (0.5 mg/1kg body weight), the mixed D1/D2 receptor agonist sulpiride (5.0 mg/kg) or the corresponding volume of the vehicle 30 minutes before testing.

***Statistical analysis***

Statistical analysis was performed using one- and two-way ANOVA analysis and post hoc with Fisher's Protected Least Significant Difference (PLSD). Probabilities<0.05 were considered significant.

**2.7. Slice culture of nervous tissue.**

I adopted a procedure for slice culture of P0 hippocampus and cerebellum by Stoppini et al (1991). Briefly, a 30-mm diameter, sterile, porous (0.4 µm), transparent and low-protein-binding membrane (Millicell Culture Plate Inserts, Millicell-CM, Millipore) was used as a support for the explant. The membranes were placed into a Petri dish containing 1.5 ml of medium. The culture medium was a mixture of MEM Hepes (50% Gibco No, 079-01012), horse serum (25%) and Hank's solution (25%) . Glucose was added to reach a final concentration of 7 mg/ml. After supplemented with penicillin (final concentration: 100 U/1 ml) and streptomycin (final concentration: 100 µg/1 ml), the medium was filtered using Sterivex-GS units (Millipore) .

After brief anesthesia with ice, the fetuses were plunged into

a 70% alcohol solution, their heads were cut with knifes, the brain was removed into MEM Hepes solution to wash out the blood and the desired areas were dissected out and placed on the Teflon stage of a tissue cutter (McILWain tissue chopper). Slices of 350 μm thickness were cut and separated. They were then transferred on the humidified membrane, 4-5 slices each.

The Petri dishes were then maintained in an incubator at 34℃ with 5% $CO_2$ and 95% air and 70% of the medium being changed about twice a week.

### 2.8. Glutamic acid decarboxylase (GAD) activity assay.

For GAD activity studies, cultured slices were separated from the membranes and homogenized in PBS containing 0.2% protease inhibitor cocktail solution (Boehringer Mannheim) and the resulting homogenate was centrifuged at 5,000 rpm for 5 min at 4 C. The pellet was discarded and the supernatant was used immediately for GAD activity assay, as well as for the later protein assay by the method of BCA Protein Assay Reagent (Pierce, Rockford, IL). GAD activity was assayed by quantitfying the liberation of $^{14}CO_2$ from [1-$^{14}$C]-l-glutamic acid (Amersham; 50-60 mCi/mM, 50 μCi/ml). Following homogenization and centrifugation, a 50 μl aliquot of the supernatant were used for this assay in a microtest tube (Assist). First a filter paper (Filter paper No.2; TOYO ADVANTEC) was soaked with 25 μl of 1M hyamine hydroxide in methanol (Sigma) and placed in a cap. Then the tube was added with 50 μl of 0.1 M potassium phosphate buffer (pH 6.8) containing 2 mM EDTA, 2% TritonX-100, 0.05% mercapto-

ethenol, 5 mM L-glutamic acid, 0.2 mM pyridoxal 5'-phosphate (PLP) and 0.5 μCi L-[1-$^{14}$C] glutamic acid and the samples were mixed by pipetting up and down several times, and the tube was closed with the cap immediately. Each sample was assayed as duplicate. To determine the PLP dependent GAD activity, each sample was examined as duplicate in the conditions of presence PLP and absence of PLP in incubation medium. Following incubation for 2 h at 37 ℃. The $^{14}CO_2$ absorbed filter papers were counted in a liquid scintillation counter. GAD activity was expressed as nM $CO_2$ released/ mg protein/2h.

### 2.9. Immunohistochemistry and histology.

Brains and slices attached to membrane were fixed by immersion in freshly prepared fixation solution (4% paraformaldehyde and 0.25% glutaraldehyde in 0.1M phosphate buffer). For immunohistochemistry the tissues were then equilibrated with 30% sucrose in PBS, frozen and 14 μm sections were prepared with a cryostat microtome. Antibodies used were anti-GABA (A-2052; Sigma, St. Louis, MO), anti-calbidin D28 (Maruyama et al.,1985), anti-neurofilament M-subunit antisera (AB 1987; Chemicon, Temecula, CA), and anti-microtubule-associated protein 2 (MAP2) monoclonal antibody (M4403; Sigma, St. Lpuis, MO). Indirect fluorescent immunihistochemistry was performed using fluorescein-conjugated anti-rabbit and mouse IgG antibodies (Cappel, West Chester, PA) were used for detection. Staining with hematoxylin-eosin or cresyl violet was done on thin sections from plastic embedded tissue

(Historesin; Reichert-Jung, Heidelberg, Germany).

### 2.10. Ornithine decarboxylase (ODC) activity assay.

The forebrains from 14-days old embryos of GAD65/GAD67 mutants were isolated and homogenized in PBS (pH 7.4). ODC activity was assayed by quantitating the liberation of $^{14}CO_2$ from [1-$^{14}$C]-l-ornithine (Amersham; 50-60 mCi/mM, 50 µCi/ml) (Packianthan et al 1993). Following homogenization and centrifugation, 100µl aliquots of the supernatant were immediately prepared for this assay in a tube (Assist) with a pyridoxal 5'-phosphate solution designed to provide a final concentration of 0.05 mM, and a cold/hot ornithine mixture with an ornithine concentration of 0.2 mM and 0.5 µCi per assay tube were added to the supernatant. The total assay volume was 170 µl in all cases. Following incubation for 2 h at 37 ºC, the $^{14}CO_2$ absorbed filter papers were counted in a liquid scintillation counter. ODC activity was expressed as nmoles $CO_2$ released/mg protein/2h.

# *3.Results:*

## 3.1. Analysis of GAD65 deficient mice

### 3.1.1. Production of GABA contents in GAD65 null mutants

Previously, our laboratory found no significant changes of GABA contents in the striatum, hippocampus and cerebellum of 1 month-old GAD65 mutant mice (Asada et al,. 1996). Here, 3 regions of brains (i. e. amygdala, hypothalamus and cerebral cortex) were examined for GABA contents in 1-4 month-old GAD65 mutant mice as shown in Table 1. GABA contents were increased after 2 month in all 3 regions of brains in the wild type mice who compared to its levels of 1 month-old mice. Compared between the genotypes, we found that the GABA contents were not significantly changed in the 1 month-old GAD65 mutant mice, in correspondence with previous observations. 2 months later, GABA contents were decreased by almost 50% in the null mice compared with wildtype mice in all three regions. Table 2 and 3 showed no differences between genotypes of the contents of glutamic acid and aspartic acid in the three different regions of brain. As shown in Table 4, GABA content in the cerebellum of 3 month-old mutant mice was also decreased about 25% but no changes in glutamic acid and aspartic acid were observed compared with the wildtype and heterozygous mice.

### 3.1.2. Extracellular GABA concentration in

## GAD65-deficient cerebellum

GABA content were reduced to some extent in GAD65-deficient mice but it might be possible that such reduction does not much affect the neurotransmission or neural functions. Extracellular GABA levels and their changes by potassium stimulation were studied in GAD65 mutant cerebellum with *in vivo* microdialysis. At 2 hr after the insertion of the dialysis probe into the cerebellum, the extracellular level of GABA could be measured stably. The mean value of the first four samples collected at 60 min prior to the administration of high potassium solution was used as the basal value for each mouse. Basal GABA levels differed from 0.08 to 0.61 pmol/10 μl among animals. Such variations were probably derived from the difference in the effective perfusion of neuron-rich area. Administration of high potassium solution produced significant increase in the extracellular GABA levels during the first 15min, followed by a decrease in the extracellular GABA levels (Fig.1). The increase in GABA concentration was also observed in GAD65-deficient cerebellum, but no significant changes were observed among genotypes. The high potassium-induced release of GABA appeared unaffected in GAD65 null mutant cerebellum in spite that GABA content was reduced to about 75% of the wild-type. Patch-clamp studies on cerebellar purkinje cells did not show any significant impairment of GABA-induced synaptic currents which were induced by low-frequency stimulation (Obata et al., in preparation).

### 3.1.3. GAD expression in amygdala of GAD65

**mutant mice**

Immunoblot analysis of the amygdala from GAD65+/+, GAD65+/- and GAD65-/- mice (from 1-months old to 4-months old) was done using anti GAD antibody which recognized a C-terminal portion common to both GAD65 and GAD67, as described previously (Asada et al., 1996) and showed complete loss of GAD65 in null mutants (Fig. 2). The expression of GAD67 protein was unchanged with age and not significantly different among GAD65 genotypes. GAD65 protein in GAD65+/- mice was reduced to about half of that in GAD65+/+ mice. In wild-type mice, the expression of GAD65 was higher in 3-4 months old mice than in younger ones. The increased GABA content was due to the increased GAD65 expression in wild-type mice. Compare with GAD65, GAD67 was absolutely stable among the ages.

### 3.1.4. Emotional behavioral changes of GAD65 deficient mice

GAD65-/- mice showed reduced freezing in the behavioral session of both contextual and cued version of a fear conditioning paradigm, accompanied by increased locomotor activity (Table 5). Preliminary data suggest that these changes were related to an increased flight tendency, rather than exploratory behavior in these animals. Both male residents of GAD65+/- and GAD65-/- genotypes showed reduced attack behavior in an intruder-aggression test ,while other social behavior (e.g. grooming of the intruder) in

GAD65+/- mice was increased (Table 6). In the forced-swim test, reduced floating was seen with GAD65-/- but not with GAD65+/- mice. At the same time, swimming activity counts were increased (Table 7). In the light/dark test, GAD65-/- mice showed increased L/D time (in the dark compartment), reduced activity in the light compartment and reduced transitions between light and dark. GAD65+/- mice displayed intermediate values in these measurements. In both genotypes total motor activity remained unchanged (Table 8).

## 3.2. Analysis of GAD67 deficient mice

### 3.2.1. GABA contents of the cultured cerebellum and hippocampus in GAD67 mutant mice

Cultured hippocampus and cerebellum slices were collected in day 0, day 7 and day 14, and GABA contents were measured. In the first day of culture, GABA contents in GAD67-/- hippocampus and cerebellum (0.15±0.02 [mean±S. D], n=2 and 0.17±0.03, n=2, respectively) were less than 5% of the levels in wildtype and heterozygous tissues (Fig. 3). After14 days in culture, GABA contents in all types of the slices were increased. The increase was much more significant in GAD67-/- tissue. This increase was due to the intact GAD65 activity, suggesting that although not high in the first day, GABA production by GAD65 is increasing postnatally.

### 3.2.2. GAD expression in cultured hippocampus in GAD67 mutant mice

To estimate the expression of GAD proteins under culture condition, immunoblot analysis was performed using anti-GAD65/67 antibody which recognized a C-terminal portion common to both GAD65 and GAD67 (Fig. 4). Before starting culture, the amounts of GAD65 in hippocampal slices were not significantly different among GAD67 genotypes, as observed previously in the cerebral cortex (Asada et al., 1997). After 14 days in culture, GAD65 proteins were increased markedly and to similar levels in all genotypes, indicating that compensatory upregulation of GAD65 protein did not occur in GAD67 deficient hippocampus. As described previously, no increased expression of GAD67 protein was observed in adult GAD65-/- mice (Asada et al.,1996). The Fig. 4 also shows that GAD67 protein in GAD67+/- hippocampus was about half of that in GAD67+/+ and that in GAD67-/- hippocampal slice, it was undetectable. After culture, GAD67 was increased, too, but the relative amounts in three genotypes were maintained similar.

### 3.2.3. Enzymatic activities of GAD in cultured hippocampus in GAD67 mutant mice.

Enzymatic activities of GAD were assayed by production of $^{14}CO_2$ from $^{14}$C-labeled glutamic acid (Fig. 5). Before culture, GAD activity in GAD67-/- hippocampal slice was about a quarter of that in GAD67+/- in the presence of PLP. GAD activities were also increased remarkably during culture. The total activities measured in the presence of PLP were increased about five times in GAD67+/+ and

GAD67+/- hippocampal slices and ten times in GAD67-/- slices after 14 days *in vitro*. At this point, GAD activities in GAD67-/- hippocampal slices were about half of that in GAD67+/- slices. Holoenzyme activity can be evaluated by incubation in the absence of the PLP and it has been reported that a substantial part of GAD67 is present as holoenzyme (Erlander et al., 1991). In the present studies, PLP-independent GAD activities were very low and appeared inappropriate for evaluation of GAD67-/- mice.

### 3.2.4. Immunohistochemical studies of cultured GAD67-/- hippocampus.

To investigate whether the decrease in GABA conditions influence the neurogenesis in the postnatal development, organotypic culture methods were used. Immunohistochemistry with anti-GABA and anti-calbidin-D28 antibodies was performed on the fixed slice preparations attached to the Millicell membrane as they were (Fig. 6). Staining patterns were similar with two anti-GABA antibodies and GABA-containing cells and fibers were demonstrated similarly in the hippocampal and cerebellar slice cultures from both controls and GAD67 null mutant mice (Fig. 6A- E). GABA staining was especially showing in the region facing the fourth ventricle. Presumably this region was derived from the deep cerebellar nuclei, a target of GABAergic Purkinje cell axons. Cerebellar Purkinje cells were selectively labeled with anti-calbindin antibody (Fig. 6 F-H). A proportion of Purkinje cells were apparently lost at the early phase of culture but the remaining cells extended their elaborate dendrites and axons. Their axons fasciculated extended to the pre-

sumable deep cerebellar nuclei (not illustrated) and formed extensive networks there (Fig. 6I). Cultured Purkinje cells appeared of similar morphology among GAD67 genotypes.

## 3.3. Analysis of GAD65-/-:GAD67-/- mice

### 3.3.1. Low frequency of GAD65/67-/- mice and GAD65/67+/+ mice

GAD65-/-:67-/- mice were obtained at reduced frequency, which were unchanged between E14 and P0. Double +/+ mice were similarly rare. The low frequencies of GAD65-/-:67-/- and GAD65+/+:67+/+ mice may be due to the fact that the two genes are located in the same chromosome and doubly mutated chromosomes will be generated only after chromosomal crossing over. The genotypes of their offspring are summarized in Fig. 7.

From their outside appearance, GAD65-/-:67-/- fetuses and pups were undistinguishable from their wild type littermates. However, they did not survive after birth because they developed a lethal cleft palate, characteristic of GAD67-/- mice.

### 3.3.2. GABA contents In E14 brains from GAD65/67 mutant mice

The GABA contents of the E14 forebrain are summarized in Table 9. These results were obtained from the analyses of 7 litters from GAD65+/-: 67+/- parents. There was no GAD65+/+: 67+/+ fetus

among these mice. Since no significant difference in GABA content was found between the wildtype and heterozygous brains in a previous analysis of GAD65-mutant fetuses (Asada et al., 1996), it was assumed that GABA content in GAD65+/+: 67+/- fetal brains would not differ much from that in GAD65+/+: 67+/+. The GABA content in the mice lacking both GAD isoforms was very low, i.e., 0.02% of the GAD65+/+: 67+/-mice, and 0.3% of the GAD65+/-:67-/- mice.

### 3.3.3. Ornithine decarboxylase (ODC) activity in E14 and P0 brains from GAD65/67 mutant mice.

Since the small amount of GABA in GAD65-/-:67-/- brain might be derived from an alternative GABA-synthetic pathway, I measured the enzymatic activity of ODC which might be involved in such pathway (Eliasson et al., 1997). As shown in Table 10, the ODC activities were detected in the E14 and P0 brains but did not differ significantly among the GAD genotypes including GAD65-/-:67-/-.

### 3.3.4. Histological and Immunohistochemical studies of GAD65/67 mutant mice

The size and shape of the GAD65-/-:67-/- brains at P0 was not distinguishable from those of their littermates with the naked eye or under a dissecting microscope. Histologically, no abnormality was detected in the neocortex (Fig. 8A and B), cerebelum (Fig. 8C and D) and hippocampus (Fig. 8E and F) of GAD65-/-:67-/- mice.

Extensive GABA-like immunoreactivity was observed in P0

brains of the wildtype mice (Fig, 9). In the cerebellum (Fig. 9A and C), Purkinje cells (cf. Fig.10A. B and H) and other cells located bellow the Purkinje cell layer were labeled by anti-GABA antibody. Some of the latter, probably Golgi cells , were intensely stained. Immunoreactive cells were also dispersed in the cerebral cortex (Fig, 9E). Fibers in the superficial layer were also labeled. The immunoreactivity was especially intense in cell bodies and processes in the superior colliculus (Fig. 9D). In contrast, GABA reactivity was never detected in GAD65-/-: 67-/- brains (Fig, 9B and F).

Immunostaining with several cell type- or neuron-specific antibodies was also performed (Fig. 10). In the cerebellum, Purkinje cells are typical GABAergic neurons (Obata, 1977) and are selectively labeled with anti-calbindin-D 28k antibody (Talamoni et al., 1993). In the P0 mice, calbindin labelled cells did not form a single-cell layer and their dendrites were just developing (Fig.10 A, B and F). The staining with anti-calbindin antibody, however, was similar between control and GAD-deficient mice.

Development of axons and dendrites was revealed by anti-neurofilament and anti-MAP2 antibodies, respectively. No defects were observed in the cerebellum, neocortex and hippocampus of P0 GAD-deficient mice (Fig.10C-E).

# 4. Discussion

## 4.1. GAD65 deficient adult mice showed reduction of GABA contents in the amygdala and hypothalamus and changes in emotional behavior

Previous investigations showed GAD65 null mutant mice had an increased susceptibility to seizures induced by chemical convulsants. But GABA contents in the striatum, hippocampus, cerebral cortex and cerebellum were not much different among GAD65-deficient and wildtype brains until its age of 2 months. Moreover, the general behavior, including locomotor activity and performance in the Morris water maze test, also appeared normal (Asada et al., 1996).

In the present experiments, I studied the spatial and temporal changes of GABA contents in GAD65-deficient mice. The results showed that GABA content was significantly increased after 2 month in wild type mice in amygdala, cerebral cortex and hypothalamus, compared to I month of animals, on the contrary, GABA contents were fairly unchanged over the examined period in GAD65-deficient mice, failing to show the increase as in controls. Compared with GAD65+/+ mice, GAD65-deficient mice showed several behavioral abnormalities, i. e, increased sensitivity to convulsants; increased anxiety; reduced intermale aggression; reduced forced swimming-induced immobility, reduced freezing and increased flight in response to a conditioned fear stimulus.

Several elements of data also provided evidence of strong

involvement of GABAergic mechanisms in the control of aggressive behavior (Miczek et al., 1995). The GABA levels were found to be lower in the olfactory bulbs of aggressive versus non-aggressive mouse strains (Early et al., 1977), as well as in muricidal rats as opposed to non-muricidal rats (Mohanan et al.,1990). Pharmacological reduction of GABAergic inhibition in rats induced an increase in muricidal behavior (Mandel et al., 1979). The concentration of GABA in the whole brain is inversely related to the level of aggressive behavior exhibited (Early et al., 1977). Using tests to measure the capacity of males to attack a passive standard opponent in a resident-intruder test and the preferences in a dark/light choice situation, it was suggested that males of more attacking strains had a higher level of anxiety but did not differ for their level of activity. The results also suggested an involvement of GABA as a mediating factor between pleiotropic genes and the two behavioral phenotypes, i.e., capacity to attack and anxiety (Guillot et al., 1996). But the present study by analysis of GAD65-deficient mice obtained an opposite result in the aggressive behavior. The amygdala, the hypothalamus and bed nucleus of the stria terminalis are brain regions enriched in androgen and estrogen receptors in males and are thought to mediate addressiveness and sexual behavior (Melis and Argiolas, 1995). The decreased GABA content was also found in the other regions of brain such as hypothalamus in GAD65-deficient mice. The regulation of hormone release by hypothalamus may have been affected, resulting in reduced intermale aggression (Early et al., 1977).

The amygdala is a critical component of the circuit that mediates fear conditioning (LeDoux et al.,1995; Davis et al.,1994).

GABA can regulate cellular excitability in the lateral and basolateral amygdaloid nuclei directly or transsynaptically (Michael et al.,1994). These direct decreases in excitability might explain the behavioral changes in response to a conditioned fear stimulus obtained from our GAD65-deficient mice. However, such emotional changes of GAD65-deficient mice might also result from an alteration in the release of noradrenaline which is known to modulate learning within the amygdala (McGaugh et al., 1992).

## 4.2. GABAergic development in organotypic culture of the hippocampus and cerebellar slices from GAD67-deficient mice

Since GAD67-deficient mice die from cleft palate early after birth, organotypic culture of the hippocampal and cerebellar slices were used to investigate the development of GABAergic structure after birth. After 2 weeks in culture, GABA contents were markedly increased and GABA-containing neurites were extensively developed in GAD67 null mutant tissue. Cerebellar Purkinje cells, typical GABAergic neurons , were normally developed in the absence of GAD67 (also see Obata and Ji. 1998). These results demonstrated that GAD65 maintained sufficient GABA synthesis in cultured GABAergic neurons without GAD67. On the other hand, GAD65 was not required in the young mice (Asada et al., 1996).

Developmental changes in GAD and GABA expression were investigated on the rat spinal cord (Szabo et al., 1994; Somogyi et al., 1995). Beginning at E11, both the GAD65 mRNA and GAD67 mRNA can

be detected by quantitive PCR (Somogyi et al., 1995). GAD65 mRNA expression undergoes an exponential growth phase, followed by a gradual increase at E15 to maximal mRNA levels at E18. The exponential phase of GAD67 mRNA induction lasts for 4 d, a gradual increase through the end of prenatal development culminats in maximal expression at P0, followed by a decline to adult levels, approximately threefold lower than maximal (Somogyi et al., 1995). The protein of GAD65 is initially detected at E17 in the spinal cord, thereafter, GAD65 protein increases, reaches its maximum level at P14; the protein of GAD67 can be detected at E13 and increases similar to GAD65 (Behar et al., 1993). Northern blot analysis of GAD mRNAs in developing striatum has shown an eightfold increase in GAD65 between P0 and P14, with the increase in GAD67 mRNA roughly linear while P21 and levels increasing 50% after this point (Greif et al., 1992).

Our cultured samples showed the same changes of GAD65 but not of GAD67 expression described above: After 14 days in culture, GAD65 proteins and GABA contents were markedly increased in GAD67 null mutant hippocampal and cerebellar slices with GAD65 protein not different between genotypes, GAD67 protein was extensively increased during the culture period in GAD67+/+ and GAD67+/- mice. Comparable levels of GAD65 among genotypes indicated that upregulation of the GAD65 protein did not occur in the GAD67- deficient hippocampus. The different results of GAD67 expression may be due to the difference between the rat and mouse or the different regions of nervous system (Ji et al., in press).

## 4.3. GABA and histogenesis in fetal and neonatal mouse brain lacking two isoforms of glutamate decarboxylase

Our results confirmed that the GABA is almost exclusively synthesized by GAD65 and GAD67 in mouse brain, and indicated that a compensatory induction of ODC did not occur for GABA synthesis in GAD-deficient tissues.It is possible that ODC could be involved in GABA synthesis by the production of putrescence (Eliasson et al., 1997), because the successive action of diamine oxidase and aldehyde dehydrogenase converts putrescine to GABA (Seiler et al., 1971). An up-regulation of ODC might occur in GAD-deficient mice, but this was not found in the present studies.

The present results also indicated that the GABA contents in the fetal brains largely depended on the GAD67, as observed in GAD67-mutant brains (Asada et al., 1997).

Previous *in vitro* investigations have suggested that GABA is involved in histogenesis in the central nervous system (Barker et al., 1998 for review) and, therefore, in the present study we elucidated the *in vivo* roles of GABA by analyzing GABA-deficient brains morphologically. In the GAD65-/-:67-/- brains at the fetal and newborn stages, GABA was scarcely detected but histogenesis progressed without any serious impairment.

No discernible defect of neural development was noted in the GAD65/67-deficient brain, macro- and microscopically. Cerebellar Purkinje cells developed morphologically, but without the expression of their neurotransmitter, GABA. Gonadotropin-releasing

hormone (GnRH)-expressing cells were distributed similarly in the preoptic area and hypothalamus of GAD-deficient and control mice similarly (Obata et al, unpublished observation), although the involvement of GABA in the cell migrations of GnRH neurons from the olfactory placode to the brain has been suggested (Fueshko et al., 1998; Tobet et al., 1996).

The present studies failed to support the trophic roles of GABA in neurogenesis which were proposed by previous *in vitro* investigations (Barker et al., 1998). The following possibilities can be considered to account for this discrepancy.

1. Most investigations suggesting the trophic roles of GABA were performed on isolated rat preparations. The functions of GABA might be different between the rat and mouse. GABA- induced migration is demonstrated on rat embryonic cells (Behar et al., 1996, 1998) but was not observed on mouse cells (J. L. Barker, personal communication).

2. *In vitro,* other substances such as glutamate, taurine and glycine could potentially compensate for the trophic action of GABA. Agonists of glutamate or GABA similarly increase the intracellular $Ca^{2+}$ concentration which is expected to activate various intracellular signaling cascades (Lin et al., 1994; LoTurco et al., 1995; Yuste and Katz., 1991). GABA and glutamate similarly depress cell proliferation in the ventricular zone isolated from embryonic rat neocortex (LoTurco et al., 1995). Similar to GABA, taurine and glycine inhibit neuronal activities in adult nervous system (Obata, 1977). Flint et al. (1998) demonstrated that these substances induce depolarization in fetal and neonatal rat cortical neurons by activat-

ing glycine receptors.

3. The trophic effects of GABA may not have a role in histogenesis at the fetal stage, but may function at the neonatal stage, during which the adjustment and refinement of synaptic organization are in progress under the control of neuronal activities (Chen and Tonegawa, 1997; Katz and Shatz., 1996). GABA-induced depolarization is observed in most neurons from neonatal animals. At this stage, AMPA-type glutamate receptors are not yet developed and GABA-induced depolarization would be essential for the increase in the intracellular $Ca^{2+}$ concentration through the voltage-dependent $Ca^{2+}$ channel and NMDA receptor (Ben- Ari et al ., 1997).

## 4.4. Conclusion

The two isoforms of GABA synthetic enzyme, GAD65 and GAD67 appear to have different roles in GABA sythesis and neural function: GABA is mainly generated by GAD67 during prenatal development, while GAD65 is not required in the young mice but seems indispensable in the adult mice. GABA contents in several brain regions of GAD65 deficient mice are reduced to nearly 50% and a largely abnormal emotional behavior is observed (see above and Obata et al., 1998; Stork et al., 1998). Although GABA was scarcely detectable in GAD65/67-deficient brains, no disernible disorders of histogenesis were detected in the brains by either histological or immunohistochemical analysis (Ji et al., in press).

# 5. Acknowledgments

I am deeply grateful to **professor K. Obata**, my supervisor for his generous support and valuable guidance throughout my work during my Ph. D. Course in The Graduate University for Advanced Studies and the Laboratory of Neurochemistry, N. I. P. S.

I thank deeply **Dr. S. Furuya** for histochemical techniques.

I am grateful to **Dr. O. Stork** for behavioral analysis.

I am also grateful to **Dr. K. Maruyama , Dr. N. Kojima** and **Dr. Y. Yamagata** for their kind encouragement and useful discussions. My sincere thank to **Dr, H. Kume** for critical discussions. I wish to thank **Ms. H. Kuzume and Ms. N. Kanbara** for technichal support.

I thank Japanese Ministry of Education, Science, Sports and Culture (Monbusho) for financial support.

# *References*


Asada, H., Kawamura, Y., Maruyama, K., Kume, H., Ding, R. G., Ji, F. Y., Kanbara, N., Kuzume, H., Sanbo, M., Yagi, K., and Obata, K. (1996), Mice lacking the 65 kDa isoform of glutamic acid decarboxylase (GAD65) maintain normal levels of GAD67 and GABA in their brain but are susceptible to seizures. *Biochem. Biophys. Res. Com.*, **229,** 891-895.

Asada, H., Kawamura, Y., Maruyama, K., Kume, H., Ding, R. G., Kanbara, N., Kuzume, H., Sanbo, M., Yagi, K., and Obata, K. (1997), Cleft palate and decreased brain γ-aminobutyric acid in mice lacking the 67 kDa isoform of glutamic acid decarboxylase. *Proc. Natl. Acad. Sci. USA.* **94,** 6496-6499.

Barbin, G., Pollard, H., Gaiarsa, J. L., Ben-Ari, Y. (1993), Involvement of $GABA_A$ receptors in the outgrowth of cultured hippocampal neurons. *Neurosci. Lett.* **152,**150-154.

Bargmann, C. I. (1998), Neurobiology of the Caenorhabditis elegans genome. *Science.* **282**:2028-2033.

Behar, T. N., Schaffner, A. E., Colton, C. A., Somogyi, R., Olah, Z., Lehel, C., Barker, J. L. (1994), GABA-induced chemokinesis and NGF-induced chemotaxis of embryonic spinal cord neurons. *J. Neurosci.* **14,** 29-38.

Behar, T. N., Li, Y. X., Tran, H. T., Ma, W., Dunlap, V., Scott, C., Barker, J. L. (1996), GABA stimulates chemotaxis and chemokinesis of embryonic cortical neurons via calcium-dependent mechanisms. *J. Neurosci.* **16,**1808-1818.

Behar, T. N., Schaffner, A. E., Scott, C. A., O'Connell, C., Barker, J. L.

(1998), Differential response of cortical plate and ventricu lar zone cells to GABA as a migration stimulus. *J. Neurosci.* **18**, 6378-6387.

Barker, J. L., Behar, T., Li, Y.-X., Liu, Q.-Y., Ma, W., Maric, D., Maric, I., Wen, X.-L., Xiao, H. (1998), GABAergic cells and signals in CNS devel opment. *Perspect. Dev. Neurobiol.* **4**,1-16.

Ben-Ari, Y., Kelly, J S. (1976), Dopamine evoked inhibition of single cells of the felline putaman and basolateral amygdala. *J, Physio., Lond.* **256,** 1-21.

Ben-Ari, Y., Khazipov, R., Leinkugel, X., Caillard, O., Gaiarsa, J. L. (1997), GABAA, NMDA and AMPA receptors: a developmentally regulated 'menage a trois'. *Trends Neuriosci.* **20**, 523-529.

Bond, R. W., Wyborski, R. J., Gottlieb, D. I. (1990), Developmentally regulated expression of an exon containing a stop codon in the gene for glutamic acid decarboxylase. *Proc. Natl .Acad. Sci. USA.* **87**, 8771-8775.

Bowery, N. G. (1993), GABAB receptor pharmacology. *Annu. Rev. Phamacol. Toxicol.* **33**, 109-147.

Bu, D. F., Tobin, A. J. (1994), The exon- intron organization of the genes ( GAD1 and GAD2) encoding two human glutamate decarboxylase ( GAD65 and GAD67 ) suggests that they derive from a common ancestral GAD , *Genomics* **21,** 222-228.

Chen, C., Tonegawa, S. (1997), Molecular genetic analysis of synaptic plasticity, activity-dependent neural development, learning, and memory in the mammlianb brain. *Annu. Rev. Neurosci.* **20**,157-184.

Christgau, S., Schierbeck, H., Aanstoot, H. J., Aagaard, L., Begley, K.,

Kofod, H., Hejnaes, K., Baekkeskov, S. (1991), Pancreatic beta cells express two autoantigenic forms of glutamic acid de carboxylase, a 65-kDa hydrophilic form and a 64-kDa am phiphilic form which can be both membrane-bound and soluble. *J. Biol. Chem.* **266,** 21257-21264 .

Christgau, S., Aanstoot, H. J., Schierbeck, H., Begley, K., Tullin, S., Hejnaes, K., Baekkeskov, S. (1992), Membrane anchoring of the autoantigen GAD65 to microvesicles in pancreatic beta-cells by palmitoylation in the NH2-terminal domain. *J .Cell. Biol.* **118,** 309-320.

Davis, M., Rainnie, D., Cassell, M. (1994 ), Neurotransmission in the rat amygdala related to fear and anxiety. *Trends Neurosci.* **17,** 208-214.

de Talamoni, N., Smith, C. A., Wasserman, R. H., Beltramino, C., Full mer, C. S., Penniston, J. T. (1993), Immunocytochemical lo calization of the plasma membrane calcium pump, calbindin-D28k, and parvalbumin in Purkinje cells of avian and mammalian cerebellum. *Proc. Natl. Acad. Sci. U S A.* **90,** 11949-11953.

Ding, R. G., Asada, H., Obata, K. (1998), Changes in extracellular glutamate and GABA levels in the hippocampal $CA_3$ and $CA_1$ araeas and the induction of glutamic acid decarboxylase-67 in dentate granule cells of rats treated with kainic acid. *Brain . Res.* **800,**105-113.

Dirkx, R .Jr., Thomas, A., Li, L., Lernmark, A., Sherwin, R. S., De, Camilli, P., Solimena, M. (1995), Targeting of the 67- kDa isoform of glutamic acid decarboxylase to intracellular

organelles is mediated by its interaction with the NH2-terminal region of the 65-kDa isoform of glutamic acid decarboxylase. *J. Biol. Chem.* **270**, 2241-2246.

Dudel, J., Hatt, H. (1976), Four types of GABA receptors in crayfish leg muscles characterized by desensitization and specific antagonist. *Pflugers Arch.* **364**:217-222.

Early, C. J., Leonard, B. E. (1977), The effect of testosterone and cyprosterone acetate on the concentration of gamma-ami nobutyric acid in brain areas of aggresssive and non-aggressive mice. *Pharmacol. Biochem. Behav.* **6**, 409-413.

Edelhoff, S., E Grubin, C. E., Karlson, A. K., Adler, D. A., Foster, D., Disteche, C. M., Lernmark, A. (1993), Mapping of glutamic acid decarboxylase (GAD) genes. *Genomics* **17**, 93-97.

Eins, S., Spoerri, P. E., Heyder, E. (1983), GABA or sodium-bromide-induced plasticity of neurites of mouse neuroblastoma cells in culture. A quantitative study. *Cell Tissue Res.* **229**, 457-460.

Eliasson, M. J. L., McCaffery, P., Baughman, R .W., Drager, U. C. (1997), A ventrodorsal GABA gradient in the embryonic retina prior to expression of glutamate decarboxylase. *Neuroscience* **79**, 863-869.

Enna, S. J., Mohler, H. (1987), Gamma-aminobutyric acid (GABA) receptors and their association with benzodiazepine recogni tion sites. In H. Y. Meltzer (ed.), *Psychopharmacology: The Third Generation of Progress*. New York: Raven Press, pp. 265-272.

Erlander, M. G., Tillakaratne N. J. K., Feldblum S., Patel N., Tobin A.

J. (1991), Two genes encode distinct glutamic acid decar boxylase, *Neuron* **7,** 91-100.

Erlander, M. G., Tobin, A. J. (1991), The structural and functional heterogeneity of glutamic acid decarboxylase: a review. *Neurochem. Res* . **16,** 215-226.

Esclapez, M., Tillakaratne, N. J., Kaufman, D. L., Tobin, A. J., Houser, C. R. (1994), Comparative localization of two forms of glutamic acid decarboxylase and their mRNAs in rat brain supports the concept of functional differences between the forms. *J. Neu rosci.* **14**,1834-1855.

Feldblum ,S., Erlander, M. G., Tobin, A. J. (1993), Different distribu tions of GAD65 and GAD67 mRNAs suggest that the two gluta mate decarboxylases play distinctive functional roles. *J. Neurosci. Res.* **34**, 689-706.

Flink, A. C., Liu, X., Kreigstein, A. R. (1998). Nonsynaptic glycine receptor activation during early neocortical development. *Neuron* **20**, 43-53.

Franklin, B. J., Paxinos George, (Eds), (1997) , (Eds) *The Mouse Brain in Stereotaxic Coordinates* . Academic Press. New York.

Fueshko, S. M., Key, S., Wray, S. (1998), GABA inhibits migration of luteinization hormone-releasing hormone neurons in embry- onic olfactory explants. *J. Neurosci.* **18,** 2560-2569.

Greif, K. F., Tillakaratne, N. J., Erlander, M. G., Feldblum, S., Tobin, A. J. (1992), Transient increase in expression of a glutamate decarboxylase (GAD) mRNA during the postnatal development of the rat striatum. *Dev. Biol.* **153**, 158-164.

Guillot, P. V., Chapouthier, G. (1996), Intermale aggression and

dark/light preference in ten inbred mouse strains. *Behav. Brain Res.* **77**, 211-213.

Ikeda, Y., Nishiyama, N., Saito, H., Katsuki, H. (1997), GABAA receptor stimulation promotes survival of embryonic rat striatal neurons in culture. *Dev. Brain. Res.* **98,** 253-258.

Ji, F. Y., Kanbara, N., Asada, H., Obata, K. (1998), GABA content and histogenesis of fetal and newborn mice lacking two isoforms of glutamic acid decarboxylase. *Soc. Neurosci. Abstr. Vol.* **24, part 1**, p.347.

Ji, F. Y., Kanbara, N., Obata, K. (1999). GABA and histogenesis in fetal and neonatal mouse brain lacking two isoforms of glutamic acid. *Neurosc. Res.* (in press).

Ji, F. Y., Obata, K. (1999), Development of the GABA system in organotypic culture of hippocampal and cerebellar slices from 67 kDa isoform of glutamic acid decarboxylase (GAD67)-deficient mice. *Neurosc. Res.* (in press).

Kaufman, D. L., Houser, C. R., Tobin, A. J. (1991), Two forms of the gamma-aminobutyric acid synthetic enzyme glutamate decar boxylase have distinct intraneuronal distributions and cofac tor interactions. *J. Neurochem.* **56,** 720-723.

Kash, S. F., Johnson, R. S., Tecott, L. H., Noebels, J. L., Mayfield, R. D., Hanahan, D., Baekkeskov, S. (1997), Epilepsy in mice deficient in the 65-kDa isoform of glutamic acid decarboxylase. *Proc. Natl. Acad. Sci. USA* **94,** 14060-14065.

Katz, L. C., Shatz, C. J. (1996), Synaptic activity and the construction of cortical circuits. *Science* **274,** 1133-1139.

Kuffler, S. W., Nicholls, J. G. (1976), *From neuron to brain: A cellular*

*approach to the function of the nervous system.* pp: 219-236.

LeDoux, J. E. (1995), Emotion: clues from the brain. *Annu. Rev. Psy chol.* **46,** 209-235.

Lernmark, A. (1996), Glutamic acid decarboxylase--gene to antigen to disease. *J. Intern. Med.* **240,** 259-277.

Lin, M.-H., Takahashi, M. P., Takahashi, Y., Tsumoto, T. (1994), In tracellularcalcium increase induced by GABA in visual cortex of fetal and neonatal rats and its disappearance with devel opment. *Neurisci. Res.* **20,** 85-94.

LoTurco, J.J., Owens, D. F., Heath, M. J. S., Davis, M. B. E., Kriegstein, A. R. (1995), GABA and glutamate depolarize cortical progenitor cells and inhibit DNA sythesis. *Neuron* **15,** 1287-1298.

Ma, W., Behar, T., Barker, J. L. (1992 ), Transient expression of GABA immunoreactivity in the developing rat spinal cord. *J. Comp. Neurol.* **325,** 271-290.

Mandel, P., Ciesielski, L., Maitre, M., Simler, S., Mack, G., Kempf, E. (1979), Involvement of central GABA-ergic systems in convulsions and aggressive behavior. *Adv. Exp. Med. Biol.* **123,**475-492.

Mandel, P., Mack, G., Kempf, E. S. (1979), Molecular basis of same models of aggressive behavior. In psychopharmacology of aggression. (M. Sandler, ed). Raven Press, New York, pp. 95-110 .

Martin, D. L and Barke, K. E. (1998), Are GAD65 and GAD67 associated with specific pools of GABA in brain? *Perspect. Dev. Neurobiol.* **5.** 119-129.

McGaugh, J. L. (1992), *In The Amygdala. Neurobiological Aspects of*

*Emotion, Memory, and Mental Dysfunction* ( Aggleton, J. P., ed) pp, 431-451.

McGaugh, J. L,, Introini-Collison, I. B., Cahill, L. F., Castellano, C., Dalmaz, C., Parent, M. B., Williams, C. L. (1993). Neuromodula tory systems and memory storage: role of the amygdala. *Behav. Brain. Res.* **58,** 81-90.

McIntire, S. L., Jorgensen, E., Kaplan ,J., Horvitz, H. R. (1993), The GABAergic nervous system of Caenorhabditis elegans. *Nature.* **364:** 337-341.

McIntire, S. L., Jorgensen, E., Horvitz, H. R. (1993), Genes required for GABA function in Caenorhabditis elegans. *Nature* **364**:334-337.

Meier, E., Drejer, J., Schousboe, A. (1983), Trophic actions of GABA on the development of physiologically active GABA receptors. *Adv. Biochem. Psychopharmacol.* **37,** 47-58.

Melis, M. R., Argiolas, A. (1995), Dopamine and sexual behavior. *Neurosci. Biobehav. Rev.* **19,**19-38.

Miczek, K. A., Weerts, E M., Vivian, J. A. & Barros, H. M. (1995), Ag gression , anxiety and vocalizations in animals: GABA A and 5-HT anxiolytics. *Psychopharmacology*, **121,** 38-56.

Mohanan, E., Maxson, S. C. (1990), Chemosignal dependent and inde pendent effects of Y chromosomal gene(s) on offense in mice. *Behav.Genet.* **20,** 735-745.

Morse, D. E., Duncan, H., Hooker, N., Baloun, A., Young, G. (1980), GABA induces behavioral and developmental metamorphosis in planktonic molluscan larvae. *Fed. Proc.* **39,** 3237-3241 .

Niranjala, J. K., Tillakaratne., Medina-Kauwe, L., Michael, K.,

Gibson, K. M. (1995), Gamma-aminobutyric acid (GABA) me tabolism in mammalian neural and nonneural tossues. *Comp. Biochem. Physiol.* **112A,** 247-263.

Obata, K., (1977), Biochemistry and physiology of amino acid trans mitters, *In: Handbook of Physiology, Section 1, The Neurous System,* Vol. 1 ( Kandel, E. R. et al., eds.) Part 1, The American Physiogical Society, Bethesda, MD 625-650.

Obata, K., Oide, M. And Tanaka, H. (1978), Excitatory and inhibitory actions of GABA and glycine on embrionic chick spinal neurons in culture. *Brain Res*. **144,**179-184.

Obata, K. (1997), Excitatory and trophic action of GABA and related substances in newborn mice and organotypic culture. *Dev. Neurosci.* **19,** 117-119.

Obata, K., Ji, F.- Y. (1998), Development of GABAergic neurons in organotypic culture of the mouse hippocampus and cerebellum lacking GAD67. *Jpn. J. Physiol.* In press .

Obata, K., Ji, F.- Y., Stork, O., Stork, S. (1998), Reduction of brain GABA contents and changes in emotional behavior in mice lacking glutamic acid decarboxylase 65kDa isoform. *Neurosci. Res. Suppl.* **22,** S72.

Packianthan, S., Cain, C. D., Stagg, R. B., and Longo, L. D. (1993), Ornithine decarboxylase activity in fetal and newborn rat brain: Responses to hypoxic and carbon monoxide hypoxia. *Dev. Brain Res.* **76,** 131-140.

Padmanabhan, R., Tchen, T. T. (1969), Nicotinamide adenine dinu cleotide and nicotinamide adenine dinucleotide phosphate-linked succinic semialdehyde dehydrogenases in a Pseu

donomas species. *J. Bacteriol.* **100**,398-402.

Petersen, J. S., Russel, S., Marshall, M. O., Kofod, H., Buschard, K., Cambon, N., Karlsen, A. E., Boel, E., Hagopian, W. A., Hejnaes, K. R. et al. (1993), Differential expression of glutamic acid decarboxylase in rat and human islets. *Diabetes* **42,** 484-495.

Porsolt, R. D., Anton, G., Blavet, N., Jalfre, M. (1978), Behavioural despair in rats: a new model sensitive to antidepressant treatments. *Eur. J. Pharmacol.* **47,** 379-391.

Redburn, D. A., Madtes, P. Jr. (1986), Postnatal development of 3H-GABA-accumulating cells in rabbit retina. *J. Comp. Neurol.* **243,** 41-57.

Redburn, D. A., Schousboe, A. (Eds). (1987), *Neurotrophic Activity of GABA during Development.* Alan R. Liss, New York, pp: 277.

Reetz, A., Solimena, M., Matteoli, M., Folli, F., Takei, K., De, Camilli, P. (1991), GABA and pancreatic beta-cells: colocalization of glutamic acid decarboxylase (GAD) and GABA with synaptic-like microvesicles suggests their role in GABA storage and secretion. *EMBO. J.* **10**,1275-1284.

Roberts, E., Chase, T. N., Tower, D. B., eds(1976), GABA in Nervous System Function. New York: Raven Press.

Satyanarayan, V., Nair, P. M. (1990), Metabolism, enzymology and possible roles of 4-aminobutyrate in higher plants. *Phytochemistry* **29,** 367-375.

Seiler, N., Wiechmann, M., Fischer, H. A., Werner, G. (1971), The incorporation of putrescine carbon into γ–aminobutiric acid in rat liver and brain *in vivo. Brain Res.* **28,** 317-325.

Sivilotti, L., Nistri, A. (1991), GABA receptor mechanisms in the central nervous system.. *Prog. Neurobiol.* **36**, 35-92 .

Somogyi, R., Wen, X., Ma, W., Barker, J. L. (1995), Developmental kinetics of GAD family mRNAs parallel neurogenesis in the rat spinal cord. *J. Neurosci.* **15**, 2575-2591.

Spoerri, P. E., Wolff, J. R. (1981), Effect of GABA-administration on murine neuroblastoma cells in culture. I. Increased membrane dynamics and formation of specialized contacts. *Cell Tissue Res.* **218**, 567-579.

Spoerri, P. E. (1988), Neurotrophic effects of GABA in cultures of embryonic chick brain and retina. *Synapse* **2**,11-22.

Szabo, G., Katarova, Z., Greenspan, R. (1994), Distinct protein forms are produced from alternatively spliced bicistronic glutamic acid decarboxylase mRNAs during development. *Mol. Cell Biol.* **14,** 7535-7545.

Stork, O., Welzl, H., Cremer, H., Schachner, M. (1997), Increased intermale aggression and neuroendocrine response in mice deficient for the neural cell adhesion molecule (NCAM). *Eur. J. Neurosci.* **9**,1117-1125.

Stoppini, L., Buchs, P. A., Muller, D. (1991), A simple method for organotypic cultures of nervous tissue. *J. Neurosci. Meth.* **37,** 173-182.

Szabo, G., Katarova, Z., Greenspan, R. (1994), Distinct protein forms are produced from alternatively spliced bicistronic glutamic acid decarboxylase mRNAs during development. *Mol. Cell. Biol.* **14,** 7535-7545.

Tanaka,C and Bowery, N. G. (Eds) (1996), *GABA: Recepors, Trans*

*porters and Metebolism.* Birkhauser Verlag, Berlin. pp: 31-43; pp: 275-282.

Tobet, S. A., Chickering, T. W., King, J. C., Stopa, E. G., Kim, K., Kuo-Leblank, V., Schwarting, G. A. (1996), Expression of γ–aminobutiric acid and gonadotropin-releasing hormone during neuronal migration through the olfactory system. *Endocri nology* **137,** 5415-5420.

Turski, W. A., Cavalheiro, E. A., Caldarazzo, F., Kleinrok, Z., Czuczwar, S. J., Turski, L. (1985), Injections of picrotoxin and bicuculline into the amygdaloid complex of the rat: and elec troencephalographic, behavioral and morphological analysis. *Neuroscience* **14,** 37-53.

Van Eden, C. G., Mrzljak, L., Voorn, P., Uylings, H. B. M. (1989), Prenatal development of GABAergic neurons in the neocortex of the rat. *J. Comp. Neurol.* **289,** 213-227.

Wee, E. L., Zimmerman, E. F. (1983), Involvement of GABA in palate morphogenesis and its relation to diazepam teratogenesis in two mouse strains. *Teratology* **28,**15-22.

Yuste, R., Katz, L. C. (1991), Control of postsynaptic $Ca^{2+}$ influx in developing neocortex by excitatory and inhibitory neuro-transmitters. *Neuron* **6,** 333-344.

# Figures and Legends

**Table 1. GABA content in GAD65 mutant brains (The ages from 1 to 4-months old).** GABA contents expressed of nmol/mg protein. Values are mean±S. D. *p<0.05 and <**p<0.005 when compared with each +/+ group (Student's test).

**Table 2. Glutamic acid in GAD65 mutants.** values are expressed as nmol/mg protein of the mean ± S. D. *p<0.05 and <**p<0.005 when compared with each +/+ group (Student's test).

**Table 3. Aspartic acid content in the brain of adult GAD65 mutant mice.** values are expressed as nmol/mg protein of the mean±S. D. *p<0.05 and <**p<0.005 when compared with each +/+ group (Student's test).

**Table 4. Amino acid contents in the cerebellum of GAD65 mutant mice.** Values are mean±S. D. *p<0.05, compared with GAD65+/+ (Student's test).

**Figure 1: Extracellular GABA Levels in GAD65 Mutant cerebellum.** Abscissa=time after perfusion of high potassium solution: ordinate=values expressed as %of the basal value obtained first 4 samples before high potassium perfusion in each mouse. Each value is mean±S. D. And numbers of animals are in parentheses.

**Figure 2. GAD Expression in GAD65 Mutants.** Immunoblot analysis of amygdala isolated from GAD65 +/+, GAD65+/- and GAD65-/- mice(from 1-month old to 4-month old) using anti-

GAD65/67 antiserum. Samples were applied to electrophoresis on 10%^ polyacylamide gel (10 µg per lane), the antibody is specifically staining GAD65 and GAD67.

**Table 5. Fear conditioning Test.** Training of GAD65 mutants was performed in a single session; One day later, auditory cued memory retrieval was tested by monitoring freezing. On the third day, contextual memory was analyzed in the shock context without presentation of the CS. Values are mean ± S. D. $*p<0.05$ and $**p<0.01$ when compared with GAD65+/+ (ANOVA).

**Table 6. Intruder-Aggression test.** After 4 weeks of single housing, the behavior displayed by male GAD mutants towards an unfamiliar male intruder to their home cage was determined. Values are mean ± S. D. $*p<0.05$ when compared with GAD65+/+ (ANOVA).

**Table 7. Forced-swim test.** GAD mutants were forced swimming in a water-filled glass cylinder (Ø 15 cm). Swimming activity and floating time were counted during the last 4 of 6 min (beam crosses). Values are mean ± S. D. $*p<0.01$ when compared with GAD65+/+ (ANOVA).

**Table 8. The effects of diazepam on GAD65 mutant mice.** Injections of diazepam (at 100µl/10g body weight) was done i.p. 30 min before assessment of anxiety-like behavior in the L/D paradigm. Values are mean ± S. D. $*p<0.05$ when compared with

control(without diazepam), (ANOVA).

**Figure 3. GABA contents in the hippocampal and cerebelar slices.** Before(0 day) and 7 and 14 days after culture, GABA contents were measured by HPLC. Blank columns, GAD67+/+ and GAD67+/- tissue and dotted columns, GAD67-/- tissue. Columns and bars are mean±S. D. No. Of experiments are shown in parentheses.

**Figure 4. Immunoblot analysis of hippocampal slices.** Before culture (0 day) and after 14 days in culture, 9 μg protein was applied to each lane. +/+, +/- and -/- describe GAD67 genotypes. Protein bands at 67 and 65 kDa were revealed by anti-GAD65/67 antibody.

**Figure 5. GAD activity of hippocampal slices.** Enzymatic activity of GAD evaluated by $^{14}CO_2$ production during 2 h incubation. mean±S. D are represented by columns and bars. Blank, in the presence and shaded, in the absence of PLP (20 μM). No. Of experiments are shown in parentheses.

**Figure 6. Immunohistochemistry of cultured slices.** A-E. GABA staining of the hippocampal (A and B) and cerebellar slices (C-E) after 20 days in culture. A and C, from GAD67+/+ mice. B, D and E, from GAD67-/- mice. C and D, close to the outer surface of the cerebellum. Positive cells are probably Purkinje cells. E, deep cerebellar nucleus region. F-I, Purkinjie cells and their processes stained with anti-calbindin-D28 antibody in organotypic culture of the

GAD67-/- cerebellum. F: 7 days, G and I: 14 days and H: 20 days in culture. I, deep cerebellar nucleus region. Purkinjie axons surround the large neurons. A bar in I represents 100 μm for A-E and 50 μm for F-I.

**Figure 7. Distribution of GAD genotypes of P0 and E14 mice obtained from GAD65+/-:67+/- parents.**
The GAD65 and GAD67 genotypes in each group is presented in succession. Figures are shown in percentages. A, 452 pups in 98 litters. The genotypes of 38 additional pups which did not have cleft palate and therefore were not GAD67-/-, were not determined because they were lost before the typing. B, 109 fetuses in 14 litters.

**Table 9. GABA contents in GAD-mutant mouse forebrain on embryonic day 14.**
Values are mean±S. D. †Insignificant difference. *$p<0.05$ and **$p<0.01$ (Student's test).

**Table 10. Ornithine decarboxylase activity in GAD-mutant mouse brain on embryonic day 14 and postnatal day 0.**
Enzymatic activity of ornithine decarboxylase evaluated by $^{14}CO_2$ production during 2 hr incubation. Values are mean±S. D. Numbers of mice are in parentheses. Differences among genotypes are non-significant.

**Figure 8. P0 brain from wild-type(A, C and E) and GAD65/67- deficient mice (B, D and F).** Cresyl violet stain A and

B, Neocortex,. C-D, Cerebellum. E and F, Hippocampus. Scale bar in A represents 100 μm for A and B, and bar in C 100 μm for C-E.

**Figure 9. GABA immunoreactivities in P0 mouse brain.** A-C, cerebellum from wild-type (A and C) and GAD65/67-deficient mice(B). A, part of the inferior colliculus is included at the right. D, superior colliculus. E and F, cerebral cortex from wildtype (E), and GAD65/67- deficient mice (F).Scale bar in F represents 100 μm for A, B, E and F, and 50 μm for C and D.

**Figure 10. Immunohistochemistry of wildtype (A, C and E) and GAD65/67-deficient mouse brains (B, D, F- H) at P0.** A, B and H, cerebellum stained with anti-calbindin-D28 k antibody. C and D, cerebellum stained with anti-neurofilament M-subunit antibody. E-G, MAP2 staining of neocortex (E and F ) and hippocampus (G). Scale bar in H represents 100 μm for A-G and 50 μm for H.

**Table 1. GABA content in GAD65 mutant brains**

| GAD65 genotype | No. of mice | Amygdala/piriform cortex | Hypothalamus | Cerebral cortex |
|---|---|---|---|---|
| | | 1 month old | | |
| +/+ | 3 | 23.10 ± 8.15 | 40.60 ± 3.52 | 15.88 ± 3.78 |
| +/- | 3 | 20.65 ± 3.66 | 45.80 ± 4.45 | 14.52 ± 2.23 |
| -/- | 3 | 13.82 ± 2.41 | 33.73 ± 6.65 | 12.44 ± 2.58 |
| | | 2 months old | | |
| +/+ | 5 | 35.83 ± 4.73 | 67.69 ± 16.69 | 21.87 ± 2.91 |
| +/- | 6 | 21.17 ± 2.88 ** | 45.15 ± 11.85 * | 17.97 ± 5.10 |
| -/- | 7 | 14.22 ± 2.18 ** | 33.28 ± 7.11 ** | 12.06 ± 3.00 * |
| | | 3 months old | | |
| +/+ | 4 | 36.10 ± 2.45 | 69.77 ± 11.28 | 21.46 ± 3.12 |
| +/- | 3 | 25.25 ± 5.47 * | 39.89 ± 9.45 * | 18.06 ± 5.79 |
| -/- | 6 | 18.19 ± 4.10 ** | 28.26 ± 7.31 ** | 11.98 ± 3.58 ** |
| | | 4 months old | | |
| +/+ | 5 | 26.93 ± 3.80 | 54.41 ± 7.33 | 23.97 ± 7.66 |
| +/- | 2 | 33.81 ± 10.94 | 64.66 ± 4.93 | 20.29 ± 0.49 * |
| -/- | 4 | 15.15 ± 2.15 ** | 33.31 ± 7.17 ** | 12.71 ± 1.09 ** |

GABA contents in tissues of 1-4-months old mice expressed as nmol/mg protein. Values are mean±S. D. *p<0.05 and **p<0.005 when compared with the respective +/+ group (Student's test). (From Ji et al.,1999).

**Table 2. Glutamic acid in GAD65 mutants**

| GAD65 genotype | No. of mice | Amygdala/piriform cortex | Hypothalamus | Cerebral cortex |
|---|---|---|---|---|
| | | 1 month old | | |
| +/+ | 3 | 49.74 ± 6.34 | 34.95 ± 4.09 | 47.26 ± 8.52 |
| +/- | 3 | 50.32 ± 11.53 | 37.80 ± 7.00 | 45.47 ± 13.94 |
| -/- | 3 | 49.62 ± 1.86 | 40.30 ± 4.92 | 40.29 ± 11.86 |
| | | 2 months old | | |
| +/+ | 5 | 75.26 ± 4.37 | 62.63 ± 11.76 | 72.20 ± 8.55 |
| +/- | 6 | 54.13 ± 12.58 | 42.46 ± 9.96 | 54.12 ± 10.05 |
| -/- | 7 | 57.83 ± 12.80 | 47.95 ± 11.16 | 53.66 ± 11.14 |
| | | 3 months old | | |
| +/+ | 4 | 58.61 ± 14.76 | 39.04 ± 8.24 | 46.01 ± 5.55 |
| +/- | 3 | 56.59 ± 5.40 | 35.07 ± 8.24 | 41.01 ± 6.09 |
| -/- | 6 | 58.44 ± 15.13 | 40.28 ± 11.08 | 42.48 ± 11.22 |
| | | 4 months old | | |
| +/+ | 5 | 108.30 ± 24.16 | 78.67 ± 14.07 | 103.84 ± 19.58 |
| +/- | 2 | 152.74 ± 10.13 | 100.16 ± 15.50 | 143.92 ± 7.29 |
| -/- | 4 | 131.35 ± 10.02 | 98.54 ± 5.65 | 119.78 ± 14.98 |

Values(mean ± S. D) expressed as nmol/mg protein. * $p<0.05$ and * * $p<0.005$ when compared with the respective +/+ group (Student's test). (From Ji et al.,1999).

**Table 3. Aspartic acid content in the brain of adult GAD65 mutant mice**

| GAD65 genotype | No. of mice | Amygdala/piriform cortex | Hypothalamus | Cerebral cortex |
|---|---|---|---|---|
| | | 1 month old | | |
| +/+ | 3 | 8.19 ± 2.59 | 9.04 ± 1.98 | 9.56 ± 1.61 |
| +/- | 3 | 8.82 ± 3.02 | 9.66 ± 2.73 | 8.73 ± 4.03 |
| -/- | 3 | 7.75 ± 1.70 | 8.43 ± 0.40 | 6.58 ± 1.78 |
| | | 2 months old | | |
| +/+ | 5 | 12.26 ± 2.02 | 16.67 ± 3.81 | 11.78 ± 2.30 |
| +/- | 6 | 7.54 ± 1.94 | 11.17 ± 1.80 | 9.43 ± 1.50 |
| -/- | 7 | 7.88 ± 1.34 | 9.87 ± 1.87 | 9.20 ± 2.51 |
| | | 3 months old | | |
| +/+ | 4 | 8.31 ± 1.49 | 10.37 ± 3.25 | 7.47 ± 1.61 |
| +/- | 3 | 10.06 ± 1.93 | 10.60 ± 2.21 | 7.64 ± 0.35 |
| -/- | 6 | 8.54 ± 2.20 | 7.83 ± 1.11 | 6.98 ± 2.26 |
| | | 4 months old | | |
| +/+ | 5 | 26.01 ± 15.03 | 30.17 ± 5.42 | 22.94 ± 3.85 |
| +/- | 2 | 30.33 ± 2.12 | 35.94 ± 6.31 | 32.85 ± 4.39 |
| -/- | 4 | 23.52 ± 2.47 | 27.30 ± 2.51 | 23.26 ± 3.54 |

Values(mean±S. D) are expressed as nmol/mg protein. $*p<0.05$ and $**p<0.005$ when compared with each +/+ group (Student's test). (From Ji et al.,1999).

**Table 4. Amino acid contents in the cerebellum of GAD65 mutant mice**

| GAD65 genotype | No. of mice | GABA | Glutamic acid | Aspartic acid |
|---|---|---|---|---|
| | | (nmol/mg protein) | | |
| +/+ | 2 | 18.60 ± 2.85 | 75.19 ± 15.54 | 16.10 ± 5.27 |
| +/- | 4 | 16.13 ± 0.67 | 72.61 ± 9.58 | 17.41 ± 3.95 |
| -/- | 5 | 13.72 ± 0.91 * | 66.40 ± 8.07 | 15.58 ± 3.33 |

Values are mean±S. D. *p<0.05, compared with GAD65+/+ (Student's test). (From Ji et al.,1999).

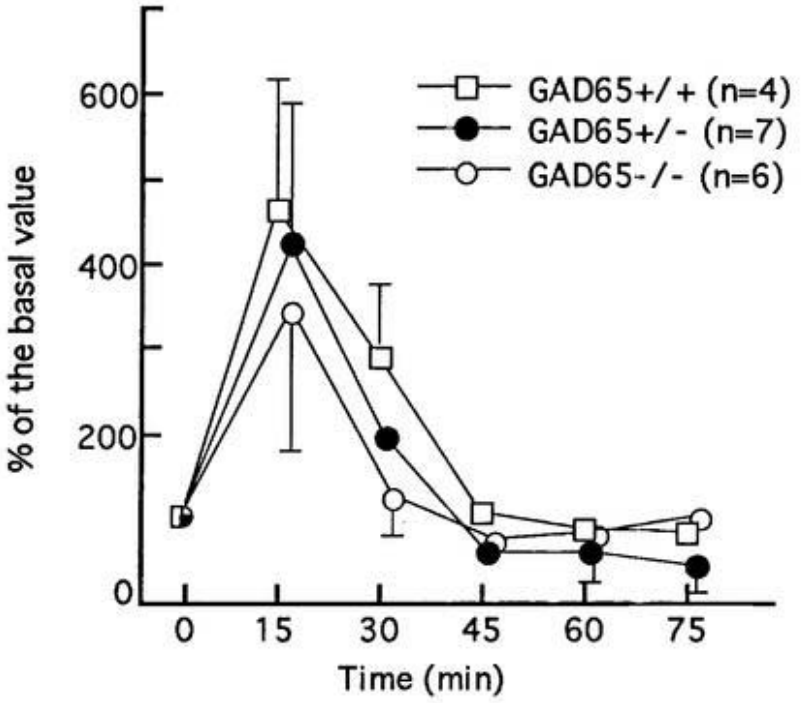


**Figure 1: Extracellular GABA Levels in GAD65 Mutant cerebellum.** The abscissa shows the time after perfusion of high potassium solution, the ordinate displays the values expressed as % of the basal value obtained in the first 4 samples before high potassium perfusion in each mouse. Each value is mean±S. D. And the number of animals are shown parentheses. (From Ji et al.,1999).

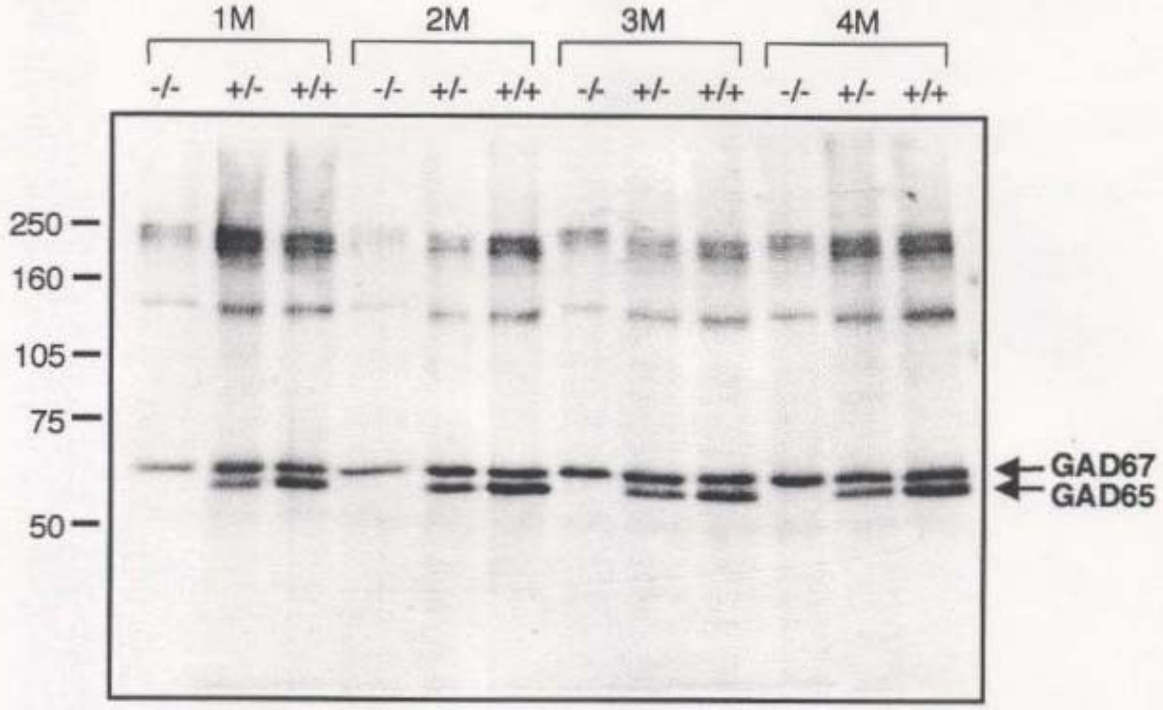


**Figure 2. GAD Expression in GAD65 Mutants**. Immunoblot analysis of amygdala isolated from GAD65 +/+, GAD65+/- and GAD65-/- mice(from 1-month old to 4-month old) using anti-GAD65/67 antiserum. Samples were applied to electrophoresis on 10% polyacrylamide gel (10 $\mu$g per lane), the antibody is specifically staining GAD65 and GAD67. (From Ji et al.,1999).

**Table 5. Fear conditioning Test**

| GAD65 | No. of mice | Contextual (without buzzer) | | Cued (with buzzer) | |
|---|---|---|---|---|---|
| | | Locomotion (cm) | Freezing (% time) | Locomotion (cm) | Freezing (% time) |
| +/+ | 5 | 84.00 ± 61.58 | 46.40 ± 36.20 | 85.70 ± 53.82 | 65.00 ± 15.86 |
| +/- | 8 | 71.81 ± 67.32 | 58.25 ± 35.03 | 111.56 ± 77.96 | 61.50 ± 30.02 |
| -/- | 6 | 287.33 ± 147.31 * | 7.00 ± 17.15 * | 269.75 ± 54.82 ** | 30.33 ± 11.52 * |

Training of GAD65 mutants was performed in a single session: One day later, auditory cued memory retrieval was tested by monitoring freezing during presentation of the CS. On the third day, contextual memory was analyzed in the shock context without presentation of the CS. Values are mean±SEM. *$p<0.05$ and **$p<0.01$ when compared with GAD65+/+ (Fisher' PLSD). (From Ji et al.,1999).

**Table 6. Intruder- Aggression test.**

| | | Attack | | Grooming |
|---|---|---|---|---|
| GAD65 | (n) | Latency (sec) | No. | No. |
| +/+ | (5) | 403.0 ± 308.3 | 9.20 ± 9.23 | 2.40 ± 2.61 |
| +/- | (8) | 706.3 ± 387.5 | 2.00 ± 3.30 * | 7.38 ± 6.23 * |
| -/- | (4) | 615.3 ± 241.1 | 1.50 ± 3.00 * | 4.50 ± 2.65 |

After 4 weeks of single housing, the behavior displayed by male GAD mutants towards an unfamiliar male intruder to their home cage was determined. Values are mean ±SEM. * $p<0.05$ when compared with GAD65+/+ (Fisher' PLSD). (From Ji et al.,1999).

**Table 7. Forced-swim test**

| GAD65 genotype | No. of mice | Swimming activity (beams crossed) | Floating time (sec) |
|---|---|---|---|
| +/+ | 5 | 163.4 ± 48.6 | 157.0 ± 38.1 |
| +/- | 8 | 235.1 ± 160.9 | 167.6 ± 36.0 |
| -/- | 7 | 502.3 ± 217.0* | 78.9 ± 22.8* |

GAD mutants were forced to swimm in a water-filled glass cylinder. Swimming activity and floating time were counted during the last 4 of 6 min. Values are mean ± SEM. * $p<0.01$ when compared with GAD65+/+ (Fisher' PLSD). (From Ji et al.,1999).

**Table 8. The effects of diazepam on GAD65 mutant mice**

| GAD65 | Without diazepam | | | After diazepam (0.5 mg/kg i.p.) | | |
|---|---|---|---|---|---|---|
| | D-time (sec) | Total activity (cm) | (n) | D-time (sec) | Total activity (cm) | (n) |
| +/+ | 215.1 ± 16.4 | 383.3 ± 50.9 | (5) | 128.3 ± 54.7 * | 505.8 ± 45.3 | (3) |
| +/- | 195.2 ± 47.2 | 359.9 ± 111.7 | (8) | 187.6 ± 63.8 | 401.0 ± 58.4 | (8) |
| -/- | 226.4 ± 32.0 | 395.0 ± 79.0 | (10) | 217.6 ± 49.1 | 413.2 ± 90.7 | (7) |

Injections of diazepam (at 100µl / 10g body weight) was done i.p. 30 min before assessment of anxiety-like behavior in the L/D paradigm. Values are mean±SEM. * $p<0.05$ when compared with vehicle-injected control, (Fisher' PLSD). (From Ji et al.,1999).

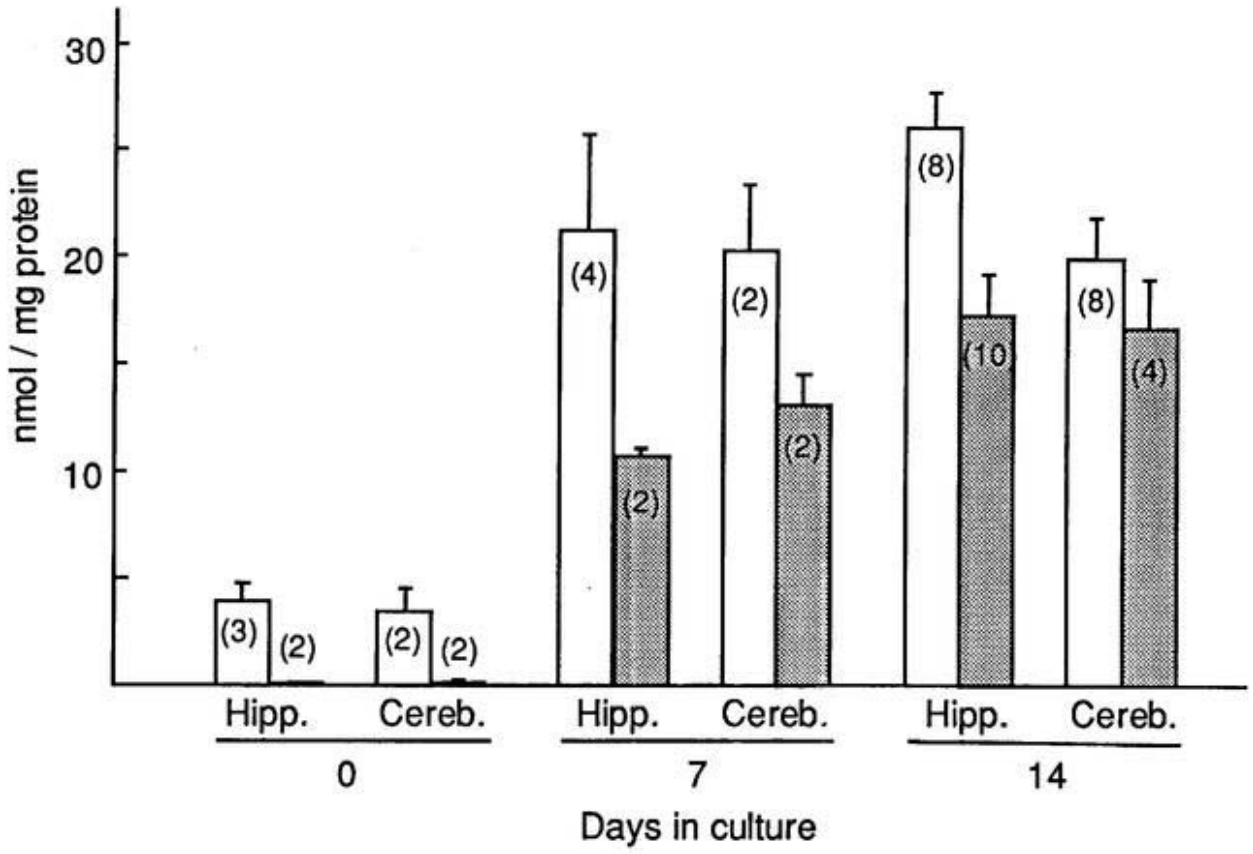


**Figure 3. GABA contents in the hippocampal and cerebelar slices.** Before(day 0) as while as 7 and 14 days after culture, GABA contents were measured by HPLC. Blank columns, controls (GAD67+/+ and GAD67+/- tissue) and dotted columns, GAD67-/- tissue. Columns and bars are mean±S. D. The number of experiments are shown in parentheses. (From Ji et al.,1999).

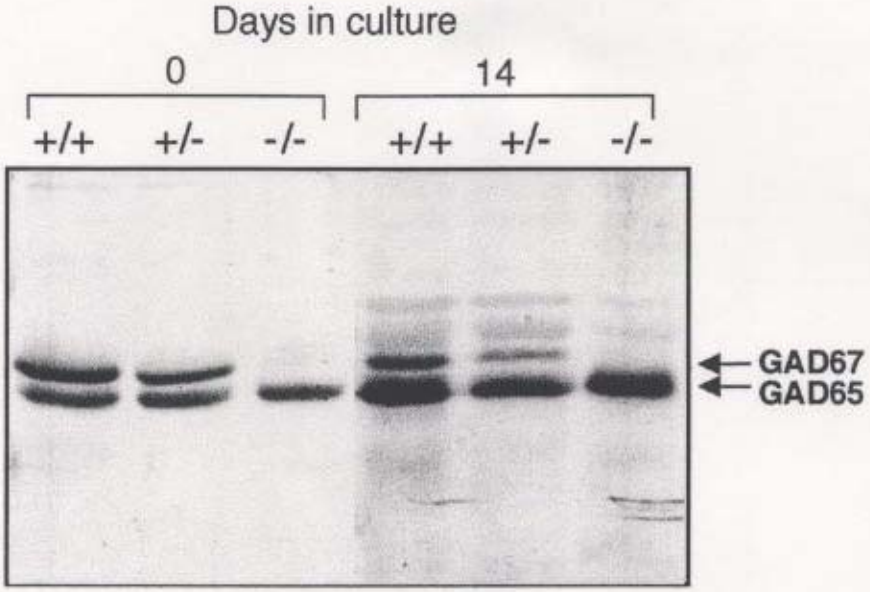


**Figure 4. Immunoblot analysis of hippocampal slices.** Before culture (day 0) and after 14 days in culture, 9 $\mu$g protein was applied to each lane. +/+, +/- and -/- describe GAD67 genotypes. Protein bands at 67 and 65 kDa were revealed by anti-GAD65/67 antibody. (From Ji et al.,1999).

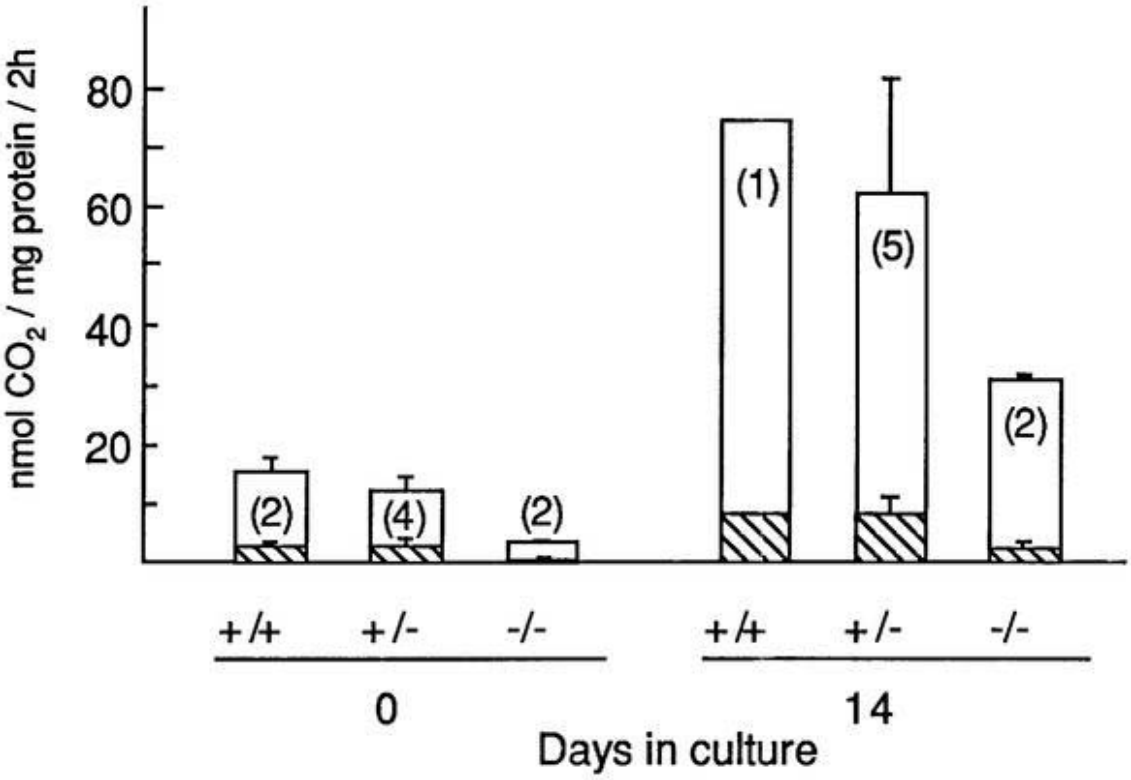


**Figure 5. GAD activity of hippocampal slices.** Enzymatic activity of GAD evaluated by $^{14}CO_2$ production during 2 h incubation. mean±S. D are represented by columns and bars. Blank, in the presence and shaded, in the absence of PLP (20 μM). The number of experiments are shown in parentheses. (Modified from Ji et al.,1999).

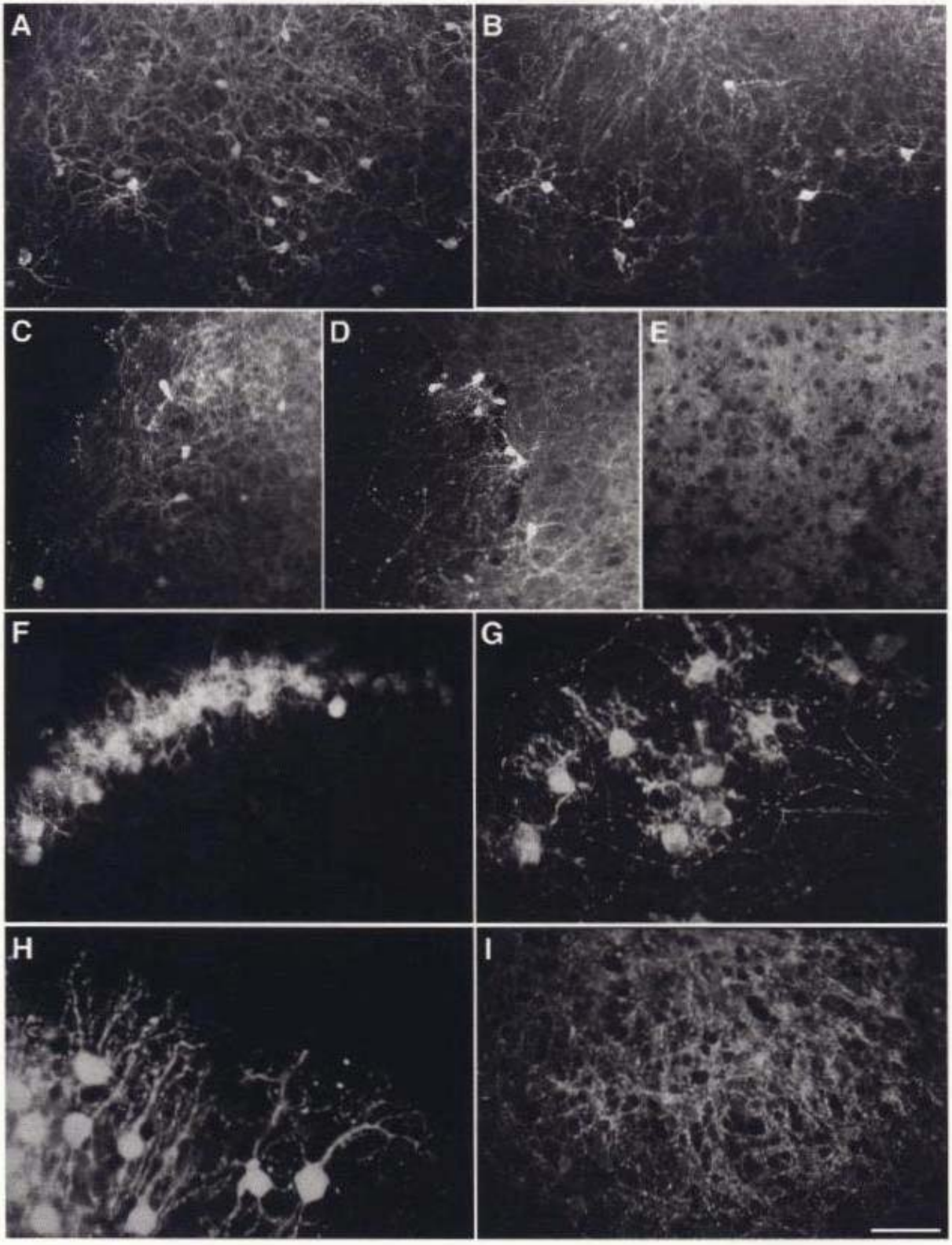


**Figure 6. Immunohistochemistry of cultured slices.** A-E. GABA staining of the hippocampal (A and B) and cerebellar slices (C-E) after 20 days in culture. A and C, from GAD67+/+ mice. B, D and E, from GAD67-/- mice. C and D, close to the outer surface of the cerebellum. Positive cells are probably Purkinjie cells. E, deep cerebellar nucleus region. F-I, Purkinjie cells and their processes stained with anti-calbindin-D28 antibody in organotypic culture of the GAD67-/- cerebellum. F: 7 days, G and I: 14 days and H: 20 days in culture. I, deep cerebellar nucleus region. Purkinjie axons surround the large neurons. A bar in I represents 100 $\mu$m for A-E and 50 $\mu$m for F-I.

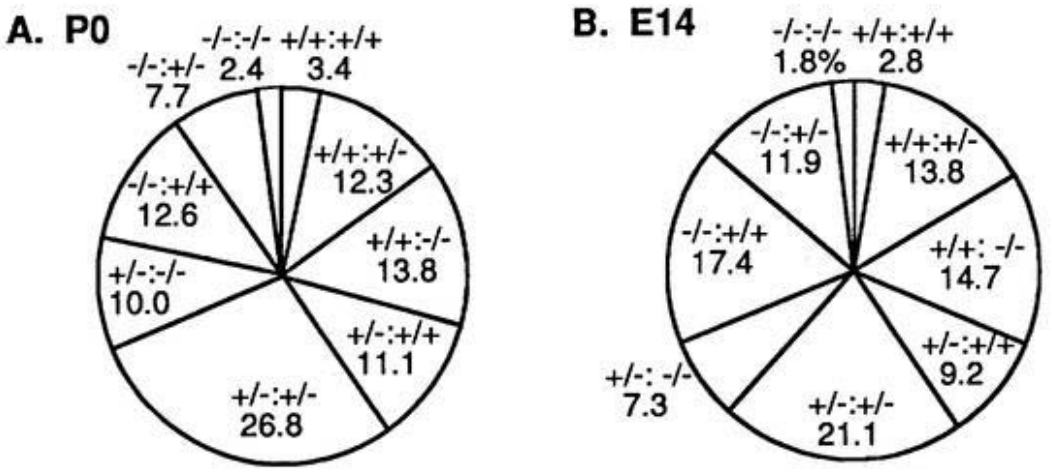


**Figure 7. Distribution of GAD genotypes of P0 and E14 mice obtained from GAD65+/-:67+/- parents.** The GAD65 and GAD67 genotypes in each group is presented in succession. Figures are shown in percentages. A, 452 pups in 98 litters.The genotypes of 38 additional pups which did not have cleft palate and therefore were not GAD67-/-, were not determined because they were lost before the typing. B, 109 fetuses in 14 litters. (From Ji et al.,1999).

**Table 9. GABA contents in GAD-mutant mouse forebrain on embryonic day 14**

| Genotype | | No. of mice | nmol GABA/mg protein (mean ± SD) | | | |
|---|---|---|---|---|---|---|
| GAD65 | GAD67 | | | | | |
| +/+ | +/+ | 0 | | | | |
| +/- | +/+ | 8 | 9.04 | ± | 4.09 | † |
| -/- | +/+ | 2 | 6.78 | ± | 1.22 | † |
| +/+ | +/- | 4 | 5.70 | ± | 1.89 | † |
| +/- | +/- | 12 | 5.06 | ± | 1.55 | * |
| -/- | +/- | 8 | 3.39 | ± | 0.23 | * |
| +/+ | -/- | 10 | 1.91 | ± | 0.72 | ** |
| +/- | -/- | 6 | 0.78 | ± | 0.24 | ** |
| -/- | -/- | 2 | 0.002 | ± | 0.003 | ** |

Values are mean ± S. D. † Insignificant difference. * $p<0.05$ and * * $p<0.01$ (Student's test). (From Ji et al., 1999).

**Table 10. Ornithine decarboxylase activity in GAD- mutant mouse brain on embryonic day 14 and postnatal day 0**

| Genotype | | Enzyme activity (nmol $CO_2$/mg protein/2h) | |
|---|---|---|---|
| GAD65 | GAD67 | E14 | P0 |
| +/- | +/+ | 0.306 ± 0.066 (4) | 0.301 (1) |
| +/+ | +/- | 0.219 ± 0.070 (4) | 0.161 ± 0.047 (2) |
| +/+ | -/- | 0.401 ± 0.067 (5) | 0.141 ± 0.101 (4) |
| +/- | -/- | 0.168 ± 0.042 (3) | |
| -/- | +/- | | 0.136 ± 0.043 (2) |
| -/- | -/- | 0.172 ± 0.036 (2) | <0.1 (1) |

Enzymatic activity of ornithine decarboxylase evaluated by $^{14}CO_2$ production during 2 h incubation. Values are mean±S. D. Numbers of mice are in parentheses. Differences among genotypes are non-significant. (From Ji et al.,1999).

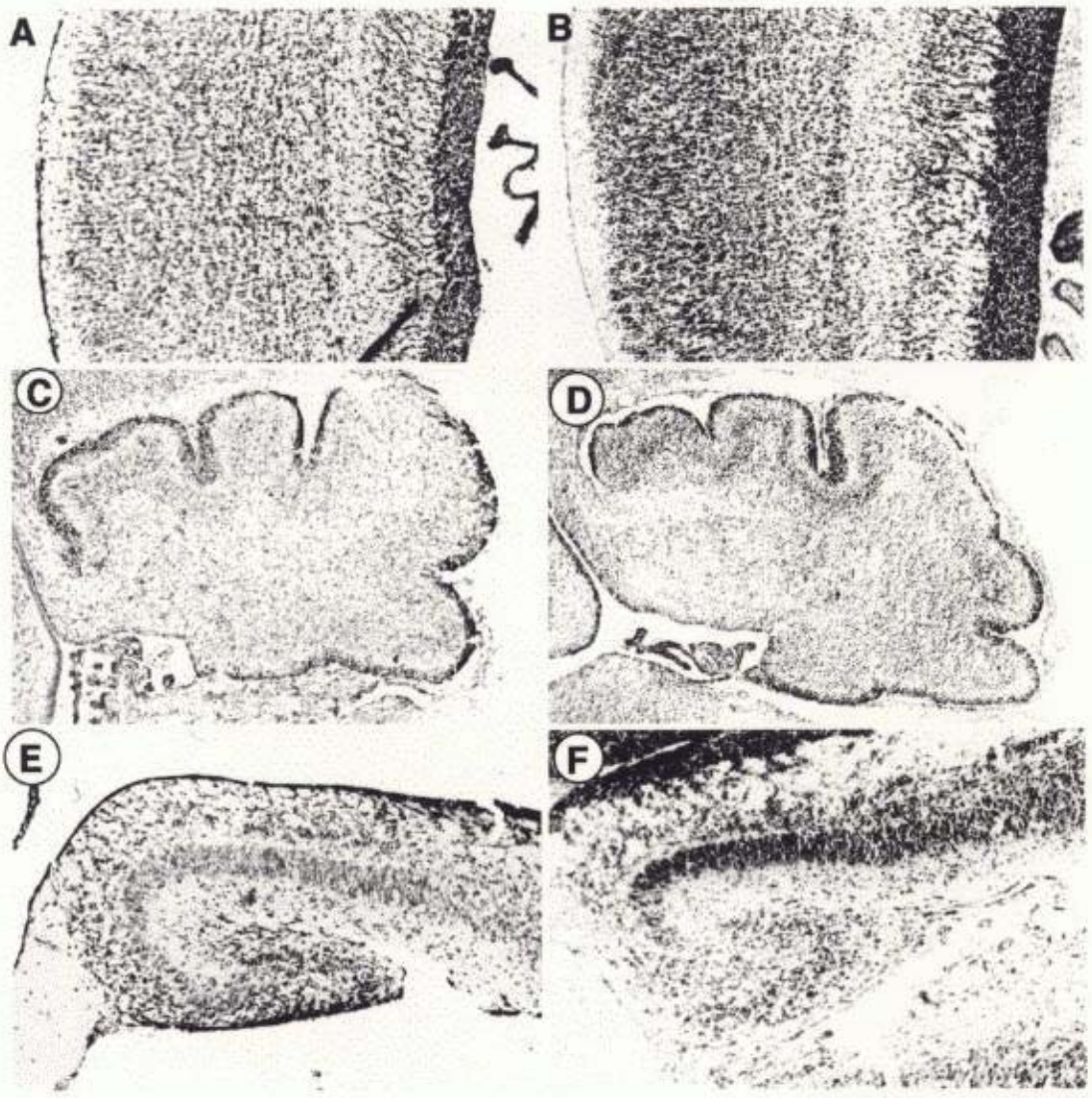


**Figure 8. E14 and P0 brain from wild-type (A, C and E) and GAD65/67-deficient mice (B, D and F).** A and B, E14 neocortex, H-E stain. C-F, P0. Cresyl violet stain. E and F, Cerebellum. Scale bar in A represents 100 $\mu$m for A and B, and bar in C 100 $\mu$m for C-E.

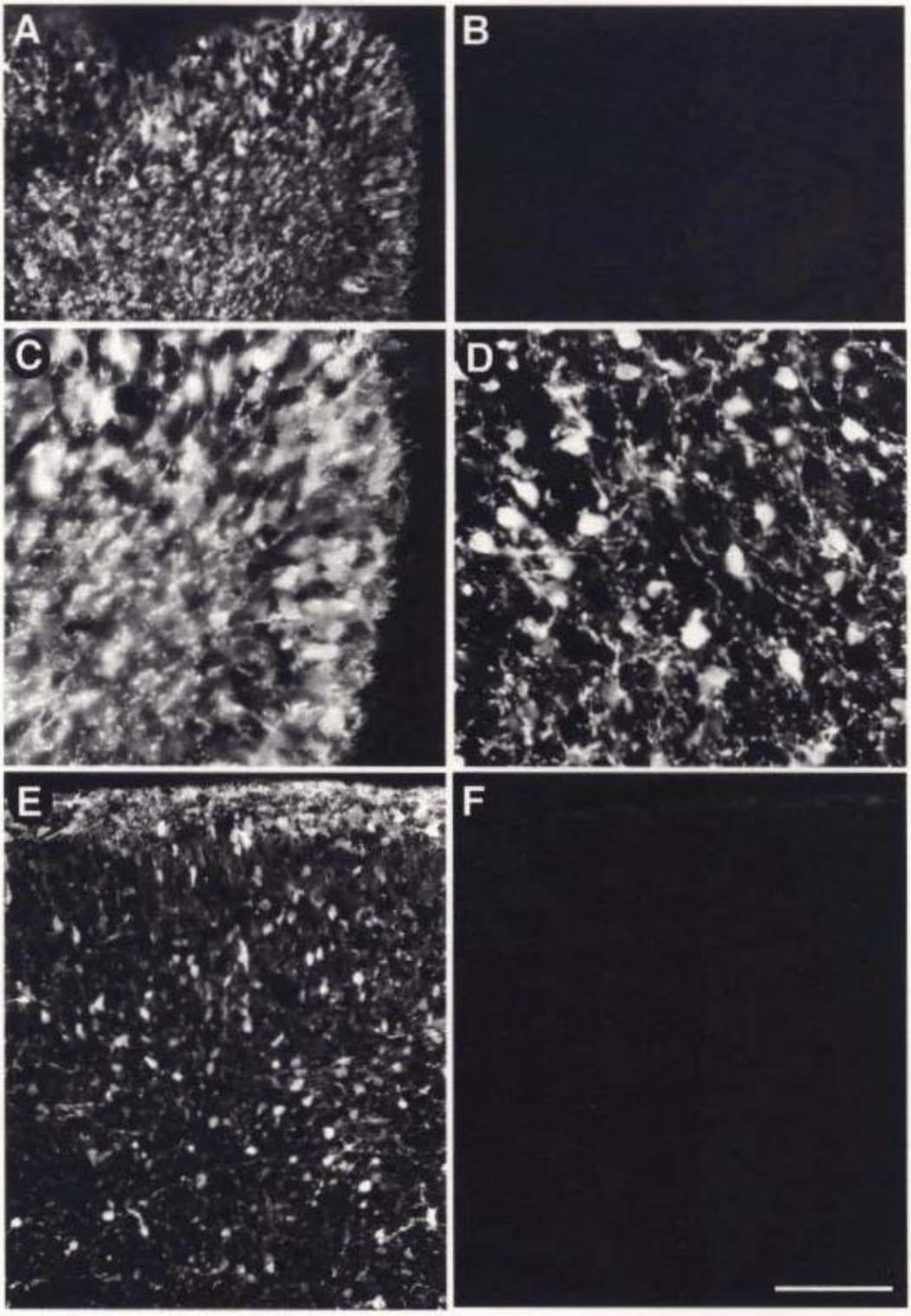


**Figure 9. GABA immunoreactivities in P0 mouse brain.** A-C, cerebellum from wild-type (A and C) and GAD65/67-deficient mice(B). A, part of the inferior colliculus is included at the right. D, superior colliculus. E and F, cerebral cortex from wildtype (E), and GAD65/67-deficient mice (F). Scale bar in F represents 100 $\mu$m for A, B, E and F, and 50 $\mu$m for C and D.

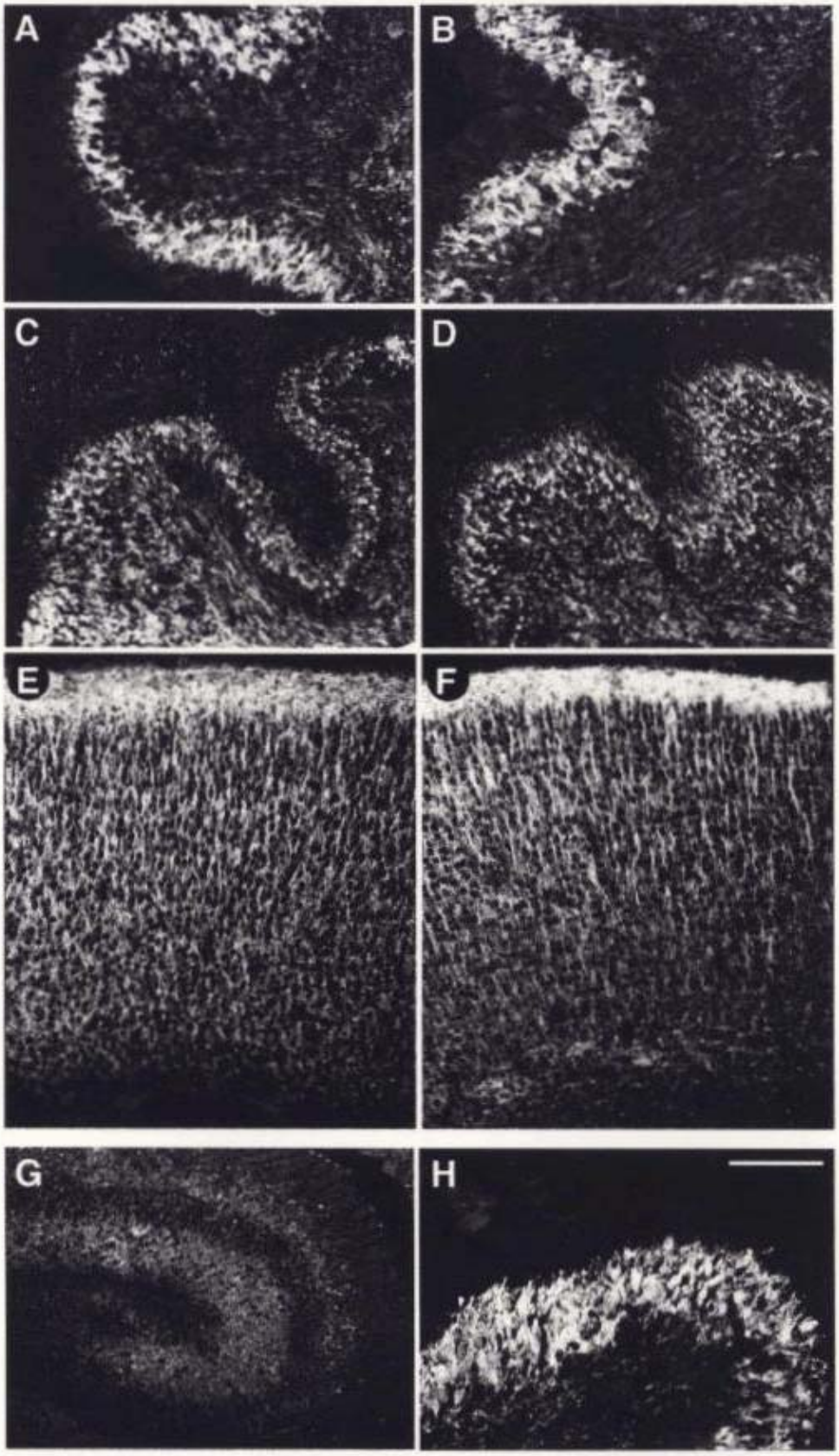


**Figure 10. Immunohistochemistry of wildtype (A, C and E) and GAD65/67-deficient mouse brains (B, D, F-H) at P0.** A, B and H, cerebellum stained with anti-calbindin-D28 antibody. C and D, cerebellum stained with anti-neurofilament M-subunit antibody. E-G, MAP2 staining of neocortex (E and F) and hippocampus (G). Scale bar in H represents 100 $\mu$m for A-G and 50 $\mu$m for H.