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# Regional Difference in Brain Benzodiazepine Receptor Carbohydrates

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**SUMMARY**

The ability to photolabel benzodiazepine receptors from various regions of the rat brain with $^3$H-flunitrazepam has allowed for the structural examination of these receptors by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions. Results for all regions studied revealed the labeled receptor to consist of a single major band of radioactivity with the apparent molecular weight of approximately 50,000. Under our conditions of labeling we do not significantly label any higher molecular weight forms of the receptor. Exposure of the benzodiazepine receptors to either of the glycosidases neuraminidase (N) and endoglycosidase-H (E) results in the specific removal of sialic acids and complete asparagine-linked carbohydrate moieties, respectively. This type of structural modification of the receptor resulted in an apparent decrease in the molecular weight, as determined by increased mobility on sodium dodecyl sulfate polyacrylamide gel electrophoresis, for all regions examined (cortex + N + E, 8,000–10,000; hippocampus + N, 7,000, + E, 12,000; cerebellum + N, none, + E, 4,000). These results point to a heterogeneity in the posttranslational glycosylation of the benzodiazepine receptor that may be due to brain region-specific differences in glycosylation. The removal of these carbohydrate moieties alters the binding of agonists and antagonists to the benzodiazepine receptor. Cortical agonist binding following either glycosidase treatment resulted in no apparent shift in the $K_d$ but a significant decrease in the $B_{max}$. The $B_{max}$ change may be the result of a large decrease in affinity or denaturation of a subpopulation of benzodiazepine receptors. Antagonist binding also showed no apparent $K_d$ shift but a significant increase in the $B_{max}$. The increase may have resulted from the activation of "hidden" benzodiazepine receptors or a shift of low affinity sites to sites of higher affinity. Cerebellar agonist or antagonist binding was not altered, in terms of either $K_d$ or $B_{max}$, by either enzyme treatment, correlating well with the small amount of carbohydrate removal seen following such treatments. The ability of these enzymes to modify the apparent molecular weight of the benzodiazepine receptors and the strong correlation to altered ligand binding, in a regional specific manner, generally parallel the description given of type 1 and type 2 benzodiazepine receptors.

The existence of a high affinity binding site in brain tissue for the BDZs as a part of the postsynaptic GABA-A receptor complex is well established (1). Major constituents of this complex include proteins with the molecular weight close to 50,000 Da. These proteins have been labeled utilizing nitro-containing benzodiazepines such as flunitrazepam (2, 3), and differential labeling of the proteins has been obtained (4). All investigators have demonstrated the labeling of a 50,000-Da protein, and some studies have also indicated the presence of higher molecular weight species (5). Labeling of the different sized fractions varies regionally, and pharmacological differences have been ascribed to the different sized fractions (4, 5). However, recent immunocytochemical evidence indicates that even the regions that did not show the higher molecular weight fractions by photolabeling possessed a protein fraction which was recognized by a series of monoclonal antibodies prepared against highly purified benzodiazepine receptors (6). The inability to label the higher molecular weight forms in these regions and the reported variability in labeling between investigators (Refs. 3–5 and others) are not understood.

One of the major underlying bases for the differences in the electrophoretic mobility of proteins is the differences in posttranslational modification. A major posttranslational modification that occurs to the cell surface molecules is glycosylation, and in contrast to amino acid sequence, there is much microheterogeneity in carbohydrate composition due to the specificity of the enzymes involved in glycosyl transfer (7). To examine the possible contribution of the carbohydrate to the apparent molecular weight and forms of the benzodiazepine receptors, we examined the action of two glycosidases, neuraminidase (EC 3.2.1.18) and endoglycosidase H, which have specificity for either sialic acid or complete asparagine-linked carbohydrate chains (8). We have found that there are regional differences in the ability of the enzymes to modify the apparent molecular

**ABBREVIATIONS:** BDZ, benzodiazepine; GABA, γ-aminobutyric acid; SDS, sodium dodecyl sulfate; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PPO, 2,5-Diphenyloxazole.

weight of the benzodiazepine receptors, and the differences can be correlated with different effects of the enzyme treatment on ligand binding. These differences generally parallel the previously described type 1 and type 2 benzodiazepine receptors (4, 5, 9, 10) and indicate that the regional differences in glycosylation may alter the binding specificity of the benzodiazepine receptor in a way that is consistent with the hypothesis that the type 1 and type 2 differences are due in part to the regionally determined patterns of glycosylation.

## Materials and Methods

All membranes were prepared from male, approximately 3-month-old Sprague-Dawley rats weighing 350–450 g. $^3$H-Flunitrazepam (77.4 Ci/mmol), $^3$H-Ro15-1788 (76 Ci/mmol), $^3$H-muscimol (20.6 Ci/mmol), and Protosol were purchased through NEN (Boston, MA). Electrophoretic grade acrylamide, Tris base, methylene bisacrylamide, *N,N,N′,N′*-tetramethylene diamine, Coomassie blue stain, and SDS were obtained from Bio-Rad. Neuraminidase, type X (<0.002% protease activity), was purchased from Sigma, and a recombinantly prepared endoglycosidase-H was purchased through Boehringer-Mannheim Biochemicals (BMB). Incubation of 0.025 units of BMB endoglycosidase-H with denatured ovalbumin for 20 hr (pH 7.0, 37°) revealed no proteolytic activity as detected via SDS PAGE. Benzodiazepines were the gift of Hoffman-LaRoche, Nutley, NJ. CL218-872 was a gift of Lederle Laboratories.

**Preparation of membranes and labeling.** Brain membranes were prepared and photolabeled as previously described (3). The benzodiazepine receptor is subject to proteolytic activity (11), and for this reason protease inhibitor, 1 mM phenylmethylsulfonyl fluoride, was added to all buffers. Cortical, hippocampal, and cerebellar tissue was obtained according to the dissection method described by Glowinski and Iversen (12). Membranes were prepared by incubation with 10 nM $^3$H-flunitrazepam in 25 mM potassium phosphate buffer (pH 7.4); $10^{-4}$ M clonazepam, which competitively inhibits photolabeling, was added to the blank incubations. This incubation was run for 60 min in the dark at 4°. Membranes were photolabeled by exposure to long wave ultraviolet light (mineral lamp, model UVGL-25) for 5 min at a distance of 5 cm while on ice and were continuously agitated with a magnetic stir bar. Following this, the membranes were washed until radioactivity in the supernatant was down to background level. Extensive washing was necessary to remove unreacted $^3$H-flunitrazepam, which under SDS PAGE could result in spurious radioactive bands.

**Enzyme treatment and assay.** Photolabeled brain membranes and nonlabeled membranes for use in binding assays were treated under identical conditions with glycosidases. Washed and pelleted membranes were resuspended at a concentration of 1 g/ml of tissue weight in 50 mM sodium phosphate and 1 mM phenylmethylsulfonyl fluoride (pH 6.8) containing either 0.5 units of neuraminidase or 0.1 units of endoglycosidase-H per ml. This mixture was then incubated with constant agitation for 12 hr at 37°. Control membranes were prepared under identical conditions excluding the enzyme. Following incubation, membranes were washed three times in 25 mM potassium phosphate buffer for use in either SDS PAGE or binding assays.

Ten per cent gels were prepared according to the method of Laemmli (13). Membrane samples were boiled for 3 min in 0.2 M Tris-HCl (pH 6.80) containing 5% 2-mercaptoethanol, 10% glycerol, 2.3% SDS, and 0.001% bromphenol blue before application onto gel. A low molecular weight standard kit (Bio-Rad) was used for reference in molecular weight determination. The amount of radioactivity in the protein bands was determined by cutting portions of the gel lanes into either 1-, 1.5-, or 2-mm slices and incubating these slices in Econofluor with 5% Protosol for 24 hr at room temperature. Slice thickness for each gel was determined by whether or not the gel was allowed to equilibrate in buffer prior to cutting. This technique allowed for the recovery of 70–80% of the radioactivity applied to the gel lanes. Following Coomassie blue staining, parallel gels were prepared for fluorography by incubation in dimethylsulfoxide for 30 min, in 23% PPO in dimethylsulfoxide for 2 hr, and finally in deionized water for 1 hr. The gel was then dried, placed against Kodak X-Omat film, and exposed at −80° for a period of 3 to 4 weeks.

Binding assays were performed as previously described (3, 14). Nonspecific binding for both $^3$H-flunitrazepam and $^3$H-Ro15-1788 assays was defined by use of $10^{-4}$ M clonazepam. Nonspecific $^3$H-muscimol binding was determined by use of $10^{-3}$ M GABA. All assays were done in a final volume of 1 ml and contained the equivalent of 10 mg of wet tissue weight. Results were analyzed by a computer-fitting program from Lunden Associates.

## Results

**SDS PAGE analysis after enzyme treatment.** The molecular weight of $^3$H-flunitrazepam photolabeled binding sites prepared from whole rat brain was determined by SDS PAGE. Utilizing either autoradiographic or gel slice techniques, $^3$H-labeled receptors from whole brain were shown to migrate as a single band with an apparent molecular weight of 50,000 and a light shadow (<10%) of slightly greater weight. Treatment of these membranes with either of the glycosidases neuraminidase or endoglycosidase-H prior to SDS PAGE analysis significantly altered the migration of the $^3$H-BDZ band (Fig. 1). Treatment of whole brain membranes with neuraminidase reduced the apparent molecular weight of the $^3$H-BDZ band to 44,000, a decrease of 6,000 Da. Endoglycosidase-H treatment of similar membranes reduced the molecular weight of the $^3$H-BDZ band by 8,000 (Fig. 2). These data suggest that carbohydrate moieties comprise a significant portion of the $^3$H-BDZ.

Rat cortex, cerebellum, and hippocampus were isolated and photolabeled in the same manner as whole brain. Again, the molecular weights of the $^3$H-BDZ receptors were determined by SDS PAGE before and after glycosidase treatment. In all cases not enzymatically treated, the $^3$H-BDZ bands migrated as a single major band with an apparent molecular weight of 50,000. However, following treatment each region displayed a distinctly different $^3$H-BDZ banding pattern (Fig. 3). Neuraminidase-treated cortical BDZ receptors and those treated with endoglycosidase-H appeared to have an apparent molecular weight between 40,000 and 42,000. The migratory pattern of cortical $^3$H-BDZ receptors before and after glycosidase treatment was consistent with the data obtained with whole brain membranes. Unlike the cortex or whole brain, a reduction in the molecular weight of the $^3$H-BDZ receptor band for the cerebellum was not seen following neuraminidase treatment. A reduction of only 4,000 Da was observed following endoglycosidase-H treatment, which is half the reduction seen in $^3$H-BDZ receptors from cortex. In direct contrast to the cerebellum or cortex, the molecular weight of hippocampal $^3$H-BDZ receptors was markedly reduced by either enzyme treatment. Neuraminidase treatment reduced the apparent molecular weight of the $^3$H-BDZ receptors to between 43,000 and 42,000, a reduction of approximately 7,000 Da. Endoglycosidase-H treatment revealed two product bands, a minor band at 40,000 Da and a major band at 38,000–37,000 Da.

**Benzodiazepine binding after enzyme treatment.** The reduction in the apparent molecular weight noted for most of the $^3$H-BDZ receptors after glycosidase treatment might result in alteration of the binding properties of these receptors. Flunitrazepam and Ro15-1788 binding studies to whole brain membranes and subsequently membranes prepared from cortical and cerebellar brain regions were examined. Initial studies

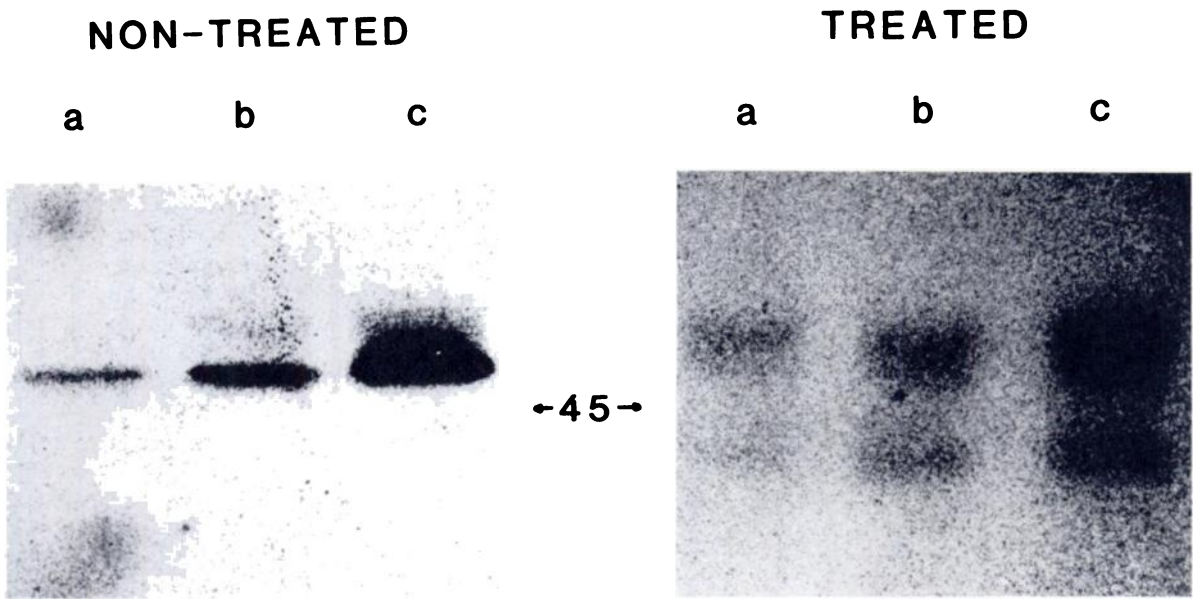


**Fig. 1.** Fluorograms of SDS PAGE endoglycosidase-treated and nontreated photolabeled whole brain membranes. Membranes were prepared and photolabeled and gels were run as described in Materials and Methods. For treated and nontreated fluorograms, *lane a* contained 200 μg of membranes; *lane b*, 300 μg of membranes, and *lane c*, 500 μg of membranes. *45* corresponds to the migration distance of the 45,000 molecular weight standard. The portion of the gel from molecular weights 66,000 to 35,000 using standard proteins as landmarks is shown. This is the region of the gel sliced in subsequent figures.

utilizing flunitrazepam revealed no significant shift in the $K_d$ for neuraminidase-treated whole brain membranes (Table 1, Fig. 4A). There was, however, a significant reduction in the $B_{max}$ of the treated membranes in comparison to nontreated membranes. Endoglycosidase-H-treated membranes followed the same trend described for the neuraminidase-treated membranes with no apparent shift in the $K_d$ but a decrease in the $B_{max}$ (Table 1). These data suggest that up to 20% of the total population of BDZ receptors are shifted to a low affinity state or denatured by either glycosidase treatment. The ability to accurately measure these low affinity sites would require a binding assay which would utilize extremely high concentrations of ligand.

Antagonist binding studies using Ro15-1788 with membranes prepared from whole brain and treated with either neuraminidase or endoglycosidase-H revealed no significant shift in the $K_d$ between treated and nontreated membranes (Table 1, Fig. 4B). Following glycosidase treatment there was a significant increase in the $B_{max}$ with both enzymes. These data can be interpreted as being the result of unmasking a population of BDZ sites, possibly of low affinity. Following glycosidase treatment these sites are converted to the affinity state which is normally associated with Ro15-1788 binding. Similar binding studies were then performed on treated and non-treated cortical and cerebellar membranes in an attempt to determine if the differences in the molecular weights noted for the $^3$H-BDZ receptors from cortex and cerebellum following glycosidase treatment would result in different binding profiles for flunitrazepam and Ro15-1788 binding. Flunitrazepam binding studies of neuraminidase treatment of membranes from both regions revealed no significant shift of the $K_d$ for either region (Table 2). However, neuraminidase treatment resulted in a reduction of the $B_{max}$ for cortical membranes of nearly 50% (Table 2) but no reduction in the $B_{max}$ of cerebellar membranes (Table 2). Flunitrazepam binding performed on endoglycosidase-H-treated membranes resulted in the same trend seen with neuraminidase-treated membranes. No shift was found in the $K_d$ from control values, and a larger reduction of the cortical $B_{max}$, as opposed to the cerebellar $B_{max}$, was observed.

Ro15-1788 binding studies of enzyme-treated and nontreated cortical and cerebellar membranes were then performed. Cortex showed no change in the $K_d$ after either neuraminidase or endoglycosidase-H treatment relative to control values. However, $B_{max}$ values were elevated almost 2-fold following either enzyme treatment (Table 2). In direct contrast, the cerebellum did not show a shift in either the $K_d$ or $B_{max}$ of receptors following either enzyme treatment (Table 2).

The above data in conjunction with the SDS gel studies indicate that the removal of carbohydrate moieties, as shown by reduction of molecular weight, alters the binding characteristics of a population of BDZ receptors. In the specific case of the cerebellum the apparent lack of enzymatic removal of carbohydrates by either neuraminidase or endoglycosidase-H correlates well with the lack of any significant change in the $B_{max}$ receptors for either ligand following enzyme treatment.

The close association and interactions between the BDZ and GABA receptors might also be dependent upon the carbohydrate moieties. The ability of GABA, muscimol, and pentobarbital to enhance BDZ binding was studied in whole brain and neuraminidase-treated whole brain membranes (Table 3). In the case of GABA and muscimol, no change in the "GABA shift" was found. The pentobarbital shift was completely eliminated in the neuraminidase-treated whole brain membranes.

Ro15-1788 displacement data, utilizing the analogs listed in Table 4 on neuraminidase and nontreated membranes, revealed no change in the ability of these compounds to displace Ro15-1788 binding after neuraminidase treatment. $IC_{50}$ values remained unaltered, and the Hill coefficient for all ligands before and after treatment remained about one (15). Ro15-1788 displacement curves, with CL218,872 used as the inhibitor, were

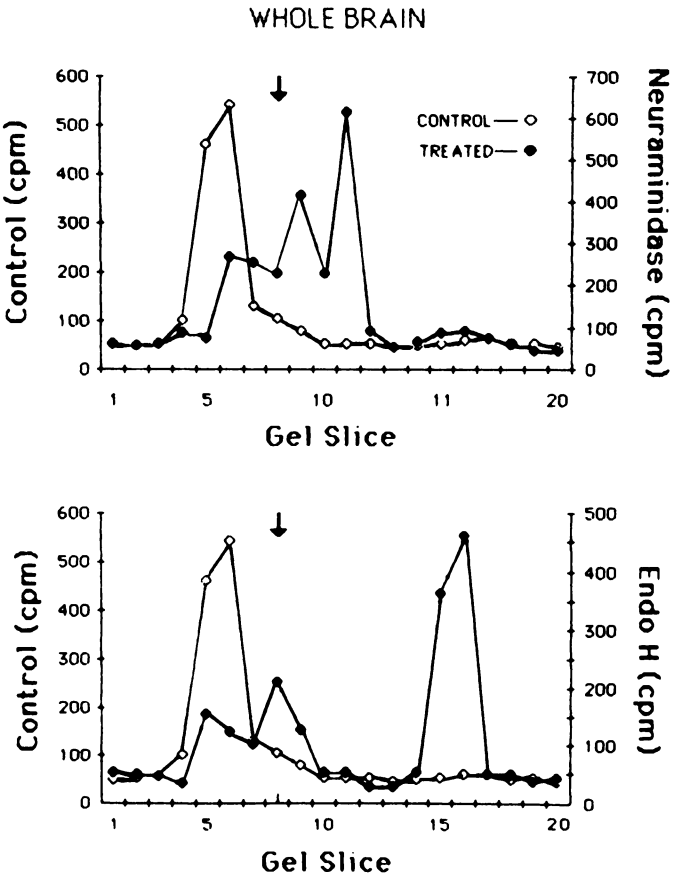


**Fig. 2.** Profiles of radioactivity eluted from SDS PAGE run on photolabeled whole brain membranes. Membranes were prepared, photolabeled, and analyzed as described in Materials and Methods. Slice number 1 corresponds to approximately a 66,000 molecular weight, while slice number 20 corresponds to approximately a 35,000 molecular weight. The *arrow* corresponds to the electrophoretic migration of the 45,000 molecular weight standard. Gels were sliced at a uniform thickness of 1 mm.

examined for both cortical and cerebellar tissue. Results for both glycosidase-treated membranes showed a slight increase in the $IC_{50}$. As previously reported (16), the Hill coefficient for the cortex was less than one, while for the cerebellum the Hill coefficient was about one (Table 4).

Another set of experiments was designed to determine the effect of neuraminidase and endoglycosidase-H treatment on muscimol binding performed on frozen whole brain membranes (Table 5). Neuraminidase treatment had no effect on the binding of muscimol to high or low affinity GABA sites as compared to control tissue. Endoglycosidase-H treatment resulted in a large increase in the $K_d$ (decreased affinity) for the high affinity site. The low affinity site was apparently not altered in its ability to interact with muscimol.

## Discussion

Studies of the BDZ receptor have recently focused on the underlying mechanism for the wide range of pharmacological actions displayed by the various BDZs. The existence of BDZ receptor subtypes, delineated by physical criteria, may be one explanation for this phenomenon. Early evidence obtained by SDS PAGE analysis of $^3$H-flunitrazepam-labeled BDZ receptors revealed two major apparent BDZ subtypes with molecular weights of 55,000 and 50,000. These possible subtypes were found in varying concentrations in several brain regions studied (5). Using similar SDS PAGE analysis, we were able to label largely (>90%) the 50,000-Da species of the coupled BDZ receptor, confirming a previously published report from this laboratory (3). However, as seen in Fig. 1, a minor band with a molecular weight of 54,000–55,000 is apparent in the lanes where large amounts of tissue were run. This minor band may be the result of incomplete electrophoretic resolution due to the possible overloading of the lanes with sample tissue or may represent a protein labeled with low efficiency. The photolabeling protocol used by a number of investigators is similar with respect to nM concentrations of flunitrazepam, equilibration, and photolabeling times (3, 5, 17). Two differences do exist in the photolabeling protocol which could account for the discrepancy in the reported findings. First, all reports of receptor heterogeneity, as determined by photolabeling and SDS PAGE, use a buffering system with a high chloride concentration, between 100 and 150 nM NaCl. However, this would not explain the failure of all investigators to label a higher molecular weight species found by immunoblots in cerebellum (6). The second difference occurs in the processing of the tissue after photolabeling (3, 5). We wash our tissue extensively, until radioactivity in the supernatant is down to background level. It has been reported that noncovalently bound ligands can result in the appearance of radioactive bands other than a band associated with the tracking dye front (18). The differences in labeling noted here do not provide clear answers to the question of BDZ receptor heterogeneity detected by photoaffinity labeling. It should be noted, however, that the use of high concentrations of $^3$H-nitro-containing BDZs (>50 nM), where nonspecific binding represents a large portion of the total, leads to the presence of a large number of radiolabeled bands which are detected on autoradiography. The inclusion of high levels of BDZ results in displacement from nonreceptor sites which are labeled when highly reactive benzo intermediates are photochemically formed (unpublished data). Quantitative immunoprecipitation and immunoblot studies of the BDZ receptor by monoclonal antibodies prepared against affinity-purified GABA/BDZ complex suggest a regionally uniform composition of the BDZ receptor rather than the heterogeneous composition implied earlier by photoaffinity labeling (6). In these studies, regions which were apparently without the higher molecular weight material measured by photolabeling contained an immunoreactive unlabeled protein. Thus, differences in photolabeling from region to region do not seem to be due to independent benzodiazepine receptor subtypes.

Several hypotheses can be formulated in an attempt to explain the BDZ receptor heterogeneity seen after photolabeling with SDS PAGE techniques. One such hypothesis could be regionally and species-specific glycosylation schemes for the BDZ receptors. Evidence has now been reported indicating that varying degrees of glycosylation result in the species heterogeneity described for the $\alpha$ subunit of the acetylcholine receptor (19) and central and peripheral insulin receptors (20). These data in association with the report that the BDZ receptor is also a glycoprotein (10) have prompted us to determine if glycosylation may account for the regional differences in BDZ receptors. The molecular weight of brain BDZ receptors apparently consists of 10–20% sialic acids and asparagine-linked carbohydrate moieties as determined after neuraminidase or

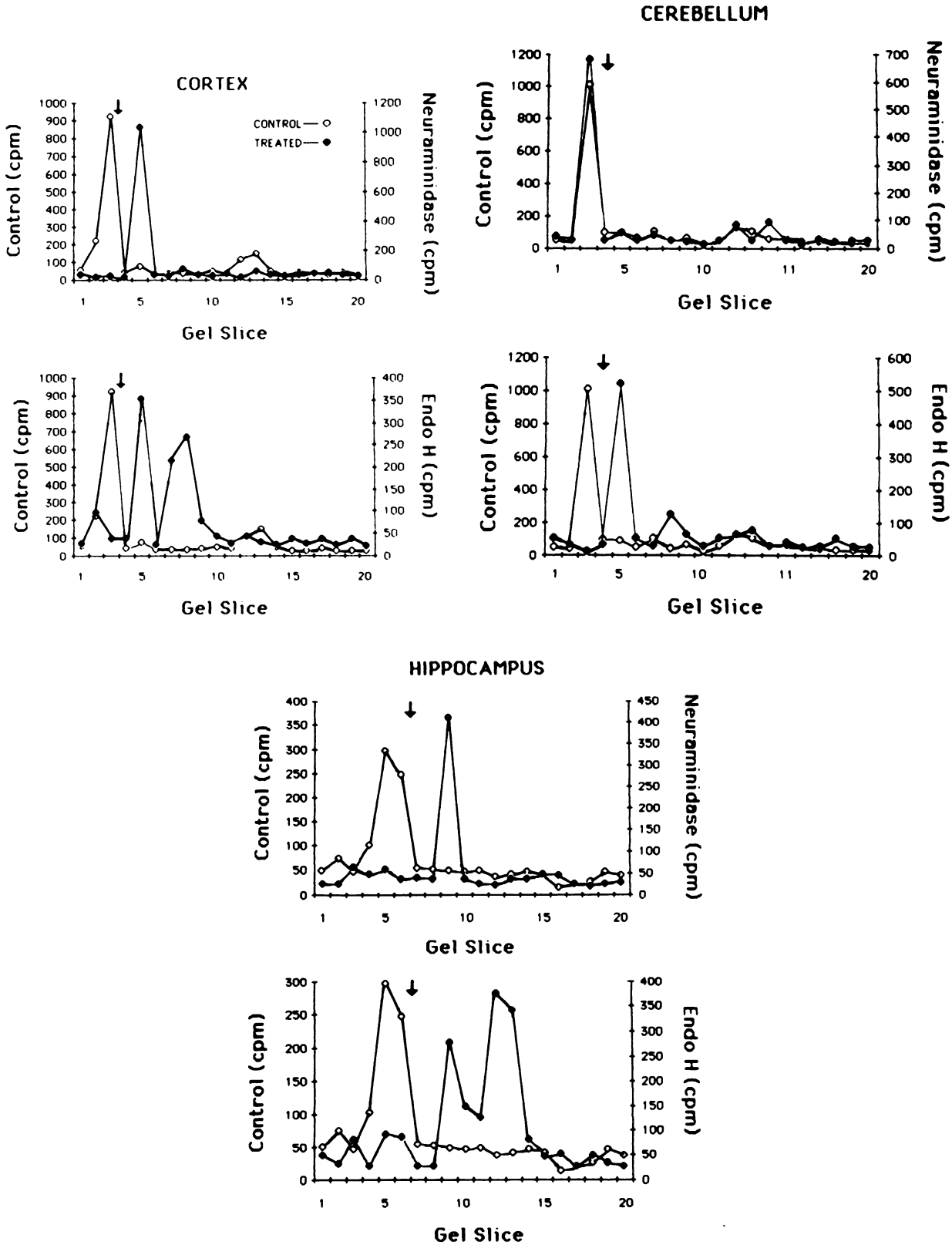


**Fig. 3.** Profiles of radioactivity eluted from SDS PAGE on photolabeled membranes from the cortex, cerebellum, and hippocampus. Membranes were prepared, photolabeled, and analyzed as described in Materials and Methods. Slice number 1 corresponds to approximately a 60,000 molecular weight, while slice number 20 corresponds to approximately a 35,000 molecular weight (with the exception of the hippocampus which ranges from 66,000 to 35,000). The *arrow* corresponds to the electrophoretic migration of the 45,000 molecular weight standard. Gels were sliced at a uniform thickness of 1.5 mm (with the exception of the cortex, which was sliced at a thickness of 2 mm).

TABLE 1

**Scatchard analysis of $^3$H-flunitrazepam and $^3$H-R015-1788 binding in glycosidase-treated whole brain membranes**

Membranes were prepared and incubated as described in Materials and Methods. Binding assays were carried out in triplicate, and ligand concentrations ranged between 0.5 and 25 nM. $B_{max}$ values are represented by the mean ± SE. Each enzyme treatment was carried out on several independently prepared membrane fractions with similar results.

| Condition and ligand | $K_d$ | $B_{max}$ |
|---|---|---|
| | *nM* | *fmol/10 mg of tissue* |
| Flunitrazepam | | |
| Control | 1.6 | 580 ± 19 |
| Neuraminidase | 1.7 | 480 ± 9[a] |
| Endoglycosidase-H | 2.3 | 430 ± 36[a] |
| R015-1788 | | |
| Control | 1.1 | 660 ± 38 |
| Neuraminidase | 1.6 | 790 ± 13[a] |
| Endoglycosidase-H | 1.2 | 910 ± 33[b] |

[a] $p < 0.05$.
[b] $p < 0.01$.

endoglycosidase-H treatment. The heterogeneity in molecular size seen among BDZ receptors from various brain regions following enzyme treatment may be due to the contribution of carbohydrate moieties which did not serve as substrates to the enzymes utilized in this study. Such carbohydrates would be O-linked oligosaccharides. Therefore, these moieties still affect the electrophoretic mobility of the receptor (e.g., in cerebellum), and such underlying causes may account in part for the previously reported receptor heterogeneity and regional drug specificity.

Sialic acids, which are negatively charged, normally occur as terminal residues of asparagine-linked carbohydrate chains and as such could be in an excellent position to alter ligand receptor interactions through charge influences or physical constraint on conformation. Neuraminidase treatment of both central and peripheral insulin receptors revealed altered affinity in the binding of $^{125}$I-insulin (20). The effect of neuraminidase treatment on BDZ binding is more complex and varies for agonist and antagonist binding. Agonist binding using $^3$H-flunitrazepam as the ligand following either enzyme treatment results in the conversion of a subpopulation of BDZ to a low affinity state as determined by a reduction in the apparent $B_{max}$. The lack of change in controls reacted under identical conditions as the enzymatically treated tissue compared to normal binding assay conditions (3) rules out the reduction in $B_{max}$ as a consequence of proteolytic inactivation of the receptor by an endogenous source. Contaminating proteases in the enzyme suspensions can also be disregarded in the case of the endoglycosidase-H, as it was the product of recombinant techniques and protease free. The use of highly purified, protease-free neuraminidase does not completely eliminate the possibility of some minute receptor inactivation due to contaminating protease activity. This would not show the regional specificity we have seen.

Opposite results were seen for antagonist binding using Ro15-1788 as the ligand where an increase was noted in the $B_{max}$ following either enzymatic treatment. This may be the result of a conversion of low affinity sites or inactive "hidden" sites to an affinity state normally associated with the Ro15-1788-labeled BDZ receptor. It is possible that the higher molecular sized receptors also contribute to increased binding by this type of unmasking phenomenon and that there are forms of the BDZ receptor in which antagonist but not agonist binding is occluded by carbohydrate.

The results of SDS PAGE analysis of BDZ receptors prepared from cortex or cerebellum correlate well with binding data obtained for these regions. $B_{max}$ changes in cortical binding for both agonist and antagonist, after either enzyme treatment, reflect results obtained for the whole brain. However, this was not the case with cerebellar binding where no significant $B_{max}$ change was seen following either enzyme treatment. These results are consistent with the small change in the electrophoretic mobility after enzyme treatment of the cerebellar BDZ receptor. In comparison with that seen for the cortical receptor, these data suggest that sialic acid is not a major constituent of receptors in cerebellum. This may explain the inability of our binding assays to uncover any variation in cerebellar tissue following either enzyme treatment. In addition, it argues against the action of possible proteases which would not show regional specificity.

The ability of either enzyme to alter the binding characteristics of one or more BDZ subtypes, delineated by degree of receptor glycosylation, could have also modified the interaction normally associated with the BDZ receptors overlapping agonist and antagonist sites. Displacement curves using Ro15-1788 were obtained for control and neuraminidase-treated whole brain by using a variety of benzodiazepine agonists, antagonists, and mixed agonists. The fact that neuraminidase did not alter

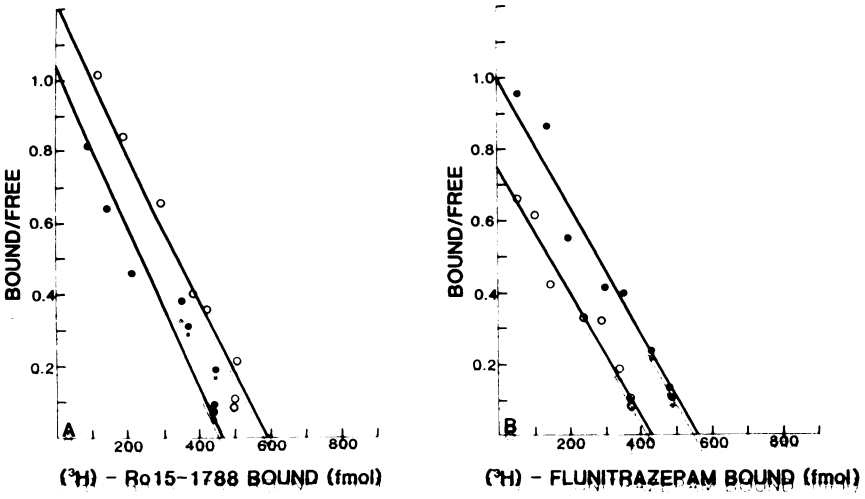


**Fig. 4.** A, Scatchard analysis of $^3$H-Ro15-1788 binding to control (●) and neuraminidase-treated (○) whole brain membranes. The ligand concentration ranged from 1 to 50 nM. B, Scatchard analysis of $^3$H-flunitrazepam binding to control (●) and neuraminidase-treated (○) whole brain membranes. The ligand concentrations ranged from 1 to 50 nM.

| | $K_d$ | $B_{max}$ |
|---|---|---|
| | *nM* | *fmol/10 mg of tissue* |
| Flunitrazepam | | |
| Control | 5.3 | 560 |
| Neuraminidase treated | 5.3 | 440 |
| Ro15-1788 | | |
| Control | 3.9 | 460 |
| Neuraminidase treated | 4.0 | 590 |

TABLE 2

**Scatchard analysis of $^3$H-flunitrazepam and $^3$H-R015-1788 binding in glycosidase-treated cerebellar and cortical membranes**

Membranes were prepared and incubated as described in Materials and Methods. Incubations were carried out in triplicate, and ligand concentrations ranged between 0.5 and 25 nM. $B_{max}$ values are represented by the mean + SE.

| Condition and ligand | $K_d$ | $B_{max}$ |
|---|---|---|
| | nM | fmol/10 mg of tissue |
| Cerebellum | | |
| Flunitrazepam | | |
| Control | 3.9 | 143 ± 14 |
| Neuraminidase | 4.7 | 105 ± 21 |
| Endoglycosidase-H | 5.2 | 135 ± 24 |
| R015-1788 | | |
| Control | 2.9 | 180 ± 18 |
| Neuraminidase | 2.6 | 260 ± 68 |
| Endoglycosidase-H | 2.1 | 215 ± 61 |
| Cortex | | |
| Flunitrazepam | | |
| Control | 3.2 | 535 ± 71 |
| Neuraminidase | 4.4 | 250 ± 70 |
| Endoglycosidase-H | 4.4 | 290 ± 33 |
| R015-1788 | | |
| Control | 1.7 | 520 ± 110 |
| Neuraminidase | 1.1 | 840 ± 42 |
| Endoglycosidase-H | 1.2 | 919 ± 62 |

TABLE 3

**The effects of GABA, muscimol, and pentobarbital on $^3$H-flunitrazepam binding to neuraminidase-treated whole brain membranes**

Membranes were prepared and incubated as described in Materials and Methods. After preincubation with no additions (nontreated) or with neuraminidase, binding assays were carried out with no additions (controls) or with a $10^{-5}$ M concentration of either GABA, muscimol, or pentobarbital. Data for incubations in the presence of drug were expressed as percentage of control for neuraminidase-treated and nontreated whole brain membranes. The control values for the specific binding of 0.5 nM flunitrazepam were 73 fmol for nontreated membranes and 64 fmol for neuraminidase-treated membranes. This decrease in binding due to neuraminidase treatment is consistent with the data shown in Table 1.

| Incubation conditions | Specifically bound |
|---|---|
| | % of control |
| Nontreated | 100 ± 2 |
| +10 μM GABA | 165 ± 8 |
| +10 μM muscimol | 141 ± 8 |
| +10 μM pentobarbital | 133 ± 8 |
| Neuraminidase treated | 100 ± 1 |
| +10 μM GABA | 175 ± 7 |
| +10 μM muscimol | 138 ± 10 |
| +10 μM pentobarbital | 100 ± 10 |

the slopes of the Ro15-1788 displacement curves or the Hill coefficients suggests that sialic acid removal does not change the BDZ receptor agonist/antagonist relationship as measured by displacement assay.

The ability of GABA and related compounds to interact with the low affinity GABA binding site and enhance benzodiazepine binding to the agonist site has been known for some time (14). It was of interest then to determine if carbohydrates might in some way participate in the GABA-mediated enhancement of BDZ binding. Neuraminidase treatment of the BDZ receptor resulted in no change in the ability of GABA or muscimol to enhance BDZ binding. The ability of pentobarbital, a sedative barbiturate, to enhance BDZ binding was abolished by neuraminidase treatment. This result suggests that the mechanism for the enhancement of benzodiazepine binding by GABA and pentobarbital could be different in terms of the degree of carbohydrate involvement in the enhancement phenomena

TABLE 4

**$IC_{50}$ and Hill coefficients for the inhibition of $^3$H-R015-1788 binding before and after neuraminidase treatment**

Nontreated and neuraminidase-treated whole brain membranes were prepared as described in Materials and Methods. The ligand concentration was 1nM. Hill plots were constructed from inhibition curves by plotting (log P/100 − P) versus I, where P is the percentage bound and I is the inhibition concentration (15). The Hill coefficient, $\eta$H, was determined by linear regression analysis. ND, not done.

| Inhibitor | Nontreated membranes | | Neuraminidase-treated membranes | |
|---|---|---|---|---|
| | $IC_{50}$ | $\eta$H | $IC_{50}$ | $\eta$H |
| | nM | | nM | |
| Clonazepam | 2.6 | 1.13 | 2.7 | 1.01 |
| Diazepam | 25.5 | 1.10 | 29.0 | 1.06 |
| R015-1788 | 5.0 | 0.93 | 5.8 | 1.3 |
| CGS 8216 | <1 | ND | <1 | ND |
| CGS 9896 | 2.1 | 0.84 | 2.8 | 0.91 |
| CL 218,872 (cortex) | 750 | 0.675 | 900 | 0.620 |
| CL 218,872 (cerebellum) | 230 | 0.91 | 256 | 0.86 |

TABLE 5

**Scatchard analysis of $^3$H-muscimol binding following endoglycosidase-H treatment of whole brain membranes**

Membranes were prepared as described in Materials and Methods. Muscimol concentrations ranged between 0.5 and 1000 nM to allow for the detection of both high and low affinity binding sites. The data were analyzed by the least squares method described in Materials and Methods. No changes were seen following neuraminidase treatment of brain membranes.

| Treatment | $K_d$ | $B_{max}$ |
|---|---|---|
| | nM | fmol/10 mg of tissue |
| Nonendoglycosidase-H Treated | | |
| High affinity | 5.4 ± 0.3 | 960 ± 71 |
| Low affinity | 360 ± 18 | 4400 ± 121 |
| Endoglycosidase-H Treated | | |
| High affinity | 16.9 ± 1.8 | 1100 ± 102 |
| Low affinity | 400 ± 14 | 4000 ± 86 |

(21). Alternatively, the site of action of the barbiturates, said to be close to the picrotoxin site (21–23), may itself be altered by enzyme treatment.

It is known that the BDZ and GABA receptor together form the major components of a neuroreceptor complex (21, 22) and may be found on the same subunit (23). This close orientation might suggest that GABA binding may be altered in the same manner as was BDZ binding by our glycosidase treatment. Data obtained from muscimol binding to neuraminidase- or endoglycosidase-H-treated whole brain membranes suggest that sialic acid and asparagine-linked carbohydrate removal does not result in modification of binding characteristics similar to those reported here for the BDZ receptor. Neuraminidase's lack of effect on muscimol binding and endoglycosidase-H's resultant increase in the $K_d$ of the high affinity GABA site were a set of enzyme effects not seen for the BDZ receptor where both enzymes altered binding in a similar direction, differing only in magnitude of effect. Carbohydrate modification of the BDZ receptor resulted only in the alteration in the number of sites, never comprising more than a portion of the total BDZ receptor population as determined by binding data. Carbohydrate modification of the GABA receptor seems to alter the affinity of a large portion, if not the entire population, of the high affinity GABA sites. The lower affinity GABA receptors, which are so intimately associated with the benzodiazepine receptor, are not influenced in the same manner, in terms of ligand-receptor interactions, by the carbohydrate chains. It is the low affinity site which is involved in the GABA/BDZ interaction.

The results obtained by this study suggest that sialic acid and asparagine-linked carbohydrates comprise a significant portion of the BDZ receptor, and one of the roles they play is a modulation of ligand-BDZ receptor interaction. Preliminary data suggest that sialic acids, if present, do not influence GABA/BDZ receptor interactions, while asparagine-linked carbohydrates play a significant part in determining the affinity of the high affinity GABA sites. Tunicamycin inhibits the formation of asparagine-linked glycoproteins and has recently been shown to alter the expression of $\beta$-adrenergic receptors in culture (24). Studies of BDZ receptor glycosylation utilizing a similar approach could further enhance our knowledge of carbohydrate moieties' contribution to this receptor's function.

Knowledge of carbohydrate's contributions to the conformational stability and function of membrane proteins is quite primitive. The results reported here suggest that other neurotransmitter receptors may be influenced in their binding properties by glycosylation. These regional differences may account for regional heterogeniety, some apparent receptor subtypes, and alteration in receptor populations which occur in development. Differences in glycosylation may also influence the pharmacology of certain receptors.

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