but no excitations of PAG neurons by both enkephalin and morphine (morphine excited one cell in the presence of naloxone). Naloxone antagonized all depressions in this area, caused by morphine or enkephalin, against which it was tested. The PAG is an area with a large number of opiate binding sites (*1, 16*). Focal electrical stimulation of this area of brain produces an analgesia which can be at least partly blocked by naloxone (*6–8*). Mayer (*6*) and also Akil *et al.* (*7*) have suggested that such stimulation-produced analgesia may result, at least in part, from the release of an endogenous morphine-like neurochemical modulator, presumably enkephalin or a closely related substance, onto the opiate binding sites on postsynaptic elements here. Furthermore, several groups (*18, 19*) have shown that focal injection of morphine into the PAG of rat, particularly the ventrolateral aspect, produces analgesia that is reversible by naloxone. Yaksh *et al.* (*19*) postulated the existence within the PAG of a morphine-sensitive inhibitory interneuron which is tonically active. According to this hypothesis, opiate action depresses these interneurons, thus disinhibiting a second-order neuron which provides ascending and descending modulation of sensory transmission. Our data are compatible with such an enkephalinergic system within the PAG providing inhibitory modulation of the inhibitory interneuron. Thus the opiate receptors at this site would actually be receptors for the endogenous morphine-like inhibitory substance. Enkephalin-responsive cells were not restricted to the PAG itself but were found also in the reticular formation ventral to the PAG.

Although a physiological role for enkephalin remains to be proved (*20*), the data that now exist make enkephalin almost as promising a neurotransmitter candidate as the biogenic amines (such as serotonin and noradrenaline) or the amino acids (such as glutamic acid and $\gamma$-aminobutyric acid). The resolution of this question requires the demonstration of the presence of enkephalin in nerve terminals from which it can be released. The cell bodies must also be located, and confirmation must be obtained that the pharmacology of the released substance is identical to that of the exogenously administered peptide. The development of a radioimmunoassay for enkephalin will facilitate such studies.

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# Neuronal-Visceral $GM_1$ Gangliosidosis in a Dog with $\beta$-Galactosidase Deficiency

Abstract. *A 9-month-old dog with a history of progressive motor dysfunction was shown to have a deficiency in brain $\beta$-galactosidase activity. The canine disease, like that of children with $GM_1$ gangliosidosis, is characterized by accumulation of $GM_1$ ganglioside in the brain, liver, and spleen, and membranous cytoplasmic bodies in neurons. The dog's pedigree suggests an autosomal recessive pattern of inheritance.*

Gangliosidoses of man comprise a group of rare inherited lipid storage diseases resulting from deficiencies in activity of glycosidases involved in the degradation of normal sphingolipids that contain *N*-acetylneuramic acid (*1, 2*). Large intracytoplasmic accumulations of $GM_1$ or $GM_2$ monsialogangliosides (*2a*) in neurons and, in some types of gangliosidoses, in cells of visceral organs, is characteristic of these ultimately fatal disorders. A few animal counterparts of some of the gangliosidoses have been described (*3*). Because of their usefulness in investigations of the pathogenesis of these diseases and as models for the development and evaluation of potential methods of therapy, current efforts are centered on the identification and biochemical characterization of gangliosidoses in nonhuman species. We now report what we believe to be the first confirmed occurrence of $GM_1$ gangliosidoses in dogs.

A crossbred male dog of predominantly beagle phenotype developed progressive signs of head tremors, hyperactivity, dysmetria, hypermetria, and visual impairment over a 4-month period, and finally an inability to stand. The dog was humanely killed with sodium barbital at 9 months of age, and the tissues were prepared for biochemical analyses and light and electron microscopy (EM) studies. At autopsy, the gross lesions were limited to the brain and consisted of thickening of the cerebrocortical gray matter with attenuation of the myelinated tracts.

Histologic examination of hematoxylin and eosin–stained tissues disclosed marked enlargement and occasional vacuolation of neurons in the brain, spinal cord, spinal root ganglia, and retina. Neuronal cytoplasmic Nissl's substance was typically reduced to a basophilic dustlike dispersion surrounding the nuclei (Fig. 1A). The remainder of the cy-

toplasm was pale and distended with a fine-grained, faintly eosinophilic material that reacted weakly to periodic acid–Schiff (PAS) reagents both before and after treatment with purified diastase; this material was PAS negative after extraction with pyridine, and stained positive with Sudan black and Luxol fast blue but not with cresyl violet. Cytoplasmic vacuolation was seen in hepatocytes, renal tubular epithelial cells, and in mononuclear cells in the germinal centers of the spleen and lymph nodes.

Examination of the cerebral cortex by EM revealed neuron soma and processes that contained lamellar membranous cytoplasmic inclusions. The number of inclusions per neuron varied. The majority were spherical and resembled membranous cytoplasmic bodies (MCB), although a few inclusions resembled "zebra" bodies (Fig. 1B). The concentric dense lamellae measured approximately 3 to 5 nm in thickness and were separated by a 2-nm clear space. Where split, the dense lamellae appeared as two less dense lamellae 2 nm in thickness. Morphologically the MCB were similar to those reported in patients with $GM_1$ gangliosidosis in the central nervous system of man (*4*).

Tissues from the gangliosidosis dog and from a normal male beagle of similar age were homogenized in distilled water and protein content of homogenates was measured (*5*). Lipids were extracted from homogenates, separated, and analyzed as in previous studies (*6*). On the basis of either protein or wet tissue weight, concentrations of total lipids, phospholipids, and sterols in tissues from the diseased dog were similar to those encountered in the same tissues from the normal animal (Table 1). Distribution patterns of individual phospholipid and neutral lipid constituents were virtually identical in counterpart tissues from the two animals (data not shown). However, in all tissues examined from the affected dog, the concentrations of ganglioside sialic acid were elevated as compared to the respective control tissue. These elevations ranged from a rather modest increase in ganglioside sialic acid in liver to a three- and fourfold increase in spleen and gray matter. Chromatographic examination showed that a ganglioside with $GM_1$ mobility was grossly increased in ganglioside fractions from affected spleen and gray matter (Fig. 2). This ganglioside was not a major constituent in normal spleen and gray matter fractions. In contrast, white matter from both animals contained a ganglioside with $GM_1$ mobility as the major

Table 1. Lipid composition of spleen, liver, and brain gray and white matter from the gangliosidosis and a normal male beagle dog of similar age; N and G refer to normal and gangliosidosis dogs, respectively. The values for gangliosides are expressed on a sialic acid basis.

| Tissue | Protein (mg/g)* | Total lipid (μg/mg)† | Phospholipid (μg/mg)† | Sterols (μg/mg)† | Gangliosides (nmole/mg)† | Ganglioside $GM_1$ (nmole/mg)† |
|---|---|---|---|---|---|---|
| | | | *Gray matter* | | | |
| N | 88 | 513 | 386 | 86 | 15.2 | 5.4 |
| G | 87 | 347 | 245 | 80 | 50.2 | 33.2 |
| | | | *White matter* | | | |
| N | 289 | 413 | 256 | 111 | 9.8 | 2.8 |
| G | 361 | 419 | 214 | 90 | 17.1 | 5.5 |
| | | | *Spleen* | | | |
| N | 129 | 159 | 119 | 21 | 2.0 | 0.06 |
| G | 146 | 131 | 87 | 23 | 9.9 | 4.3 |
| | | | *Liver* | | | |
| N | 125 | 310 | 150 | 12 | 1.2 | 0.15 |
| G | 185 | 210 | 100 | 10 | 1.4 | 0.7 |

*Wet weight. †Concentration per milligram of protein.

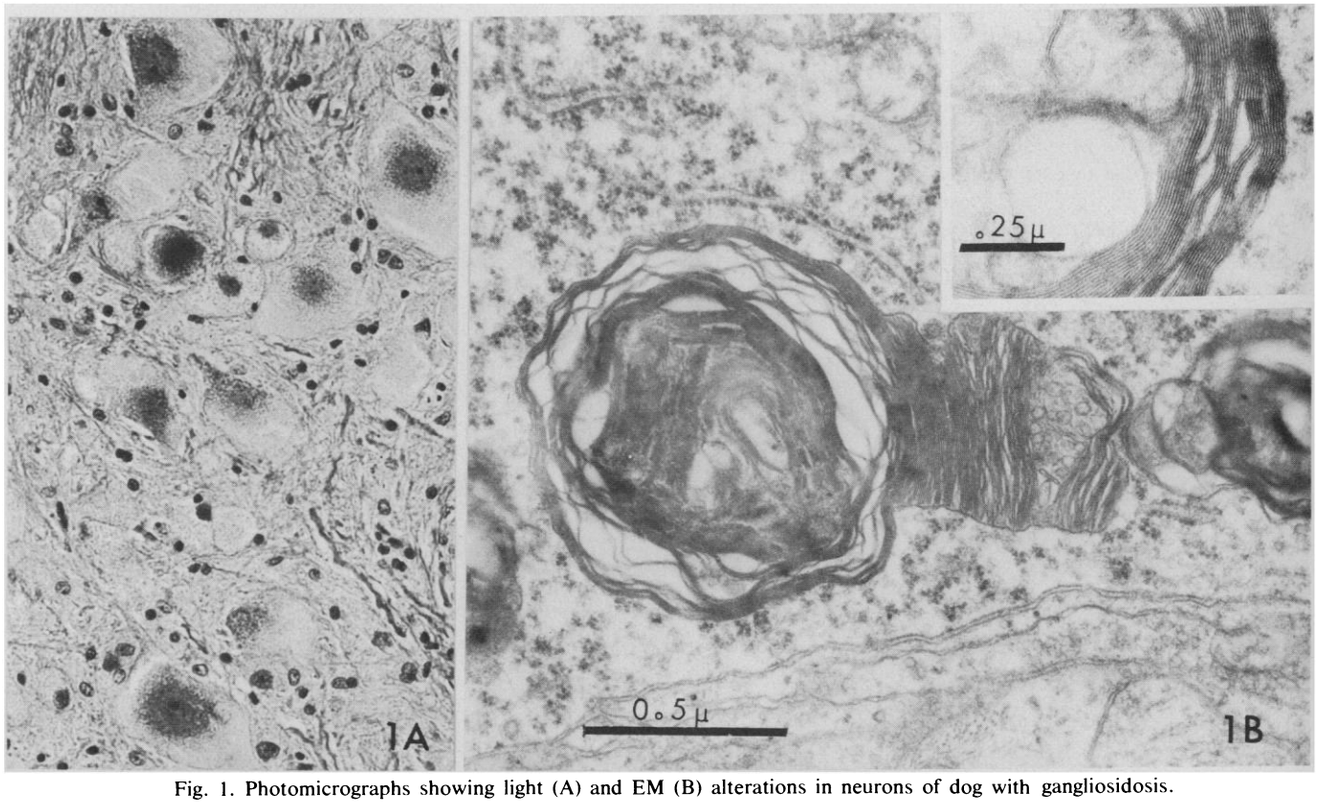


Fig. 1. Photomicrographs showing light (A) and EM (B) alterations in neurons of dog with gangliosidosis.

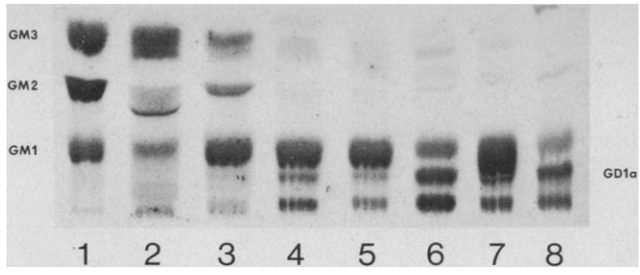


Fig. 2. Thin-layer chromatographic separation of ganglioside fractions from normal and gangliosidosis dog tissues. (**1**) Reference gangliosides $GM_3$, $GM_2$, and $GM_1$; (**2**) normal spleen; (**3**) gangliosidosis spleen; (**4**) normal white matter; (**5**) gangliosidosis white matter; (**6**) normal gray matter; (**7**) gangliosidosis gray matter; and (**8**) reference bovine brain gangliosides showing the mobility of the disialoganglioside $GD_{1a}$. Approximatly 50 nmole of ganglioside sialic acid from each tissue was applied to the plate. The silica gel G thin layer was developed in a chloroform, methanol, and 28 percent ammonium hydroxide system (60 : 35: 10, by volume) and the separated constituents were visualized with resorcinol spray.

constituent. Recovery of the presumed $GM_1$ from thin-layer plates and measurement by sialic acid assay confirmed that this constituent was greatly elevated in all tissues sampled from the afflicted dog. While not measured quantitatively, thin-layer chromatographic examination of neutral glycosphingolipid fractions also gave evidence of large increases in constituents with mobilities identical to lactosyl ceramide and tetrahexosyl ceramide (asialo-$GM_1$) in the spleen and white and gray matter.

Repeated preparative thin-layer chromatography was used to purify the major ganglioside in lipid extracts from afflicted gray matter for component characterization. As was expected for $GM_1$, this ganglioside was not labile to neuraminidase but was converted by mild acid hydrolysis to a glycosphingolipid with thin-layer chromatographic mobility identical to that of tetrahexosyl ceramide (*6*). Analysis of the constituents (*7*) showed that the ratios of sphinogosine to sialic acid to glucose to galactose to *N*-acetylgalactosamine were very close to 1 : 1 : 1 : 2 : 1. These results left little doubt that the ganglioside which accumulated was $GM_1$.

The above observations demonstrated that the dog had a $GM_1$-type gangliosidosis and suggested, by analogy with the human disease, that the enzymatic basis for this defect may have been a deficiency in $\beta$-galactosidase activity. Supernatant fractions from normal and gangliosidosis gray matter were assayed for $\beta$-galactosidase activity with *p*-nitrophenyl-$\beta$-D-galactopyranoside as the substrate (*8*). Specific activity of the supernatant fraction from normal gray matter was 8.5 nmole of *p*-nitrophenol released per hour per milligram of protein, and the corresponding value for lipidosis gray matter supernatant was 0.4 nmole per hour per milligram of protein. With lactosyl ceramide or $GM_1$ as substrates (*9*), the specific activities obtained with normal gray matter supernatant were 473 and 280 pmole of galactose released per hour per milligram of protein, respectively, and the corresponding specific activities with afflicted gray matter supernatant were 34 and 2 pmole per hour per milligram of protein. It was thus clear that there was a deficiency in $\beta$-galactosidase activity in gray matter from the gangliosidosis dog.

The pedigree of the dog that we studied is shown in Fig. 3. Dogs 1 and 2 were siblings which were mated and had a litter of six. Two of the litter (Nos. 4 and 6) died suddenly of unknown causes at 7 weeks of age. Another two, Nos. 5 and 7, developed a slight ataxia, starting at 7 and 16 weeks of age, respectively. Dog No. 7, however, disappeared from its owner's residence a short time after the onset of signs. Its littermate (No. 5) showed signs of purposeless crying, continued development of ataxia with progressive paresis, and became aggressive when disturbed. Humane killing and rabies examination was recommended at 20 weeks of age because the dog had bitten a child. Evaluation for rabies was negative, but histologic studies revealed a neuronal-visceral type "lipidosis." A fifth clinically normal female sibling (No. 8) died unexpectedly during routine surgery for ovarian-hysterectomy at 24 weeks of age. Hydrocephalus was diagnosed postmortem, but no evidence of gangliosidosis was found histologically.

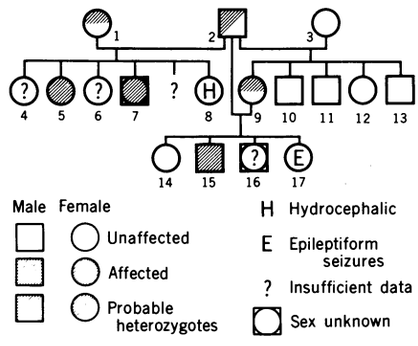


Fig. 3. Pedigree of canine gangliosidosis family.

Male No. 2 was later bred to an unrelated cross, beagle × collie (No. 3), and sired five offspring (Nos. 9 to 13) all of which were clinically normal. This male (No. 2) was subsequently mated with one of his daughters from this litter (No. 9) and sired four offspring (Nos. 14 to 17). The gangliosidosis dog (No. 15) reported here was one of these four offspring. Dog No. 16 died of unknown causes at 6 weeks of age while another sibling (No. 17) from the same litter developed epileptiform-type seizures at 26 weeks of age and was killed. At autopsy, no histologic evidence of gangliosidosis was found. The remaining dog from this litter, No. 14, was still alive and clinically normal at 2 years of age.

Two forms of $GM_1$ gangliosidoses are recognized, both of which are transmitted as an autosomal recessive: type 1 (generalized $GM_1$ gangliosidosis) and type 2 (juvenile $GM_1$ gangliosidosis) (*2*). Type 1 gangliosidosis is essentially a disease of infants. Clinical signs of impaired psychomotor development are almost always evident at birth or develop within a few weeks postpartum. Frontal bossing, depression of the nasal bridge, and various other facial abnormalities are characteristic. Hepatosplenomegaly and bone deformities are also seen in 85 to 95 percent of all cases. In contrast, children with type 2 $GM_1$ gangliosidosis develop normally until approximately 1 year of age, at which time initial symptoms of locomotor ataxia appear. Neither abnormal facies nor enlargement of the liver or spleen are seen, and bone deformities are slight or absent. Comparison of the above features with those observed in the propositus can be interpreted as being compatible with those of juvenile (type 2) $GM_1$ gangliosidosis, the symptoms being normal postpartum development until approximately 5 months of age with subsequent appearance of progressive motor dysfunction and absence of hepatosplenomegaly and bone and facial abnormalities at necropsy. Results of our tissue ganglioside analyses suggest, however, that such an interpretation is not entirely satisfactory. While both human forms of gangliosidosis are accompanied by significant elevations in brain $GM_1$ ganglioside and a deficiency in $\beta$-galactosidase activity similar to those seen in the dog we describe here, marked accumulations of $GM_1$ ganglioside in visceral tissues are

not usual in the type 2 human disease but can be consistently demonstrated in individuals with the type 1 disease. In our dog the gangliosides in the spleen were increased approximately fivefold, and 43 percent of the total was identified as $GM_1$. The splenic concentrations of the $GM_1$ fraction was also shown to be more than 70 times greater than those found in the spleen of a normal dog of comparable age. Similar but less marked changes were found in the liver. These results are consistent with a type 1 human gangliosidosis.

Findings in this dog are of special interest because the disorder appears to have features of both types of $GM_1$ gangliosidosis seen in children. As in the human conditions, an autosomal recessive pattern of inheritance is also probable. Evidence for this include (i) pedigree consanguinity, (ii) phenotypically normal parents, and (iii) clinical or morphologic observations that indicate that both male and female siblings are affected. Data on the ratio of normal to diseased dogs are fragmentary, but would be expected to approach unity if enough litters were available for evaluation.

On the basis of the genetic, clinical, morphologic, and biochemical studies presented here, we have identified this disease as neuronal-visceral $GM_1$ gangliosidosis with $\beta$-galactosidase deficiency. Since canine examples of $GM_1$ gangliosidosis have not been reported, dogs from this family show promise as models for the study of gangliosidosis in man.

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8. Gray matter was homogenized in ten volumes of 0.25*M* sucrose containing 1 m*M* EDTA, *p*H 7; the homogenate was treated with ultrasound for 30 seconds with cooling and then centrifuged at 4000*g* for 10 minutes. The resultant supernatant was used as the enzyme source. Complete incubation mixtures contained, in a final volume of 0.35 ml, the following: acetate buffer, *p*H 4.5, 100 moles; *p*-nitrophenyl-$\beta$-D-galactopyranoside, 2.5 moles; and 0.4 to 0.6 mg of enzyme protein. Incubations were at 37°C for 1 hour. Reactions were terminated with trichloroacetic acid and the amount of *p*-nitrophenol liberated was determined [S. Gatt, *Methods Enzymol.* **14**, 156 (1969)].
9. Tritium was introduced into the terminal galactose of $GM_1$ and lactosyl ceramide by oxidation with galactose oxidase and reduction with $NaB^3H_4$ [Y. Suzuki and K. Suzuki, *J. Lipid Res.* **13**, 687 (1972)]. Specific activities of the resultant products were 2000 and 420 count/min per nanomole for $GM_1$ and lactosyl ceramide, respectively. Complete incubation mixtures contained 20 nmole of acetate buffer, *p*H 4.5, 100 nmole of glycosphingolipid, 1 mg of sodium taurocholate, 0.5 mg of Triton X-100, and 4 to 6 g of gray matter supernatant protein in a final volume of 0.2 ml. Incubations were carried out at 37°C and reactions were terminated with 0.2 ml of ethanol. Liberated galactose was separated on thin-layer chromatography plates, and radioactivity was determined by standard methods.
10. Purdue University Agricultural Experiment Station Paper No. 6256. Supported in part by NSF grant PCM75-11908. T.W.K. is supported by NIH research career development award GM-70596.
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## Halictine Social Evolution: The Australian Enigma

Abstract. *Australian halictines belong to the primitive genus* Lasioglossum *or related subgenera. The underground nests have lined cells in series or clusters and sometimes at the end of laterals. Two full generations per year are produced in the communal nests. Overwintered and newly emerged females form unique "pseudosocieties" rather than matrifilial societies along Holarctic patterns. Several* Chilalictus *species produce a "male caste" of big-headed, flightless males, in addition to normal individuals. Oviposition of unfertilized eggs on large pollen balls causes such allometric bees.*

Halictine bees form a rich assemblage of species that embraces every conceivable degree of presocial and fully social behavior. The studies of many observers have contributed greatly to our understanding of the evolution of bee societies (*1, 2*). North America and Europe were especially active centers of research, since the two major halictine groups, *Lasioglossum* and *Halictus*, reach their greatest development there. *Lasioglossum* is found all over the world, with the related species of *Evylaeus* and *Dialictus* dominating the Holarctic region. *Halictus* is mostly confined to the Northern Hemisphere, where solitary and primitively social members occur. Their nests consist of burrows with sessile cells, except for the combs that are constructed by *H. quadricinctus* in Europe.

Nothing illuminates the diversity of nest architecture and the range of behavior better than the many species of the genus *Evylaeus*. One generation per year is produced by *E. lucidulus*, *E. rufitarsis*, and *E. minutissimus*, whereas two independent generations occur in *E. villosulus* and *E. quadrinotatulus*. Primitive as well as highly evolved societies abound in most regions; sometimes several social levels are represented by a few related species. *Evylaeus pauxillus*, *E. linearis*, *E. laticeps*, and *E. malachurus*, for example, show a gradual increase in caste differences, nest population, and number of worker broods produced, but at the same time they delay the production of males until their appearance coincides with that of the future queens (*3*). Much lower levels of sociality are found in the siblings *E. calceatus*, *E. albipes*, and *E. duplex* where caste differences are not distinct, males are produced throughout the summer, and nest populations remain small (*4*). *Evylaeus marginatus* is the only perennial social halictine whose huge societies last for up to 6 years but where queens and workers are morphologically identical (*5*).

Great variety is also found in the nest architecture of *Evylaeus*. Solitary species usually have a simple burrow from which lateral tunnels lead to a single terminal cell or, as in *E. nitidiusculus*, to a few cells in series. Social species have sessile cells or combs surrounded by a cavity. The most advanced halictine societies of *E. marginatus*, *E. linearis*, *E. malachurus*, and *E. cinctipes* leave the brood cells open for greater interaction between the two generations and better nest sanitation (*6*). No communal nests are known from *Evylaeus*, but several