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Radiation Inactivation Studies of the Dopamine Reuptake Transporter Protein

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SUMMARY

Using radiation inactivation, we have estimated the target size for the neuronal dopamine transporter protein. The specific binding of several radioligands previously shown to label the dopamine transporter was determined in an irradiated striatal membrane preparation. The apparent target size of the 1-[1-(2-[^{3}H]benzo[*b*]thienyl)cyclohexyl]piperidine site was approximately 98 kDa. However, the apparent target size of the "cocaine binding site," as measured with the cocaine analogue 2β-[^{3}H]carbomethoxy-3β-(4-fluorophenyl)tropane in the same assays, was approximately 140 kDa. Radiation inactivation of the binding of other ligands (GBR-12935 and mazindol) led to target size estimates in the same range (94 kDa and 133 kDa, respectively). All of these target sizes are significantly larger than the estimate of 70 kDa derived from the deduced amino acid sequence for the cloned dopamine reuptake transporter cDNA. Larger target sizes than expected have also been reported for ligand binding to the sodium-dependent serotonin transporter and glucose transporter. The estimated sizes for the ligand binding site(s) associated with the dopamine transporter protein are difficult to reconcile with a single transporter protein of 70 kDa. We conclude that the dopamine transporter protein is a homo- or hetero-oligomer when occupied *in situ* by uptake-blocking drugs like cocaine.

The DA reuptake transporter protein is responsible for the Na^+-dependent reuptake of DA, which is the principle inactivation mechanism for synaptically released DA. This DA transporter also appears to be an important site of action for psychomotor stimulants, including cocaine (1). By inhibiting DA reuptake, cocaine leads to increased synaptic levels of DA in areas of the brain such as the nucleus accumbens (2), where DA is believed to be reinforcing. Tritiated ligands such as [^{3}H]cocaine (3), the cocaine derivative [^{3}H]CFT (4), [^{3}H]GBR-12935 (5), [^{3}H]mazindol (6), [^{3}H]nomifensine (7), [^{3}H]RTI-55 (8), and [^{3}H]methylphenidate (9) have been used to characterize the binding of DA reuptake inhibitors to the transporter protein. Covalent labeling of the DA transporter protein with radioligands has also been accomplished (10–12). More recently, Amara and colleagues (13), as well as Uhl and coworkers (14), have cloned a DA transporter cDNA. When expressed, it results in a functional DA transporter protein that is sensitive to cocaine and related drugs. *In situ* hybridization has revealed that the DA transporter gene is expressed solely in dopaminergic neurons. DA reuptake is inhibited with similar pharmacological specificity at the recombinantly expressed DA transporter protein and the native transporter, suggesting the presence of a single gene product. The estimated molecular mass of the DA transporter protein, based on the deduced amino acid sequence of the cloned cDNA, is approximately 70 kDa, which is similar to results of recent studies of the photolabeled protein using 3-azido-[^{3}H]GBR-12935 (12) or ^{125}I-1-[2(diphenymethoxy)ethyl]-4-[2-(4-azido-3-iodophenyl)ethyl] piperazine (15).

Although these studies strongly suggest that the apparent molecular mass of the fully glycosylated DA transporter is approximately 70–80 kDa, little is known about the size of the functional transporter *in situ*. Conceivably, the DA transporter may exist as a homo-oligomer, as proposed for the sodium-dependent glucose transporter (16) (which is similar in tertiary structure to the DA transporter). Alternatively, the DA transporter protein may exist as a hetero-oligomer analogous to the dihydropyridine-sensitive calcium channel (17).

Radiation inactivation has proved to be a useful method for estimating the molecular size of many enzymes (18) and, more recently, of various receptors, including the nicotinic and muscarinic cholinergic (19, 20), opiate (21), DA (22), and benzodi-

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ABBREVIATIONS: DA, dopamine; BTCP, 1-[1-(2-benzo[*b*]thienyl)cyclohexyl]piperidine; CFT, 2β-carbomethoxy-3β-(4-fluorophenyl)tropane; GABA, γ-aminobutyric acid.

azepine (23) receptors, as well as the serotonin transporter (24, 25). This method requires measuring the amount of activity surviving increasing doses of radiation. The functional target size can then be calculated based on certain experimentally validated assumptions (26). Radiation inactivation of proteins is an "all or none event," i.e., one high-energy "hit" results in complete destruction of the functional target. Because larger "targets" (i.e., proteins of higher molecular mass) are more likely to be "hit" than smaller targets, larger proteins are inactivated at lower doses of radiation than are smaller proteins. From the exponential decrease in protein activity, one can calculate the target size of the functional protein. Because radiation inactivation is performed *in situ*, without protein solubilization or denaturation by detergents, the method has proven particularly useful for estimating the approximate sizes of proteins that are required for "functional" ligand binding or enzyme activity. Using radiation inactivation, we have calculated the target size of the DA transporter protein labeled with three different radioligand uptake inhibitors. Our results suggest that the binding of reuptake inhibitors to native proteins requires native structures whose mass is considerably greater than the estimate of 70 kDa derived from the deduced amino acid sequence of the cloned DA reuptake transporter cDNA.

Experimental Procedures

Materials

[^{3}H]CFT (specific activity, 82.1 Ci/mmol), [^{3}H]flunitrazepam (specific activity, 77.4 Ci/mmol), [^{3}H]GBR-12935 (specific activity, 53 Ci/mmol), and [^{3}H]mazindol (specific activity, 25.3 Ci/mmol) were obtained from New England Nuclear (Boston, MA). [^{3}H]BTCP (specific activity, 29.8 Ci/mmol) was synthesized by deCosta and colleagues as described previously (27). All other drugs and reagents were obtained from commercial sources.

Methods

Preparation of striatal (P_2) synaptosomal membranes. Male Sprague-Dawley rats (100–200 g), housed with free access to food and water, were used in all experiments. After decapitation, the corpus striatum was dissected on ice (approximately 100 mg, wet weight/striatal pair). A crude synaptosomal membrane preparation was obtained as described previously (5). Briefly, tissue pooled from several rats (approximately 1.5 g, wet weight, of striatal tissue) was homogenized in 10 volumes of ice-cold sucrose (0.32 M) with 100 μM phenylmethylsulfonyl fluoride, using a Teflon/glass homogenizer, and was centrifuged at 1000 × *g* for 10 min. The resulting supernatant was centrifuged at 23,000 × *g* for 20 min, the supernatant was discarded, and the pellet (P_2) was resuspended in 8 ml of ice-cold 0.32 M sucrose. Tissue samples were frozen in sucrose rather then lyophilized, because past studies suggested that lyophilization can lead to erroneously high target size estimates (23). Aliquots (500 μl) were then placed in thin-walled, 2-ml, glass vials, which were immediately frozen on dry ice, sealed with an oxygen gas flame, and stored at −70°.

Radiation inactivation. Radiation inactivation was carried out as described previously (23). The samples were irradiated with 10-MeV electrons from a linear accelerator (Armed Forces Radiobiology Research Institute, Bethesda, MD). Radiation dose measurements were made with thermoluminescent dosimeters. During irradiation the samples were maintained at −135° with a stream of cold nitrogen gas. The irradiated samples were stored at −70° until assays were performed.

Binding assays. Before assay, the irradiated and nonirradiated, frozen, striatal P_2 membrane aliquots were quickly thawed by immersion in a water bath maintained at 25°. [^{3}H]BTCP, [^{3}H]CFT, [^{3}H]mazindol, and [^{3}H]GBR-12935 binding to P_2 membrane preparations was determined as follows. The incubation mixture contained 100 μl of radioligand (final concentration, 1–10 nM), 20 μl of the striatal aliquots described above, and 100 μl of buffer (50 mM Tris, 120 mM NaCl, pH 7.4). For each assay, an unlabeled drug at a concentration of 10 μM was used to define nonspecific binding. Because target sizes might vary for different ligands, it was conceivable that the ligand chosen to define nonspecific binding could affect target size determinations. Therefore, as noted below, for some ligands we measured a target size estimate using both the same ligand and a different ligand to define nonspecific binding. Additional buffer was added to bring the final assay volume to 2 ml. After incubation at 5° for 60 min, the incubation mixture was rapidly filtered by vacuum filtration through Whatman GF/B filter discs (which had been presoaked for 30 min in 0.1% polyethyleinimine). Each filter was rinsed three times with 4 ml of ice-cold incubation buffer. The filters were solubilized in Aquasol scintillation fluid (New England Nuclear, Boston, MA) and the radioactivity was measured in a Beckman scintillation counter at a counting efficiency of 40%. Specific binding was defined as the difference between total and nonspecific binding and was at least 75% for each assay. Identical assay conditions were used for all radioligands, to facilitate comparison with previously reported data and comparison of estimated target sizes for the different radioligands.

Specific [^{3}H]flunitrazepam binding to the $GABA_A$/benzodiazepine receptor was assayed as described in our earlier radiation inactivation study (23), with minor modifications. These included the use of frozen striatal aliquots (prepared as described above) and a final incubation volume of 0.5 ml. Nonspecific binding was defined using 10 μM diazepam. All assays were done in triplicate.

Target size calculations. Target sizes were calculated by expressing the specific binding in irradiated samples (A) as a function of that observed in the nonirradiated controls (A_o). A least-squares linear regression was calculated from $\ln (A/A_o) = -KD$, where D is the dose of radiation (in megarads). A direct measure of the target size can be obtained using the following equation: target mass (in daltons) = $6.4 \times 10^5 S_t/D_{37}$, where $S_t = 2.8$ for irradiations performed at 135° and D_{37} ($= K^{-1}$) is the radiation dose (in megarads) required to reduce activity to 37%.

Statistics. Differences between groups were analyzed by Student's t test.

Results

When crude synaptosomal (P_2) membranes prepared from rat striatum were frozen and irradiated with high-energy electrons, the binding of [^{3}H]mazindol, [^{3}H]CFT, and [^{3}H]BTCP to the DA transporter protein decreased as a simple exponential function of radiation dose (Figs. 1–3). The simple exponential rate of decline we observed for ligand binding at these concentrations is consistent with binding to a single site. From these data (Table 1) a target size of approximately 133 ± 28 kDa was estimated for the mazindol binding site (seven experiments), 98 ± 14 kDa for the [^{3}H]BTCP binding site (eight experiments), 143 ± 27 kDa for the [^{3}H]CFT site (six experiments), and 94 ± 36 kDa for the [^{3}H]GBR-12935 site (five experiments). Because we obtained an unexpectedly high value for the [^{3}H]CFT site, we also determined the target size (140 ± 20 kDa, five experiments) when nonspecific binding was defined with unlabeled mazindol rather than CFT. The estimated target size for the [^{3}H]BTCP binding site differed significantly from that for the [^{3}H]CFT binding site when both assays were undertaken under similar conditions, on the same day, and with the same irradiated tissue samples. This difference was greater than what would be expected due to variation in the radiation inactivation technique between different assays. In three independent experiments, BTCP binding and CFT binding were assayed simultaneously with the same irradiated material. Pairwise com-

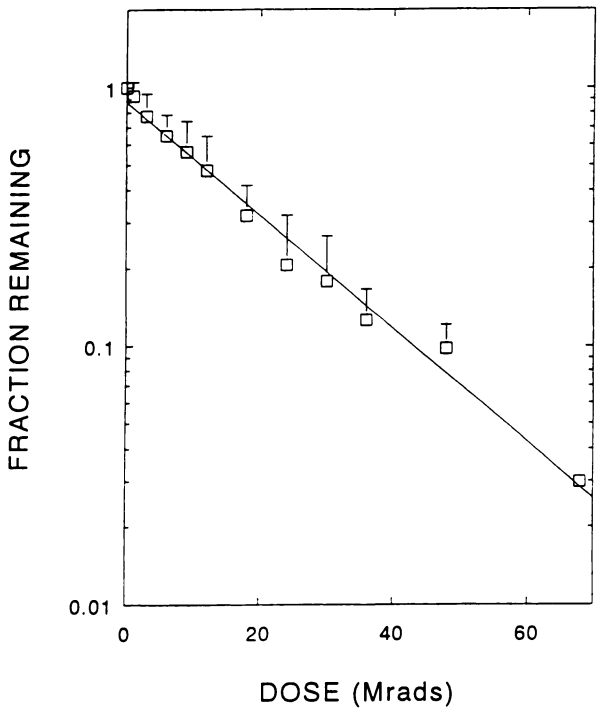

Fig. 1. Surviving specific binding of [^{3}H]BTCP to rat brain striatal membranes exposed to increasing doses of radiation. Membrane suspensions frozen in 0.32 M sucrose, in sealed glass vials, were irradiated at −135° with 10-MeV electrons at a dose rate of 15–30 Mrad/hr. Thawed samples were assayed at 50° as described. Unlabeled mazindol was used to define nonspecific binding. Data were combined from eight independent experiments.

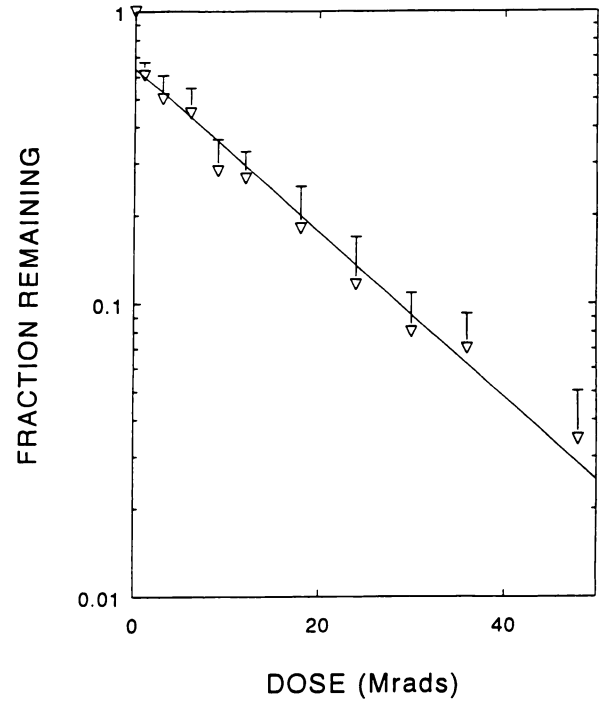

Fig. 2. Surviving specific binding of [^{3}H]mazindol to rat brain striatal membranes exposed to increasing doses of radiation. Conditions were as described in the legend to Fig. 1. Unlabeled mazindol was used to define nonspecific binding. Data were combined from seven independent experiments.

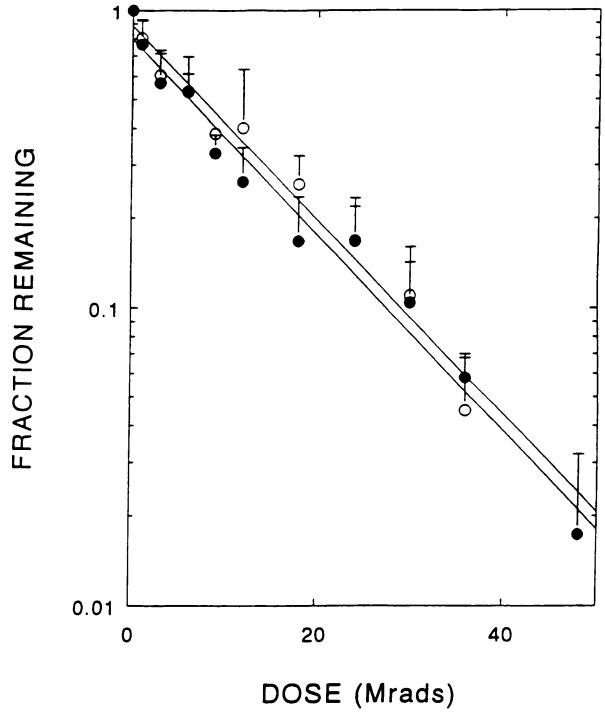

Fig. 3. Surviving specific binding of [^{3}H]CFT to rat brain striatal membranes exposed to increasing doses of radiation. Conditions were as described in the legend to Fig. 1. ○, Data were combined from five independent experiments in which unlabeled mazindol was used to define nonspecific binding. ●, Data were from six independent experiments in which unlabeled cocaine was used to define nonspecific binding.

TABLE 1

Target size measurements in rat brain striatum

The nonradioactive compound used in each case to define nonspecific binding is shown in parentheses. Values are averaged ± standard deviations.

Ligand	Target size	No. of experiments
	kDa	
GBR-12935 [(*cis*)-Flunitrazepam]	94 ± 36	5
BTCP (mazindol)	98 ± 14	8
CFT (mazindol)	140 ± 20	5
CFT (cocaine)	143 ± 27	6
Mazindol (mazindol)	133 ± 31	7

parison showed that the target size for CFT was 155 ± 8% of that for BTCP, showing that the two targets are significantly different. The target size estimates for binding of the other ligands (GBR-12935 and mazindol) were in a similar range as those for BTCP and CFT, although direct comparison is difficult because these assays were not undertaken simultaneously. The mazindol binding inactivation curve began at 60% rather than 100% of total because that curve was the best least-squares fit to all of the data. This phenomenon has been seen frequently in past studies, but extensive research has failed to identify the cause (28). Beginning the curve at 60% did not significantly change the target size, in comparison with beginning the curve at 100%. Scatchard analysis of the specific binding of each radioligand revealed that the decrease in binding due to increasing radiation dose reflected a decrease in the number (B_{max}) and not the apparent affinity (K_d) of ligand binding sites (Table 2). Although a component of low affinity binding is often observed with these ligands, we specifically chose ligand concentrations to characterize the high affinity binding.

TABLE 2
Analyses of binding data for control and irradiated samples

Ligand	Radiation dose	B_{max}	K_d
	Mrad	*pmol/mg*	*nM*
Mazindol	0	6.81	7.89
	6	2.31	6.01
BTCP	0	5.25	3.23
	6	1.47	4.13
CFT	0	4.30	12.75
	6	2.31	6.5

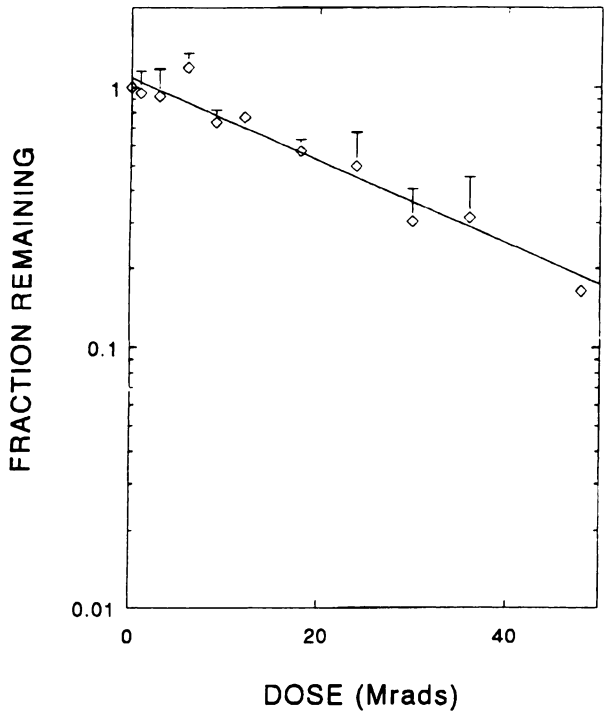

Fig. 4. Surviving specific binding of [^{3}H]flunitrazepam to rat brain striatal membranes exposed to increasing doses of radiation. Conditions were as described in the legend to Fig. 1. Unlabeled diazepam (10μM) was used to define nonspecific binding. Data were combined from three independent experiments.

As an internal control, the target size for flunitrazepam binding to the $GABA_A$/benzodiazepine receptor was also determined in these experiments (Fig. 4). As reported previously (23), a single-exponential decline in flunitrazepam binding was observed, yielding a target size of 62.0 ± 17.9 kDa, which is consistent with previous radiation inactivation studies (23) and the recent data derived from the cloning of the GABA receptor (29).

Discussion

In the present study, radiation inactivation analysis of radioligand binding to the DA transporter protein yields target size estimates of 94–143 kDa, considerably higher than the molecular size estimates of the protein determined by photoaffinity labeling (11, 12) or that based on the deduced amino acid sequence of the cloned cDNA (13, 14). Target size estimates from radiation inactivation analyses have yielded higher molecular mass estimates, compared with those determined by other methods, for a number of proteins. For example, photoaffinity labeling of the DA (D2) receptor with [^{3}H]azidospiperone resulted in a radiolabeled protein of approximately M_r 90,000 (30), whereas the target size of the D2 receptor measured with [^{3}H]spiroperidol was approximately 150 kDa, as determined by radiation inactivation (22). It is possible that proteins in addition to those that are covalently labeled are required for ligand binding to occur *in situ*. If the native DA transporter is a hetero-oligomer *in situ*, then another polypeptide may be needed for optimal ligand binding. Alternatively, an additional protein, distinct from the DA transporter protein itself, may modulate ligand binding to the transporter. For example, Eldefrawi and colleagues (31) have reported stimulation by purinergic agonists of both ligand binding to the DA transporter protein and DA uptake.

Our target size estimates for both the [^{3}H]BTCP and [^{3}H]GBR-12935 binding sites (98 kDa and 94 kDa, respectively) associated with the DA transporter are consistent with a possible hetero-oligomeric structure for the DA transporter. An additional 30-kDa protein might be needed for ligand binding to the 75-kDa protein. Conceivably, the allosteric interactions in ligand binding to the DA transporter reported independently by several groups (3, 4) could reflect allosteric interactions between different proteins associated with the DA transporter. Although it is possible that a common sodium-transporting protein is shared among a variety of structurally diverse sodium-dependent transporters, chemical modification of the sodium-dependent glucose transporter suggests that a single protein contains both the sodium and glucose binding sites (16). Moreover, expression of the recently cloned DA transporter cDNA alone is sufficient to result in functional DA uptake that is inhibited by either mazindol or cocaine (13), although Caron and co-workers (32) have demonstrated differences in the affinity of the transporter for substrates when it is expressed in neuronal versus non-neuronal cell lines. Only in neuronally derived cell lines did 1-methyl-4-phenylpyridinium have affinity for the cloned transporters comparable to that for DA uptake into rat striatal synaptosomal membranes. Conceivably, an additional subunit affecting substrate affinity might be present in the neuronal cell lines. If the native protein in the striatum contains additional proteins accounting for the higher target size estimate, then radiation inactivation of cells expressing the cloned transporter might result in a lower target size estimate than what we now report as the target size estimate for the native striatal protein. Studies are in progress to determine the target size of the DA transporter protein in cells expressing the cloned DA transporter cDNA and to compare these results in neuronal and non-neuronal cell lines.

Given the similar target size estimates for the protein(s) labeled by both [^{3}H]BTCP and [^{3}H]GBR-12935 (approximately 100 kDa), it is possible that both drugs inhibit DA transport by interacting with binding sites associated with the same protein or, alternatively, with different proteins of similar molecular weights. It is difficult to reconcile the estimated target size of the DA transporter labeled with either BTCP or mazindol with the molecular mass of the cloned DA transporter (70 kDa). The target size estimate for CFT binding to the transporter, 143 kDa, would be consistent with this ligand binding to a homodimer of the cloned 70-kDa protein. However, other oligomeric structures could also account for the observed target size. Conceivably, CFT or cocaine could bind to an entirely different protein than does BTCP, GBR-12909, or mazindol. However, more recent studies have revealed that the

azido derivatives of both CFT and GBR-12909 label the same 70-kDa protein (33). A recent "rapid report" by Milner *et al.* (34) reported a target size for inactivation of [^{3}H]GBR-12935 binding to canine striatal membranes of 278 kDa, considerably higher than the value we obtained with this or any other DA transporter ligand. The larger target size could be due to the use of lyophilized samples, which have been previously shown to yield artifactually large target sizes (23, 35), in comparison with tissue frozen in buffer. Another significant difference in methodology involves the species used in our study and the study of Milner *et al.* (rat versus canine).

The differences in observed target size estimates for different radioligands that bind to the DA transporter are also consistent with an earlier radiation inactivation study of the closely related serotonin transporter protein (25). In that study, a different target size for the [^{3}H]paroxetine binding site (70 kDa), compared with the [^{3}H]imipramine binding site (90 kDa), on the serotonin transporter was observed. To date, most of the cloned sodium-dependent transporter cDNAs encode proteins of approximately 70 kDa. However, radiation inactivation of ligand binding to these proteins frequently yields larger targets sizes, e.g., [^{3}H]phlorizin binding to the glucose transporter [110 kDa (36) or 230 kDa (37)], [^{3}H]imipramine binding to the serotonin transporter (90 kDa) (25), and our own data on radioligand binding to the DA transporter. Even though the cloned sodium-dependent glucose transporter, like the DA transporter, was functional when expressed, a second gene (encoding a protein that further stimulated glucose uptake) was eventually cloned by Koepsell and co-workers (37). In an earlier study before the cloning of the additional subunit, Koepsell *et al.* (38) cited the glucose transporter radiation inactivation study as important evidence for an oligomeric structure. Our virtually identical data for the DA transporter also are consistent with an oligomeric structure. In our view, the differences in target sizes for the binding of various ligands to the DA transporter suggest that the functional size of these sodium-dependent transporters *in situ* may be greater than that predicted for the polypeptides from the amino acid sequence deduced from the corresponding cloned cDNA nucleotide sequences. Whether these differences reflect dimerization of DA transporter polypeptides *in situ*, the presence of associated unrelated proteins, or extensive post-translational modification will require further investigation.

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