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THE NUCLEAR LOCALIZATION OF A PUTATIVE NEUROTRANSMITTER RECEPTOR

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A high affinity strychnine binding site has been identified within a membrane fraction prepared from partially purified rat brain nuclei. This interaction appears similar in its characteristics to that occurring in the non-nuclear membrane fraction which is thought to occur at the synaptic glycine receptor complex. Both the nuclear and non-nuclear membrane binding of tritiated strychnine is greater within the pons-medulla region than in the cerebral cortex. Nuclear membrane binding sites for dopamine, norepinephrine (β -adrenergic), acetylcholine (muscarinic), GABA, and diazepam were not detected.

INTRODUCTION

The mechanism by which neurotransmitters act following their liberation from nerve terminal synapses is thought to involve binding at specific postsynaptic sites. This allosteric interaction causes changes in the configuration of the receptor protein which may then effect alterations in the permeability of the membrane to ions, causing fluxes of the local membrane potential. Transmembrane intracellular effects such as activation of adenylyl cyclase can then be induced resulting in modulation of the postsynaptic cell. These processes are thought to occur without internalization of the transmitter-receptor complex.

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We have examined the high affinity binding properties of a series of labeled compounds which are thought to interact with some selectivity to specific sites within nerve tissue. The binding of these radioactive ligands has been compared in nuclear, cytoplasmic, and total membrane fractions of brain. The nuclear membranes generally bound ligands to a much lesser extent than did cytoplasmic membranes. However, a specific high affinity strychnine binding site has been identified within the nuclear membrane fraction. The specific binding of strychnine appears to involve an interaction with the receptor complex for glycine (26).

EXPERIMENTAL PROCEDURES

Male Fischer rats (6–8 weeks old) were decapitated and whole brains or brain regions were dissected, weighed, and homogenized in a glass-Teflon homogenizer in 19 volumes 0.32 M sucrose. The homogenate was spun at 1000 g for 10 min. The resulting supernatant was centrifuged (20,000 g, 10 min) and the pellet constituted the crude mitochondrial fraction. The pellet from the low speed centrifugation was vigorously shaken with 2.47 volumes of 2.4 M sucrose, 3 mM CaCl_2 , 50 mM Tris-HCl, pH 7.4; the final sucrose concentration was therefore 1.8 M. This suspension was centrifuged (35,000 g, 30 min) (4). A pinkish white pellet was obtained and this was the nuclear fraction. The material rising to the surface of the high density sucrose is called the cell debris fraction and probably consists largely of nuclei and incompletely homogenized cells. In some studies a total membrane fraction was prepared by centrifugation (20,000 g, 10 min) of a 5% (w/v) tissue homogenate in 0.32 M sucrose. Fractions were stored frozen (-20°C) and membranes were prepared from them by homogenizing precipitates in cold distilled water corresponding to 19 times the original tissue wet weight. Centrifugation (20,000 g, 10 min) was followed by a further homogenization in 40 mM tris-HCl pH 7.4. Protein was estimated by the method of Lowry et al. (15) and membrane suspensions were diluted with tris buffer to a final concentration of 1–2.5 mg/ml.

The nucleic acid content of the original brain homogenate and nuclear fraction was measured by suspending tissues or precipitates in ethanol and centrifuging at 20,000 g for 10 min. This procedure was repeated and the final precipitate was incubated with 0.3 N KOH (37° , 2 hr). DNA and protein were precipitated by neutralization of the solution with 60% (w/v) HClO_4 (perchloric acid). The absorption of the supernatant was determined spectrophotometrically at its 260 nm maximum in order to estimate RNA (A_{260} of 1 mg/ml hydrolyzed RNA was 30.0). The precipitate was incubated with 0.5 N HClO_4 (90°C , 20 min) and centrifuged. The absorbance in the supernatant of hydrolysed DNA was measured at its absorbance maximum (266 nm). The A_{267} of 1.0 mg hydrolyzed DNA/ml was 32.3. The final precipitate was incubated with 0.3 N NaOH (60°C , 1 hr) and recentrifuged. The resulting supernatant was used for protein determinations (15).

Binding assays were performed by a glass fiber disc filtration method previously described (25). Saturability, specificity, regional distribution, and reversibility characteristics of ligands were delineated prior to commencing this study (1, 3). Briefly the method consisted of incubation of membranes (100–250 μg protein, 37° 15 min) together with $0.7\text{--}6.0 \times 10^{-9}$ M of a tritiated ligand in 40 mM Tris HCl pH 7.4. The receptor-ligand complex was separated by filtration through 0.3 μ pore size glass fiber discs (Gelman Inc., Ann Arbor, Michigan) washed three times with 4 ml Tris buffer. Dried discs were then counted in a scintillation

counter (38–43% efficiency) in order to determine the total amount of bound ligand. Binding was also determined in a series of parallel incubations in the presence of 10^{-6} M of an unlabeled competing pharmacological agent. Those counts which remained in the presence of this excess of the competitor were taken to represent nonspecific binding. Specific binding could then be calculated by subtraction of the nonspecific binding from total binding.

The assay of the dopamine receptor was performed using 10^{-9} M [1-phenyl- 4^3H]spiroperidol (23 Ci/mmol) as the binding ligand and haloperidol as the competing compound in control tubes. In a parallel manner 10^{-9} M DL-[benzilic-4, 4'- 3H]quinuclidinyl benzilate (29 Ci/mmol, QNB) was used to measure muscarinic sites with atropine as a competitor. Benzodiazepene sites were estimated with 10^{-9} M [methyl- 3H]diazepam (73 Ci/mmol) and the unlabeled compound as competitor. 0.9×10^{-9} M L-[propyl-2,3- 3H]dihydroalprenolol (34 Ci/mmol) and unlabeled alprenolol were used in β -adrenergic binding site assays. For the glycine site, 10^{-5} M non-radioactive strychnine was used to compete with 5.6×10^{-9} M [$G^3\text{H}$]strychnine sulphate (13 Ci/mmol). Isotopes were obtained from New England Nuclear Corporation, Boston, MA. and Amersham-Searle Inc., Arlington Heights, Illinois.

RESULTS

The nuclear fraction when estimated by phase contrast microscopy appeared to consist largely of morphologically intact nuclei. In view of the difficulty of estimating nuclear purity by morphological criteria alone, the purity of the nuclear fraction was quantitated by a method taking advantage of the fact that most of the cellular DNA is nuclear. The RNA/DNA and protein/DNA ratios have previously been proposed as indices of purity of cerebral nuclei (2, 21). The RNA/DNA ratio of the nuclear pellet was 0.56 and the protein/DNA ratio was 8.34 (results of two independent determinations within 5% of each other). Since purified brain nuclei have been reported to have an RNA/DNA ratio of 0.4 and a protein/DNA ratio of 4.8 (8, 17), our preparation of nuclei was estimated to be 58–71% pure. Since we found the protein/DNA ratio of whole brain to be 96 (mean of two determinations within 5% of each other), this represents an 11.5-fold purification. While these nuclei were not devoid of cytoplasmic membranes, this method resulted in a preparation of a reliable and definable degree of purity.

The binding of several labeled ligands to membranes prepared from the nuclear and crude mitochondrial fraction was compared. Values for the nuclear binding of ligands representing dopamine, benzodiazepine, muscarinic cholinergic, and α -adrenergic sites were 9–43% of those found in the crude mitochondrial fraction (Table I). This could not have been due to the destruction of receptors by high density sucrose, since membranes prepared from the 'cell debris' fraction exposed to the 1.8 M sucrose were found to have binding capacities intermediate between those of the total brain membrane preparation and those of purified nuclei (Table II).

TABLE I
EXTENT OF SPECIFIC BINDING OF LABELED LIGANDS TO BRAIN MEMBRANES
PREPARED FROM NUCLEAR AND CRUDE MITOCHONDRIAL FRACTIONS

Putative receptor	Nuclear
	crude mitochondrial
Glycine	1.15 $\pm$ 0.02
Dopamine	0.43 $\pm$ 0.02
Benzodiazepine	0.14 $\pm$ 0.01
Muscarinic acetylcholine	0.09 $\pm$ 0.03
β -adrenergic	0.35 $\pm$ 0.07

Data are the mean of 3–7 separate experiments; standard errors are shown. Results are presented as the extent of binding (pmol bound per 100 mg protein) of nuclear membranes relative the membranes from the crude mitochondrial fraction.

In contrast to all other ligands studied, the binding of strychnine to the nuclear membranes was as great as its binding to the crude mitochondrial membranes (Table I).

In order to study this phenomenon more closely, some characteristics of the nuclear strychnine binding site were determined. The binding of [^3H]strychnine to whole and nuclear membranes was compared in two regions known to have differing strychnine binding capacities (26, 3). Binding was 2.9 ± 0.4 times higher in nuclei derived from pons-medulla than from cortex. This paralleled the intensity of binding for whole tissue membranes. In the case of total brain membranes specific binding defined by using 10^{-5} M glycine as competitor was $90 \pm 7\%$ of the value obtained using 10^{-5} M strychnine. For whole brain nuclear membranes, the corresponding efficacy of glycine relative to strychnine was $86 \pm 6\%$. The

TABLE II
BINDING OF LABELED LIGANDS TO MEMBRANES PREPARED FROM NUCLEAR, CELL
DEBRIS, AND BRAIN MEMBRANES

Ligand	Nuclear	Cell fraction Total	Cell debris
Strychnine	180 $\pm$ 34	181 $\pm$ 9	147 $\pm$ 11
Spiroperidol	96 $\pm$ 25	253 $\pm$ 22	121 $\pm$ 29
Diazepam	4.5 $\pm$ 1.4	28.9 $\pm$ 4.0	29.5 $\pm$ 2.5
QNB	252 $\pm$ 4	753 $\pm$ 83	414 $\pm$ 41
Dihydroalprenolol	20 $\pm$ 8	75 $\pm$ 2	51 $\pm$ 3

Results expressed as pmol bound/total gm protein together with standard errors. Each value represents mean of three experiments.

similarity of the nuclear and cytoplasmic receptors was further suggested when Scatchard plots (22) for the binding of strychnine at a variety of concentrations between $3\text{--}100 \times 10^{-9}$ M were determined.

The binding capacity and dissociation constant (K_D) for membranes of the crude mitochondrial fraction were 100 ± 23 pmol/100 mg protein and $(42 \pm 14) \times 10^{-9}$ M, respectively. The corresponding values for nuclear membranes were 87 ± 13 pmol/100 mg protein and $68 \pm 25 \times 10^{-9}$ M, and thus not significantly different from cytoplasmic values.

DISCUSSION

There are several reports of a nonsynaptic binding site for some neurotransmitter receptors. Cholinergic receptors may exist within the axon plasma membrane (16). Varga et al. (23) have some evidence for a mitochondrial binding site for GABA and extrasynaptic GABA systems have been described in the peripheral nervous system (7). The appearance of high affinity binding sites often precedes the development of functioning synapses (24, 13). The internalization of β -adrenergic receptors within frog erythrocytes has been proposed as a mechanism for receptor desensitization (10). Internalization of receptors for some peptide hormones and soluble cytoplasmic receptors for steroids have been described (9, 20, 14, 11) and the specific nuclear binding of [^3H]flunitrazepam has been reported (6). However, there are no previous reports of a nuclear receptor site for neurotransmitter species. The implication from the data presented here is that the postsynaptic glycine receptor may become internalized and migrate to the neuronal nucleus. In a manner analogous to steroids, the glycine-receptor complex may be capable of directly causing gene derepression.

The possibility of an activated glycine receptor having the capacity to alter RNA synthesis is suggested by the report that strychnine, at levels known to cause subtle behavioral changes, will increase the RNA concentration within certain brain areas (12, 19). Strychnine may act as an agonist of glycine in the intracellular role of the receptor. The concentration of free glycine within the brain is around 10^{-3} M (18). Thus the nuclear receptor could be extensively occupied and perhaps activated without any migration of glycine receptor complexes from the external cell membrane. However, the regional distribution of the nuclear receptor suggests that it is related to neurotransmission rather than to the general metabolic roles of glycine. However, the evidence for glycine as a neurotransmitter is somewhat equivocal. It may be that glycine has a hor-

monal role in those brain regions where high affinity binding sites for glycine are found.

The data presented here do not permit distinction between binding to chromatin or to nuclear membranes. Furthermore, the nuclear fraction is unquestionably contaminated with cytoplasmic components. The use of detergents in order to further purify nuclear was precluded since these may modulate receptor sites. However, strychnine bound to the nuclear fraction to a much greater extent than all other labeled receptor-probe ligands. This anomalous binding is best explained in terms of a glycine receptor with a nuclear location.

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