*SEROTONIN RECEPTORS, VII. ACTIVITIES OF VARIOUS PURE GANGLIOSIDES AS THE RECEPTORS**

By D. W. Woolley and B. W. Gommi

THE ROCKEFELLER INSTITUTE

Communicated March 19, 1965

Evidence has recently been produced to indicate that the hormonal receptors for serotonin in susceptible tissues are lipids,[1, 2] and in particular, that they are gangliosides.[3, 4] The hormone seems to exert its effects on susceptible tissues by combination with its specific receptors, thereby displacing, by an ion exchange mechanism,[5, 6] the calcium ions normally in combination with these lipids. In this way calcium ions are carried through the ion-impermeable cell membranes and are then able to activate enzymic processes inside the cells. Such activation seems to be responsible for the effects of the hormone on its target cells.

The direct evidence that the receptors were gangliosides was the following. A serotonin-susceptible tissue such as a strip of a rat stomach was treated with a mixture of purified neuraminidase and EDTA. This treatment rendered the tissue insensitive to the action of serotonin, but did not destroy its susceptibility to other hormones such as acetylcholine and bradykinin. By subsequent treatment of the inactivated tissue with a purified sample of gangliosides, the sensitivity to serotonin was restored to it. This indicated that the enzyme had destroyed the serotonin receptor rather specifically, and that the thing which had been so destroyed was a ganglioside, not a neuraminic-acid-containing mucoprotein.[3]

Another line of evidence to suggest that the receptors are gangliosides arose from the study of rats reared from infancy on diets high in galactose.[4] The stomachs of such animals were specifically deficient in serotonin receptors, probably because the galactose had caused inhibition of the biosynthesis of some galactolipid which was the serotonin receptor. The deficiency of such tissues could be corrected, and their sensitivity to serotonin thereby increased by addition to them *in vitro* of tissue lipids which had been fractionated in such a way as to enrich them in gangliosides.

The aim of the work to be described in this paper was to test samples of various highly purified gangliosides for serotonin receptor activity. In this way it could be determined whether the receptor was a single specific chemical substance or whether many different gangliosides each exhibited activity. Furthermore, by use of two different kinds of assay procedure (the one based on neuraminidase-treated stomachs and the one based on galactosemic stomachs), one could find whether both kinds of assay gave the same answer.

Materials and Methods.—A much-purified sample of beef brain gangliosides was purchased from Sigma Chemical Company. This material (Sigma Type II) consisted of at least five gangliosides which could be separated in thin-layer chromatography. Chromatographically pure samples of brain gangliosides were provided by Drs. E. Klenk,[7-9] R. H. McCluer,[10] and L. Svennerholm.[11] These were the mono- (1G and A_2), di- (2G, 3G, B_1, and C), and trisialo- (4G) tetrose gangliosides, which are the most abundant gangliosides in brain. Apparently 1G = A_2 = G_1, 2G = B_1 = G_2, and 3G = C (?) = G_3 if the various systems of nomenclature are equated. The 1G had been purified beyond the stage of column chromatography[10] by ether precipitation. This proved to be important because without this step the column-purified monosialotetrose-ganglioside labeled 1Ga showed high potency which proved to be due to an impurity. There was also the

TABLE 1

ACTIVITIES OF VARIOUS SPHINGOLIPIDS IN THE SEROTONIN RECEPTOR ASSAY BASED ON NEURAMINIDASE-TREATED STOMACHS

Ganglioside or sphingolipid	Source	Sugar Components: Glucose	Galactose	Acetylgalactosamine	Sialic	Detectable at (γ/4 ml)	Unit*
1G	McCluer[10]	1	2	1	1	>1000	—
$1G_a$	McCluer[10]	.....(Impure 1G).....				5	50
A_2	Klenk[8]	1	2	1	1	>250	—
B_1	Klenk[8]	1	2	1	2	>1000†	—
2G	McCluer[10]	1	2	1	2	30	—‡
C	Klenk[9]	1	2	1	2	100	>375‡
3G	McCluer[10]	1	2	1	2	30	500§
4G	McCluer[10]	1	2	1	3	>400	—
FM	McCluer[10]	1	1	1	1	>100	—
Kerasin	—	0	1	0	0	>4000	—
Cytolipin H	Rapport[12]	1	1	0	0	>2000	—
GM_3	Svennerholm[11]	1	1	0	1	200	800
New G	—	1?	1?	0	2?	1.5	15

All lipids contain, in addition to sugars shown, a higher fatty acid in amide linkage with sphingosine or a related amine.

The symbol > means no activity detectable at highest level tested.

* One unit represents the amount of material required to cause an increase of 1 cm in the response to the challenging dose to serotonin.

† In some experiments B_1 was detectable at 10γ, but when the amount of lipid was increased, the response failed to increase and a response of 1 cm could not be obtained.

‡ Although doses of 100γ were detectable, larger doses failed to give larger responses.

§ In one assay 3G showed a higher activity (50γ/unit).

monosialotriose-ganglioside (FM) lacking the final galactose residue, presumably the Tay-Sachs' ganglioside,[10] and the monosialobiose-ganglioside (GM_3) obtained from human spleen by Svennerholm.[11] Samples of cytolipin H were supplied by Dr. M. Rapport,[12] and kerasin was isolated by chromatographic methods[13] from rat brain. The carbohydrate components of all of these substances are outlined in Table 1.

Measurements of the receptor activity were carried out in two ways. In one, strips of rat stomach were treated twice with neuraminidase plus EDTA to render them specifically deficient in serotonin receptors. The ability of various gangliosides to restore serotonin sensitivity to them was then measured quantitatively. Great care was taken to observe all of the details described for this method by Woolley and Gommi.[14] In the other method of assay, strips of stomachs from galactosemic rats were prepared as previously described,[4] and the ability of each lipid to increase the sensitivity to serotonin was measured. The only change from the method described by Woolley and Gommi was that the lipid was allowed to remain in contact with the tissue for only 90 sec, and was then washed away. The increased sensitivity to a predetermined dose of serotonin was then measured. Care was taken to test graded doses of each lipid in order to establish the minimal amount required for activation as well as the maximal amount needed for maximal activity.

Thin-layer chromatography (TLC) of serotonin receptor lipids: Because the Sigma gangliosides (Type II) were quite active in both kinds of assay, they were fractionated on silicic acid TLC with chloroform-methanol-water 60:35:8 in order to learn about the migration of the receptor activity. Five bands visualizable with iodine vapor were found with R_f's 0.15, 0.25, 0.53, 0.60, and 0.90.[15] When these were separately eluted and assayed, the only active fractions were those of R_f's 0.15 and 0.25. These had the R_f's of 3G and 1G run alongside. It was thus clear that the receptor activity migrated as did mono- and disialotetrose-gangliosides.

Isolation of a highly active impurity in the monosialotetrose-ganglioside $1G_a$: Assays by the "neuraminidase method" showed that the most active substance was the monosialotetrose $1G_a$ of Johnson and McCluer.[10] However, the ether-purified material (1G) and the corresponding compound (A_2) obtained from Klenk[7,9] were essentially inactive. In addition, digestion with neuraminidase inactivated $1G_a$ despite the known resistance of the monosialotetrose-ganglioside to this enzyme.[8,16] It was plain that the activity was due to an impurity present in $1G_a$. By intensive TLC in chloroform:methanol:water, a new ganglioside (new G) with the highest potency was separated in small yield from $1G_a$, leaving behind inactive 1G. New G migrated slightly faster than 1G in chloroform:methanol:water and very slightly slower in propanol:water (7:3).

TABLE 2

ACTIVITIES OF VARIOUS GANGLIOSIDES IN THE RECEPTOR ASSAY WITH GALACTOSEMIC STOMACHS

Substance	Amount Required (γ/4 ml) to Cause Increase in Serotonin of 0.5 cm	2.0 cm
Ganglioside mixture (Sigma)	—	80
$1G_a$	1.0	—*
B_1	1.0	5.0
C	0.05	—*
4G	>30	—

* Doses of 10 γ were apparently deleterious because they reduced response to challenging dose.

Results of the Assay Based on Neuraminidase with Pure Gangliosides.—The data of Table 1 will show that all gangliosides did not show activity. Some, such as 1G, 4G, and FM, were inactive. The most active material was the new ganglioside, new G.

Table 1 also shows that the disialogangliosides 2G, 3G, and C had receptor activity, but that this was much less than that found in new G.

Inhibition Caused by Certain Gangliosides.—Whenever, in the assay based on neuraminidase, a ganglioside proved to be inactive, the patency of the tissue was always tested by application to it of an active ganglioside and determination of whether the tissue then showed increased sensitivity to serotonin. Details of how this was done are given in the paper which described the method.[14] With the gangliosides A_2, B_1, and FM there was clear evidence of inhibitory activity, especially when large doses (1 mg/4 ml) were used. Presumably these inhibitory gangliosides were able to combine with the deficient tissues, and to occupy the sites where the receptor would have combined. They thus blocked the position so that the real receptor, subsequently applied, could not combine, and hence the sensitivity to serotonin was not restored by an active compound. These inhibitory gangliosides might thus be regarded as true antimetabolites of the serotonin receptor.

Results of the Assays Based on Galactosemic Tissues.—The data of Table 2 will show that in this kind of assay all gangliosides were not active, just as had been the case in the assays based on neuraminidase. $1G_a$ was active, but now the disialogangliosides B_1 and C showed much more activity relative to $1G_a$ than they had in the assay based on neuraminidase.

When dose-response curves were plotted, a novel point emerged. The only compound which gave a large reactivation of the galactosemic tissues was ganglioside B_1. With it, the maximal increase to the challenging dose of serotonin was about 5 cm. With all of the other gangliosides the maximal increase in response to the challenging dose of serotonin was less than 2 cm. Larger doses of these gangliosides gave no larger increments in response. Ganglioside C was outstanding because of the very small amounts which produced some increase in sensitivity to serotonin. However, just as with $1G_a$ and A_2, the maximal reactivation with this ganglioside was of the order of 1 cm. The results with the galactosemic tissues therefore pointed to ganglioside B_1 as the one with most outstanding potency, but left some doubt about the significance of $1G_a$ and C.

Discussion.—Perhaps the most striking feature of the present findings was the specificity observed among gangliosides. Several of the principal gangliosides of tissues were inactive. Only one ganglioside showed high potency in the "neur-

aminidase method" of assay. This was the new ganglioside (new G) which was separated as an impurity from $1G_a$.

The results indicated that the serotonin receptor lipid was the new ganglioside, and that minor degrees of activity were associated with a few other gangliosides such as those containing disialotetroses (2G, 3G, or C, and the disialobiose GM_3).

Although the chemical structure of new G has not yet been established, its behavior during thin-layer chromatography suggested that it may be identical with a ganglioside named G'_{lact} which was described very briefly recently by Kuhn and Weigandt.[17] G'_{lact}, like new G, moved just slightly faster than 1G ($=G_1$) with chloroform-methanol-water, and very slightly slower than 1G with propanol-water 7:3. Kuhn and Weigandt assigned a structure to G'_{lact} of a disialobiose-ganglioside containing 1 mole each of glucose and galactose and 2 moles of sialic acid. Shortage of material and extreme difficulty of separation from 1G apparently prevented them, as it has us, from firm establishment of the structure. Present results, however, indicate that the principal serotonin receptor lipid may be a disialobiose-ganglioside of such a structure.

The reasons why the results from the neuraminidase assay did not correspond perfectly with those from the assay based on galactosemic tissues require some discussion. The correspondence was fair, but not perfect. The "neuraminidase method" said that the most active compound was an impurity present in $1G_a$ which was separated and called new G, and that the disialogangliosides 3G and C had some, but less potency. The "galactosemic method" said that the one with most outstanding potency was B_1, and that $1G_a$ and C had a less impressive showing. In thinking about these results, we must remember that the assay based on neuraminidase may be the more specific. In this one the normal tissue was treated with the enzyme just prior to test. The serotonin receptor was destroyed but compensatory changes in the relative amounts of other gangliosides did not have an opportunity to take place in the tissue.

By contrast, in the assay based on galactosemic tissues, the presumed interference with galactolipid biosynthesis was imposed very early in the life of the animal. The rat grew up with its receptor deficiency, and therefore had a chance to make adjustments to it. In the light of this consideration it was impressive that the correspondence was as good as it was. In both assays there were several inactive gangliosides and only a few with activity. This indicated that considerable specificity was involved.

The finding of some receptor activity in more than one ganglioside should not be perplexing. In the first place we must remember that the separation of these compounds is extremely difficult. Only within the last few years has it been possible to obtain relatively pure gangliosides. It is conceivable that the methods of separation are not yet good enough to ensure complete chemical purity of each substance. The small activities in 2G and 3G, for example, might be due to a slight contamination with the highly active new G. However, if we take the point of view that 3G is completely pure, its activity still should not be surprising. From pharmacological experiments it has been possible to recognize at least two kinds of receptors for serotonin.[18] It may be that each of these is a separate ganglioside such as the ones found to have activity in the present work.

With regard to the question of whether the serotonin receptors actually are

gangliosides, one other point needs mentioning. In his work on the identification of the gangliosides as the substances in brains which fixed tetanus toxin, Van Heyningen[19] showed that purified gangliosides *in vitro* would in fact bind serotonin in aqueous solution. Such binding of the hormone in a reversible fashion with its tissue receptor is one of the requisites for a hormonal receptor.

Summary.—A series of gangliosides and related sphingolipids have been assayed for serotonin receptor activity by two different methods of bioassay. The most active compound was a new ganglioside called new G. Very much less activity was found in three other known gangliosides, but no activity was present in such common gangliosides as G_1, G_4, and the Tay-Sachs' ganglioside. These results indicated that the serotonin receptors are definite and specific chemical substances, possibly one or more gangliosides such as new G.

* This work was supported in part by grant A3386 of the U.S. Public Health Service.

[1] Woolley, D. W., these PROCEEDINGS, **44**, 1202 (1958).

[2] Woolley, D. W., *Biochemical Bases of Psychoses, or The Serotonin Hypothesis About Mental Diseases* (New York: John Wiley and Sons, 1962).

[3] Woolley, D. W., and B. W. Gommi, *Nature*, **202**, 1074 (1964).

[4] Woolley, D. W., and B. W. Gommi, these PROCEEDINGS, **52**, 14 (1964).

[5] Dombro, R. S., L. S. Bradham, N. K. Campbell, and D. W. Woolley, *Biochim. Biophys. Acta*, **54**, 516 (1961).

[6] Woolley, D. W., and N. K. Campbell, *Biochim. Biophys. Acta*, **57**, 384 (1962).

[7] Klenk, E., and W. Gielen, *Z. Physiol. Chem.*, **330**, 218 (1963).

[8] Klenk, E., and W. Kunau, *Z. Physiol. Chem.*, **335**, 275 (1964).

[9] Klenk, E., and W. Gielen, *op. cit.*, **326**, 158 (1961).

[10] Johnson, G. A., and R. H. McCluer, *Biochim. Biophys. Acta*, **70**, 487 (1963).

[11] Svennerholm, L., *Acta Chem. Scand.*, **17**, 860 (1963).

[12] Rapport, M., L. Graf, and J. Yariv, *Arch. Biochem. Biophys.*, **92**, 438 (1961).

[13] Payne, S. N., and B. S. Platt, *J. Neurochem.*, **7**, 5 (1961).

[14] Woolley, D. W., and B. W. Gommi, *Arch. Intern. Pharmacol. Therap.*, in press.

[15] R_f's are given to help in the visualization of relative rates of migration, but it must be remembered that these values differ in each run and are only valuable for identification when knowns and unknowns are run on the same plate.

[16] Kuhn, R., and H. Weigandt, *Chem. Ber.*, **96**, 866 (1963).

[17] Kuhn, R., and H. Weigandt, *Z. Naturforsch.*, **19b**, 256 (1964).

[18] Gaddum, J. H., and K. A. Hameed, *Brit. J. Pharmacol.*, **9**, 240 (1954).

[19] Van Heyningen, W. E., *J. Gen. Microbiol.*, **31**, 375 (1963).

---

*IN VITRO SYNTHESIS OF BRAIN PROTEIN**

BY ALBERT L. RUBIN AND KURT H. STENZEL†

ROGOSIN LABORATORIES, DEPARTMENT OF MEDICINE,
THE NEW YORK HOSPITAL–CORNELL MEDICAL CENTER

Communicated by F. O. Schmitt, March 29, 1965

Neurons are active protein-synthesizing cells as indicated by their highly developed, ribosomal-rich endoplasmic reticulum and by results of metabolic studies on whole brain and brain slices. Cell-free systems derived from brain tissue are also active in protein synthesis.[1–6] These systems provide an *in vitro*