POSTNATAL DEVELOPMENT OF γ -AMINO BUTYRIC ACID (GABA) NEURONS IN THE CEREBELLUM OF JAUNDICED GUNN RATS: A STUDY WITH ANTI-GABA ANTISERUM

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ABSTRACT

In the cerebellum of jaundiced homozygous (*jj*) Wistar Gunn rats, specific activity of glutamate decarboxylase (GAD) on a wet weight basis on and after postnatal day 10 was significantly lower than in non-jaundiced heterozygous (*j+*) rats (2). In the present study, we have examined the postnatal development of GAD activity in another strain of Gunn rats (Sprague-Dawley Gunn rat). The *jj* rats of this strain also showed a considerably low rate of increase in GAD activity from days 10 to 15 compared with *j+* rats. To clarify a histological basis of the impaired development of GAD activity in *jj* rats, the postnatal development of cerebellar γ -aminobutyric acid (GABA)ergic neurons was studied immunocytochemically using anti-GABA antiserum raised in rabbits. Golgi and Purkinje cells were stained with the anti-GABA antiserum at the day of birth in both *jj* and *j+* rats. Many Purkinje cells, which showed some degenerative changes already at day 1 in *jj* rats, suffered from severe damages at day 10, and disappeared before day 15. Golgi cells also showed some degenerative changes at day 15, though they still maintained their immunoreactivity to the antiserum. Basket cells, which appeared at day 10, were small in number in *jj* rats at day 15, suggesting that they had been severely damaged during this period. The results suggest that bilirubin sensitivity of GABAergic neurons is heterogeneous, and destruction by bilirubin of Purkinje and basket cells before day 15 is mainly responsible for a low rate of increase in GAD activity from postnatal days 10 to 15.

Homozygotes (*jj*) of Gunn rats, a mutant strain of Wistar rats (6), exhibit lifelong nonhemolytic jaundice soon after birth as a result of bilirubin: UDP-glucuronyltransferase deficiency, whereas heterozygotes (*j+*) do not (11, 15). Jaundiced rats of this strain also show a marked cerebellar hypoplasia, which becomes evident after postnatal day 10 (7, 8, 13). We previously examined the postnatal development of several cerebellar

enzymes located in synapses and subcellular components in *jj* and *j+* rats (2). The activity of glutamate decarboxylase (EC 4.1.1.15; GAD), a rate-limiting enzyme in the formation of γ -aminobutyric acid (GABA) known as a major inhibitory neurotransmitter in the central nervous system, was lower in the hypoplastic *jj* rat cerebellum, and remained unchanged from days 10 to 15, in contrast to other enzymes including acetylcholinesterase.

In the present communication, the postnatal development of cerebellar GAD activity was examined with another strain of Gunn rats. Also,

Abbreviations: BSA, bovine serum albumin; GABA, γ -aminobutyric acid; GAD, glutamate decarboxylase; GAL, glutaraldehyde; RSA, rabbit serum albumin

an immunocytochemical study using anti-GABA antiserum visualized several types of GABAergic neurons in the developing cerebellum. The type of GABAergic neurons which may account for the disturbance of cerebellar GAD development from days 10 to 15 in *jj* rats will be discussed.

MATERIALS AND METHODS

Sprague-Dawley (SD) Gunn rats, established in our laboratory by transferring the *j* gene of a Wistar Gunn rat to SD rats (JCL:SD) (18), were used. Complete Freund's adjuvant was purchased from Difco Laboratories, U.S.A., and anti-rabbit IgG (goat) and peroxidase-antiperoxidase complex (rabbit) were from Miles-Yeda, Israel. Glutaraldehyde (GAL) and L-[1-¹⁴C]glutamic acid (55 mCi/mmol) were obtained from TAAB Laboratories and Amersham, U.K., respectively. GABA, L-glutamic acid and taurine were products of Nakarai Chemicals, and aspartic acid and rabbit serum albumin (RSA) were from Seikagaku Kogyo, Japan. Nihon white rabbits were supplied from Chubu Kagaku, Japan. All other chemicals were of the purest grade available.

Enzyme Preparation and Assay

Each cerebellum, removed immediately after decapitation and weighed, was homogenized with 9 vol of ice-cold 0.32 M sucrose in a Potter-Elvehjem glass homogenizer with a motor-driven Teflon pestle. GAD activity in the homogenate from *jj* and *j+* rats at days 5, 10 and 15 was assayed according to the method of Wu and Roberts (21), with a minor modification (2).

Production of Anti-GABA Antiserum

Anti-GABA antiserum was produced essentially according to the procedure of Storm-Mathisen *et al.* (20), by immunizing rabbits with GABA covalently conjugated to RSA with GAL. Purity of GABA was ascertained by TLC developed with three solvent systems, *i.e.* 96% ethanol/distilled water, 63/37, v/v; *n*-butanol/acetic acid/distilled water, 60/20/20, v/v; and 96% ethanol/34% ammonia, 67/23, v/v. GABA (100 μ mol) and RSA (12 mg) were dissolved in 2 ml of 0.2 M sodium phosphate buffer, pH 7.4, and GAL (100 μ mol) was added to initiate the reaction. The mixture was shaken for 24 h at room temperature, and the reaction was terminated by adding 0.2 ml of 1 M lysine solution, pH 7.0. The

conjugate (GABA-GAL-RSA) was dialysed for 48 h at 4°C against 2 l of 0.1 M sodium phosphate buffer, pH 7.4, and emulsified with an equal volume of complete Freund's adjuvant. The emulsion was repeatedly injected intracutaneously into the multiple sites on the back of the rabbit.

Double Immunodiffusion Test

The specificity of the anti-GABA antiserum was checked by the Ouchterlony double diffusion test on 1% agar plates. The center well was filled with 10 μ l of undiluted anti-GABA antiserum, and the respective outer wells contained 10 μ l each of 50-fold diluted solutions of the immunogen (GABA-GAL-RSA) and three different amino acid conjugates (glutamate-GAL-RSA, aspartate-GAL-RSA and taurine-GAL-RSA), prepared similarly as the GABA conjugate. Plates were kept in a closed humidified chamber at room temperature.

Immunocytochemical Procedures

Two or three *jj* and an equal number of *j+* littermates at days 0, 1, 5, 8, 10, 15, 20 and 30 were used for immunocytochemical examinations. Rats were perfused via the heart with a mixture of 1% GAL, 4% formaldehyde, 0.2% picric acid and 2% sucrose in 0.1 M sodium acetate buffer, pH 6.0 (14). Each cerebellum was sectioned in slices of about 3 mm thickness, fixed in the same solution for 4–5 h, rinsed 2 times with 50 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl and kept overnight in this Tris-buffered solution at 4°C. After intrinsic peroxidase activity was eliminated by H₂O₂ treatment, cerebellar slices were embedded in polyethylene glycol (9). Three- μ m-thick sections cut with a glass knife (17) were floated on the surface of the anti-GABA antiserum (dilution, 1:500), and incubated overnight at 4°C. The location of GABAergic neurons was visualized by the peroxidase-antiperoxidase method (19) with diaminobenzidine as chromogen. To ascertain the location of immunonegative cells, some sections were counterstained with toluidine blue (not shown). Control sections were prepared by using the antiserum exhaustively preabsorbed with GABA-GAL-RSA complex which was immobilized on Sepharose 4B.

Table 1 *Changes in Cerebellar GAD Activity during Postnatal Development of Homozygous (jj) and Heterozygous (j+) Gunn Rats*

Age (days)	Genotype	Number of animals	Cerebellar wet weight (mg)	Specific activity (nmol/min/g wet weight)
5	jj	3	35.4±3.2	20.4±3.1
	j+	3	34.2±1.5	22.9±9.1
10	jj	4	58.3±5.1*	35.4±4.2
	j+	7	80.9±10.4	47.0±11.1
15	jj	3	44.6±1.6*	58.9±6.7*
	j+	3	138.4±3.1	92.0±5.9

Values are expressed as the average ±SD. *P<0.01 by Student's *t*-test

RESULTS

GAD Activity

Cerebellar wet weight and GAD activity on a wet weight basis of *jj* and *j+* rats were summarized in Table 1. Both the cerebellar weight and GAD activity showed no statistical differences between *jj* and *j+* rats at day 5. Values in *jj* rats at day 10 were about 75% of those in *j+* rats. At day 15, cerebellar weight in *jj* rats was decreased to 32% of that in *j+* rats, and GAD activity to 64%.

Double Immunodiffusion Test with Anti-GABA Antiserum

A single precipitin band was observed only between the GABA-GAL-RSA complex and its

antiserum. No precipitin lines were formed against glutamate, aspartate and taurine complexed with RSA by GAL (Fig. 1).

Immunocytochemical Observations

By using the anti-GABA antiserum, both somata and processes of putative GABA neurons were clearly visualized as have been shown by using anti-GAD antisera (5, 10, 12). In the cerebellar cortex, Golgi, basket, stellate, and Purkinje cells were immunoreactive even without colchicine pretreatment. At the day of birth, Golgi and Purkinje cells were stained in both the *jj* and *j+* rat cerebella. The staining of Purkinje cells was weaker than in Golgi cells (Fig. 2). No pathologi-

Fig. 1 Ouchterlony double diffusion test of the antiserum obtained from a rabbit immunized with GABA-GAL-RSA. The center well contained the anti-GABA antiserum, and the outer wells various amino acids (a, GABA; b, glutamate; c, taurine; and d, aspartate) conjugated to RSA by GAL. A single precipitin band is observed only between the GABA-GAL-RSA complex and the antiserum.

Fig. 2 Heterozygous Gunn rat cerebellum immunostained with the anti-GABA antiserum at the day of birth. Golgi (G) and Purkinje (P) cells are already immunoreactive to the antiserum. All the following photographs are in the same magnification.

Fig. 3 Homozygous Gunn rat cerebellum immunostained 24 h after birth. Purkinje cells contain small cytoplasmic vacuoles (arrows), an early histological manifestation of bilirubin intoxication.

cal changes were observed in the cerebellar interneurons of jaundiced rats at this age.

Twenty-four hours after birth, Purkinje cell somata were dispersed over the cortex forming a monolayer. Golgi cells, some of which began to extend short processes, showed a strong reactivity to the antiserum. In *jj* rats, morphological

abnormalities were first detected at this age. Small cytoplasmic vacuoles appeared in some Purkinje cells (Fig. 3).

At day 5, Purkinje cells in *j+* rats had a number of processes and thorns. In *jj* rats, Purkinje cells with cytoplasmic vacuoles increased in number, but Golgi cells exhibited no pathological changes.

In 10-day-old *j+* rats, Purkinje cells in the nodulus and in the ventral part of the uvula, which belong to early-maturing lobules (1), showed characteristic branchings of tertiary dendrites (Fig. 4A), whereas those cells in the tuber and declive, late-maturing lobules (1), were still immature in dendritic arborization (Fig. 4B). The staining intensity of Purkinje cells was moderate. Basket cells in the nodulus and ventral uvula began to be stained in the soma and processes, but those in the tuber and declive did not. Immunoreactivity of basket cells to the anti-GABA antiserum seemed to appear soon after the cells migrated to their destination, the lower molecular layer, and changed their position from vertical to parallel to the Purkinje cell layer. The processes of Golgi cells developed well and were stained heavily by the antiserum in both rats (Fig. 5). In all the lobules of *jj* rats except the nodulus and ventral uvula, almost all of Purkinje cells were severely damaged and disarranged, and showed a markedly disorganized cytoplasm (Fig. 6). Golgi cells in *jj* rats seemed unaffected in spite of a high serum bilirubin level (18).

A

B

Fig. 4 Dendritic branchings of Purkinje cells in a 10-day-old homozygote. A: nodulus with well-developed branchings. B: declive with still immature branchings

Fig. 5 A Golgi cell (G) of a 10-day-old homozygote. Processes are well-developed and stained heavily by the antiserum.

Fig. 6 Purkinje cells of a 10-day-old homozygote. Their cytoplasm is markedly disorganized. P, Purkinje cell; G, Golgi cell

At day 15, the intensity of staining of Purkinje cells in *j+* rats was nearly the same as that at day 10. Dendrites and axons of basket cells were stained, and around Purkinje cell bodies were seen numerous punctate deposits of the reaction products, which were considered to correspond to the axon terminals of basket cells. Processes of Golgi cells became more elongated and slender,

Fig. 7 Granular layer of a heterozygote at postnatal day 15. Processes of Golgi cells (G) are more elongated and slender than in 10-day-old rats (*cf.* Fig. 5), and glomeruli (gl) are stained distinctly. P, Purkinje cell

and glomeruli were also stained at this age, showing the typical configuration of matured Golgi cells (Fig. 7). In *jj* rats, most of Purkinje and basket cells underwent degeneration and often disappeared especially in the declive, which seemed to be one of the lobules most sensitive to bilirubin (Fig. 8A). However, Golgi cells remained largely intact, and maintained the immunoreactivity to the antiserum, though some were affected (Fig. 8B).

Fig. 9 shows immunostained pinceaux, which were formed in 20-day-old *j+* rats by the axon collaterals of basket cells around the initial segments of Purkinje cell axons. Stellate cells were also stained intensely. The staining intensity of GABAergic neurons of *j+* rats at day 30 was almost the same as that at day 20, suggesting that these cells had already been matured before day 20 as far as the GABA-synthesizing ability was concerned. In the lobules of *jj* rats except the nodulus and ventral uvula, which were similar in immunostaining to those of *j+* rats, most of GABAergic neurons other than Golgi cells disappeared and the rest underwent degenerative changes. The immunoreactivity to the antiserum and the morphology of Golgi cells in *jj* rats were, though more or less vacuolated, nearly the same as those in *j+* rats. Purkinje cells could be classified into two groups depending on the degree of immunoreactivity to the antiserum, *i.e.*

Fig. 8 Cerebellum of a 15-day-old homozygote. A: Many of Purkinje and basket cells have been lost. P, Purkinje cell with degenerative changes. B: Golgi cells remain largely intact (arrows); some are affected (arrowhead) though they still preserve the reactivity to the antiserum.

Fig. 9 Purkinje cell layer of a heterozygote at postnatal day 20. Axon collaterals of basket cells (B) are immunostained around the Purkinje cell somata (P) and form the pinceaux (arrowheads). Stellate cells (S) are also stained intensely.

a part of Purkinje cells were moderately stained whereas others remained almost pale (Fig. 10). Such a heterogeneity was evident even at the day of birth (not shown). Bilirubin intoxication was manifested to the same degree in both Purkinje cell populations.

DISCUSSION

We have previously reported that the specific activity of GAD on a wet weight basis in the cerebellum of jaundiced (*jj*) Wistar Gunn rats is significantly lower than that of non-jaundiced (*j+*) rats at postnatal day 10, and remained unchanged through day 15 (2). SD Gunn rats used in the present study are superior to Wistar Gunn rats in that the variation of cerebellar weight in *jj* rats is smaller, the serum bilirubin concentration in *jj* rats is higher and the litter size is larger. Developmental features of GAD activity in this new strain of Gunn rats were essentially the same as those in Wistar Gunn rats (Table 1; 2).

Storm-Mathisen *et al.* first raised antiserum against GABA by immunizing rabbits with GABA coupled to bovine serum albumin (BSA) via GAL and visualized GABAergic neurons immunocytochemically (20). We produced anti-GABA antiserum according to their method with a minor modification; RSA was employed instead of BSA, because rabbits would be immunologically tolerant to RSA. In fact, preabsorption of the anti-albumin antibody (20) for the preparation of the anti-GABA-GAL-RSA anti-

Fig. 10 Heterogeneity of Purkinje cells in terms of immunoreactivity to the anti-GABA antiserum in a 20-day-old heterozygote. Immunopositive (arrows) and immunonegative (arrowheads) Purkinje cells are clearly observed.

serum was not necessary in the present study, because the antiserum did not react with albumin complexed with other amino acids. There are three lines of evidence to account for the mono-specificity of our anti-GABA antiserum. First, positive immunoreactivity in the cerebellar cortex was found in four neurons, *i.e.* Purkinje, Golgi, basket, and stellate cells, where the putative transmitter is GABA. This observation is consistent with the result of Storm-Mathisen *et al.* (20) and of previous reports using anti-GAD antisera (10, 12, 5). Second, preincubation of the antiserum with the GABA-GAL-RSA complex completely abolished positive stainings. Third, in an Ouchterlony double diffusion test, a single precipitin band was observed only between the GABA-GAL-RSA complex and the antiserum.

Histological studies on the development of GABAergic neurons have not been reported. We have investigated the developmental changes in GABA-immunoreactive neurons in both apparently normal and hypoplastic cerebella of Gunn rats. Chan-Palay *et al.* suggested that Purkinje cells might acquire capability of synthesizing their transmitters, including GABA, at a critical stage or on about the 4th postnatal day (3). But, judging from the reactivity to the anti-GABA antiserum, Purkinje cells as well as Golgi cells contain GABA already at the day of birth (Fig. 2), suggesting an earlier onset of transmitter synthesis in GABAergic Purkinje cells. The present study has also revealed that pathological

changes in GABA-immunoreactive cells in *jj* rats appear within 24 h after birth (Fig. 3), whereas a decrease in cerebellar GAD activity is manifested as late as 2 weeks (Table 1). These are consistent with the observation that GABA neurons, even though they contain cytoplasmic vacuoles, do not lose their immunoreactivity to the anti-GABA antiserum, suggesting the persistence of GAD activity in these cells. Schutta and Johnson (16) reported that Purkinje cells of *jj* rats from *j+/jj* matings began to show abnormalities from 5 days after birth. An earlier manifestation of degenerative changes found in the present study might be due partly to a strain difference. Expression of bilirubin toxicity was pronounced in Purkinje and basket cells, whereas it was far less in Golgi cells, suggesting a high resistance of Golgi cells to bilirubin. Thus, it is considered that destruction by bilirubin and subsequent disappearance of Purkinje cells and basket cells before day 15 is largely responsible for a low rate of increase in GAD activity from days 10 to 15 (Table 1; 2).

Chan-Palay *et al.* (4) have shown a chemical heterogeneity in cerebellar Purkinje cells, that is, coexistence of anti-GAD immunopositive and immunonegative cells. A similar observation was made with our anti-GABA antiserum (Fig. 10), which would probably reflect a difference in the intracellular concentration of GABA synthesized by GAD. In our study, the heterogeneous immunoreactivity of Purkinje cells was seen as

early as at birth. We initially expected that GABA-positive Purkinje cells might be more sensitive to bilirubin, because GAD was most markedly reduced in the *ji* rat cerebellum among several enzymes including acetylcholinesterase (2). However, no evidence was obtained that the immunoreactivity of Purkinje cells to the anti-GABA antiserum was associated with the susceptibility of these cells to bilirubin toxicity.

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REFERENCES

1. ALTMAN J. (1972) Postnatal development of the cerebellar cortex in the rat. II. Phases in the maturation of Purkinje cells and of the molecular layer. *J. Comp. Neurol.* **145**, 399-464
2. AOKI E., KATOH-SEMBA R. and KASHIWAMATA S. (1982) Cerebellar hypoplasia in Gunn rats: Effects of bilirubin on the maturation of glutamate decarboxylase, Na, K-ATPase, 2',3'-cyclic nucleotide 3'-phosphohydrolase, acetylcholine and aryl esterase, succinate and lactate dehydrogenase, and arylsulfatase activities. *J. Neurochem.* **39**, 1072-1080
3. CHAN-PALAY V. (1984) Multiple chemical messengers in the cerebellum: Presence, ontogeny and function. In *Proceedings of the 7th International Congress of Histochemistry and Cytochemistry* (Helsinki), p. 67
4. CHAN-PALAY V., NILAVER G., PALAY S. L., BEINFELD M. C., ZIMMERMAN E. A., WU J.-Y. and O'DONOHUE T. L. (1981) Chemical heterogeneity in cerebellar Purkinje cells: Existence and co-existence of glutamic acid decarboxylase-like and motilin-like immunoreactivities. *Proc. Natl. Acad. Sci. USA* **78**, 7787-7791
5. CHAN-PALAY V., PALAY S. L. and WU J.-Y. (1979) Gamma-aminobutyric acid pathways in the cerebellum studied by retrograde and anterograde transport of glutamic acid decarboxylase antibody after *in vivo* injections. *Anat. Embryol.* **157**, 1-14
6. GUNN C. K. (1938) Hereditary acholuric jaundice in a new mutant strain of rats. *J. Hered.* **29**, 137-139
7. KATOH-SEMBA R., AOKI E. and KASHIWAMATA S. (1982) Cerebellar hypoplasia in Gunn rats: Effects of bilirubin on postnatal changes in cerebellar constituents. *Biomedical Res.* **3**, 319-329
8. KEINO H. (1982) Enzyme histochemical studies on acid phosphatase in the cerebellum of young Gunn rats. *Cell Tissue Res.* **223**, 593-601
9. MAZURKIEWICZ J. E. and NAKANE P. K. (1972) Light and electron microscopic localization of antigens in tissues embedded in polyethylene glycol with a peroxidase-labeled antibody method. *J. Histochem. Cytochem.* **20**, 969-974
10. McLAUGHLIN B. J., WOOD J. G., SAITO K., BARBER R., VAUGHN J. E., ROBERTS E. and WU J.-Y. (1974) The fine structural localization of glutamate decarboxylase in synaptic terminals of rodent cerebellum. *Brain Res.* **76**, 377-391
11. NAKATA D., ZAKIM D. and VESSEY D. A. (1976) Defective function of a microsomal UDP-glucuronyltransferase in Gunn rats. *Proc. Natl. Acad. Sci. USA* **73**, 289-292
12. SAITO K., BARBER R., WU J.-Y., MATSUDA T., ROBERTS E. and VAUGHN J. E. (1974) Immunohistochemical localization of glutamate decarboxylase in rat cerebellum. *Proc. Natl. Acad. Sci. USA* **71**, 269-273
13. SAWASAKI Y., YAMADA N. and NAKAJIMA H. (1976) Developmental features of cerebellar hypoplasia and brain bilirubin levels in a mutant (Gunn) rat with hereditary hyperbilirubinaemia. *J. Neurochem.* **27**, 577-583
14. SCHMECHEL D. E., BRIGHTMAN M. W. and MARANGOS P. J. (1980) Neurons switch from non-neuronal enolase to neuron-specific enolase during differentiation. *Brain Res.* **190**, 195-214
15. SCHMID R., AXELROD J., HAMMAKER L. and SWARM R. L. (1958) Congenital jaundice in rats, due to a defect in glucuronide formation. *J. Clin. Invest.* **37**, 1123-1130
16. SCHUTTA H. S. and JOHNSON L. (1971) Electron microscopic observations on acute bilirubin encephalopathy in Gunn rats induced by sulfadimethoxine. *Lab. Invest.* **24**, 82-89
17. SEMBA R. (1979) Contributions to semithin sectioning on a conventional rotary microtome. *Stain Technol.* **54**, 251-255
18. SEMBA R. and KATO K. (1982) Increased nervous system-specific enolases in rat plasma and cerebrospinal fluid in bilirubin encephalopathy detected by an enzyme immunoassay. *J. Neurochem.* **39**, 360-365
19. STERNBERGER L. A. (1974) The unlabeled antibody enzyme method. In *Immunocytochemistry* (ed. STERNBERGER L. A.) Prentice-Hall, Englewood Cliffs, pp. 129-171
20. STORM-MATHISEN J., LEKNE S. A. K., BORE A. T., VAALAND J. L., EDMINSON P., HAUG F.-M. S. and OTTERSEN O. P. (1983) First visualization of glutamate and GABA in neurones by immunocytochemistry. *Nature* **301**, 517-520
21. WU J.-Y. and ROBERTS E. (1974) Properties of brain L-glutamate decarboxylase: Inhibition studies. *J. Neurochem.* **23**, 759-767