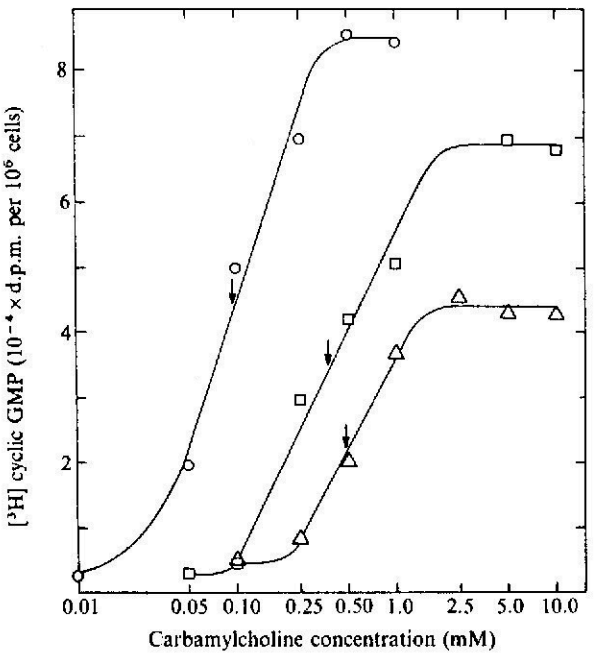

Fig. 2 Effect of preincubation of cultured nerve cells with carbamylcholine on the carbamylcholine dose-response curve. Mouse neuroblastoma clone NIE–115 cells (subculture 19) were preincubated with carbamylcholine (0.1 mM final concentration), washed three times and then assayed in duplicate for carbamylcholine-stimulated [³H]cyclic GMP formation at the indicated concentrations of carbamylcholine, as described in the legend to Fig. 1. The data are increases over basal levels of radioactivity, and were, in d.p.m. per 10^6 cells, 4,200, 5,200 and 5,100 for control, and 3- and 6-min carbamylcholine-treated cells, respectively. Arrows indicate the concentration of carbamylcholine at half-maximal stimulation. There were approximately 2.6×10^5 cells per well, as determined by an electronic cell counter (Model Z_F, Coulter Electronics) and 690 μg protein, as determined by a modification of the method of Lowry *et al.*[15], with bovine serum albumin as a standard. ○, control; □, carbamylcholine preincubated cells, 3 min; △, carbamylcholine preincubated cells, 6 min.

response (E.R., unpublished data), the antagonist binding site may not be involved in this desensitisation.

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LSD labels a novel dopamine receptor in molluscan nervous system

ELECTROPHYSIOLOGICAL studies on molluscan ganglia have provided evidence for the presence of two pharmacologically distinct dopamine responses—a hyperpolarising response which is inhibited by low concentrations of ergot alkaloids and related drugs, and a depolarising response which is sensitive to neuroleptics such as haloperidol[1–3]. Although dopamine receptors in the mammalian central nervous system (CNS) have been detected directly by the binding of a number of ^{3}H-labelled ligands, including the lysergic acid derivatives lysergic acid diethylamide (LSD) and dihydroergocryptine[4–8], no such studies have been reported with molluscan nervous system. We report here that ^{3}H-LSD binds to both dopamine and serotonin (5-HT) receptors in molluscan ganglia, but that conditions can be obtained which allow the two receptors to be studied separately. This is the first demonstration that dopamine and 5-HT receptors may be investigated individually in the same tissue using a single ligand. The pharmacological characteristics of the dopamine-sensitive ^{3}H-LSD binding are compatible with the properties of the dopamine receptor which mediates hyperpolarisation in certain molluscan neurones. These receptors are, however, different from the dopamine receptors previously described in the mammalian CNS.

Binding studies were carried out on a particulate fraction derived from the circumoesophageal ring of ganglia, which comprises the CNS of the Roman snail *Helix pomatia*. Specific

Table 1 Effect of putative neurotransmitters on specific [³H]-LSD binding to snail ganglia particulate fraction

Experiment	Agent	Concentration	Inhibition of specific [³H]-LSD binding (%)	
1	5-HT	10 μM	32	
	Dopamine	10 μM	35	
	Noradrenaline	10 μM	0	
	Octopamine	10 μM	0	
	Histamine	10 μM	0	
2	5-HT	3 μM	29	
	Dopamine	3 μM	32	
	5-HT + dopamine	3 μM	67	(61)
	5-HT	10 μM	39	
	Dopamine	10 μM	57	
	5-HT + dopamine	10 μM	100	(96)
	5-HT	30 μM	58	
	Dopamine	30 μM	64	
	5-HT + dopamine	30 μM	100	(100)
	5-HT	500 μM	100	
	Dopamine	500 μM	93	
	5-HT + dopamine	500 μM	100	(100)

Helix pomatia were decapitated and the circumoesophageal ring of ganglia dissected. Ganglia were homogenised (60 mg wet weight per ml) in cold buffer (0.2 M sucrose, 2 mM Tris-HCl, *p*H 7.3) using a Polytron (30 s; setting 5). The homogenate was centrifuged at 750*g* for 15 min at 4 °C and the supernatant decanted. The pellet was resuspended in the same volume of cold buffer and recentrifuged. The combined supernatants were centrifuged at 4 °C for 30 min at 15,000*g*, and the resulting pellet washed once in 2 mM Tris-HCl, *p*H 7.3. The pellet was finally resuspended in this solution to a protein concentration of 1–2 mg per ml and stored at −70 °C until use. Control experiments demonstrated that there was no difference between fresh and pre-frozen membranes in terms of the amount of specific or nonspecific [³H]-LSD binding observed, and that the ability of 5-HT or dopamine to compete with this binding was unaltered. The binding reaction mixture contained membrane protein (20–100 μg per tube), 50 mM Tris-HCl, *p*H 7.3, 1.8 mM ascorbic acid, [³H]-*d*-LSD (specific activity 11.1 Ci mmol^{-1}), and various drugs as indicated in a volume of 500 μl. In experiments 1 and 2 the [³H]-LSD concentrations were 1.0 nM and 0.2 nM, respectively. After 10 min at 37 °C, the reaction was terminated by rapidly adding 5 ml of ice-cold buffer (50 mM Tris-HCl, *p*H 7.3) and filtering immediately through a Whatman GF/B glass fibre filter mounted in a suction apparatus. The filter was then washed 4 times with 5 ml volumes of the same buffer. The time taken for filtering and washing was 5–10 s. Radioactivity bound to trapped particles on the filter was counted in a liquid scintillation counter using a Triton X-100–toluene-based scintillation fluid. Values in parentheses are those expected theoretically from an additive effect of 5-HT and dopamine. Specific [³H]-LSD binding in the absence of neurotransmitters was 298 ± 13 c.p.m. in experiment 1 (6% of added label) and 213 ± 8 c.p.m. in experiment 2 (18.4% of added label).

binding of ^{3}H-LSD was defined as the difference between the radioactivity bound in the absence and presence of 10 μM unlabelled *d*-LSD. The binding remaining in the presence of this concentration of *d*-LSD ('nonspecific') was linearly related to the amount of radioactivity added, and consisted largely of binding to the glass fibre filter. Using ^{3}H-LSD concentrations below 2.0 nM, nonspecific binding was less than 10% of specific binding; it rose to 30% with 8.0 nM ^{3}H-LSD. The conditions used for the measurement of specific binding were optimal with respect to time of incubation, pH and ionic conditions; these data will be described in detail elsewhere (A. H. D., F. B. and I. B. I., in preparation).

In initial studies using a ^{3}H-LSD concentration of 1 nM, various putative neurotransmitters were tested for their ability to decrease specific ^{3}H-LSD binding. At concentrations of 10 μM or less, only dopamine and 5-HT were effective inhibitors; noradrenaline, octopamine and histamine were inactive (Table 1). Moreover, dopamine and 5-HT were probably displacing ^{3}H-LSD from different receptors, as, at concentrations below 100 μM, their effects were additive (Table 1). With higher neurotransmitter concentrations ($\geq$ 500 μM), binding was essentially abolished by either agent acting alone, suggesting that the two sites exhibit specificity for 5-HT or dopamine only at lower transmitter concentrations. Subsequent experiments were directed towards finding conditions in which ^{3}H-LSD labelled only the dopamine-sensitive site (D site) or the 5-HT-sensitive site (S site). We attempted to discriminate between the D and S sites by finding, for example, a concentration of 5-HT which would largely abolish binding of ^{3}H-LSD to the S-site, without affecting binding to the D-site. Preliminary experiments indicated that a concentration of 10^{-4}M 5-HT would be appropriate. As shown in Fig. 1*a*, the ^{3}H-LSD binding displaced by this concentration of 5-HT was that which was insensitive to dopamine. Comparable results were obtained when the experiment was reversed, that is, 10^{-4}M dopamine displaced that portion of ^{3}H-LSD binding which was insensitive to 5-HT (data not shown).

The dependence of the ^{3}H-LSD binding on LSD concentration is shown in Fig. 1*b*. Total specific binding was saturable with a K_D of 1.0 nM (determined by Scatchard analysis, not shown). The K_D for ^{3}H-LSD binding to the D-site (measured in the presence of 10^{-4}M 5-HT) was 0.5 nM, and for the S-site (measured in the presence of 10^{-4}M dopamine) 1.2 nM. When both agents were present simultaneously, the resultant inhibition of ^{3}H-LSD binding was that expected on an additive basis (Fig. 1*b*), confirming that these neurotransmitter concentrations were appropriate for dissecting the two sites. Figure 1*b* also reveals that there are approximately equal numbers of D and S sites in this preparation.

Fig. 1 Inhibition of specific [^{3}H]-LSD binding to *Helix* particulate fraction by dopamine and 5-HT. *a*, Effect of different concentrations of dopamine on the binding of 2 nM [^{3}H]-LSD in the absence (●) or presence (○) of 100 μM 5-HT. *b*, Saturation of specific [^{3}H]-LSD binding: ●, control; ▲, plus 100 μM 5-HT; ○, plus 100 μM dopamine; □, plus 100 μM 5-HT and 100 μM dopamine.

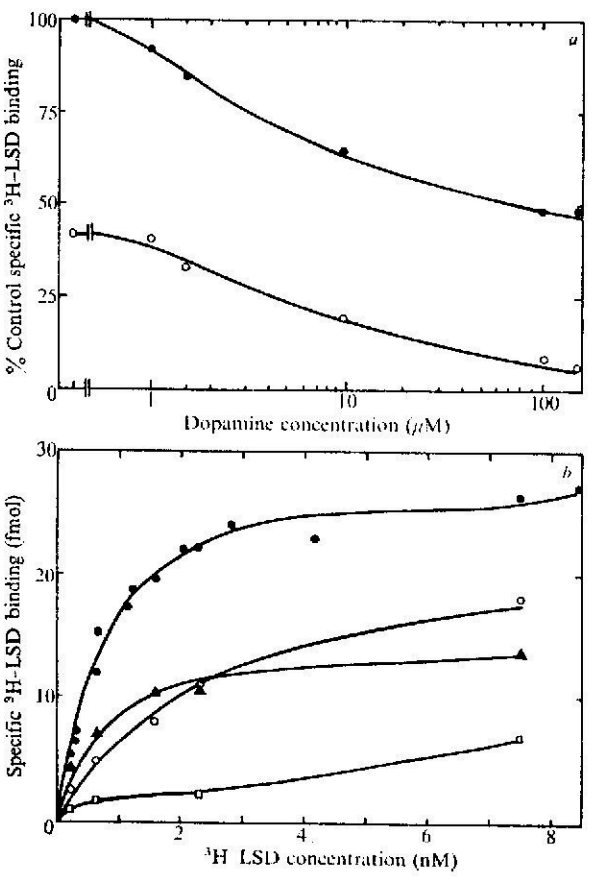

Table 2 Effect of various agents on dopamine-sensitive [^{3}H]-LSD binding

Agent	Inhibiton of dopamine-sensitive [^{3}H]-LSD binding K_i (μM)
Dopamine and related compounds	
Epinine	2.7
Dopamine	4.0
DPI	14.0
L-noradrenaline	54.0
L-isoproterenol	100.0
β-phenylethylamine	> 200.0
5-HT and related compounds	
N-methyltryptamine	0.34
Bufotenine	2.0
6,7-dihydroxytryptamine	4.0
Tryptamine	13.0
6-hydroxytryptamine	22.0
5-methoxytryptamine	50.0
N-methyl 5-hydroxytryptamine	60.0
5,6-dihydroxytryptamine	100.0
5-hydroxytryptamine	> 200.0
Ergot alkaloids and derivatives	
d-LSD	0.0005
l-LSD	10.0
Ergotamine	0.0003
Ergometrine	0.0008
Dihydroergocryptine	0.010
Methysergide	0.010
Neurolepties	
d-butaclamol	0.0046
l-butaclamol	54.0
Chlorpromazine	0.16
cis-chlorprothixene	0.20
trans-chlorprothixene	0.46
Spiroperidol	0.34
Haloperidol	0.38
cis-flupenthixol	10.0
trans-flupenthixol	6.0
Pipamperone	20.0

Helix ganglia particulate fractions were assayed with 4–8 concentrations of drug in duplicate or triplicate. All tubes contained 100 μM 5-HT to restrict [^{3}H]-LSD binding to the dopamine site. The concentrations of drugs required to inhibit binding by 50% (IC_{50}) were calculated and converted to K_i values according to the equation $K_i = IC_{50}/(1 + c/K_D)$, where c is the concentration of radioactive ligand (2 nM) and K_D is its dissociation constant (taken as 0.5 nM for dopamine-sensitive [^{3}H]-LSD binding).

The results of a preliminary pharmacological investigation of the dopamine receptor (D-site) are summarised in Table 2. Epinine (*N*-methyldopamine) and dopamine were the most potent of the catecholamines tested as competitors of dopamine-sensitive ^{3}H-LSD binding (Table 2). *l*-Noradrenaline and *l*-isoproterenol were respectively 20 and 37 times less active than epinine. This order of potency is similar to that seen for both inhibitory and excitatory physiological responses to dopamine in molluscs[3,9] (although isoproterenol is generally inactive as a dopaminergic agonist on the inhibitory dopamine receptor, and 300 times less active than dopamine on the excitatory receptor[3]). Furthermore, (3,4-dihydroxyphenylamino)-2-imidazoline (DPI)

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was a potent inhibitor of dopamine-sensitive ^{3}H-LSD binding (Table 2), and electrophysiological studies have shown it to be a potent dopaminergic agonist at hyperpolarising (but not depolarising) sites[10]. Several tryptamine derivatives (with the exception of most 5-substituted compounds) were effective inhibitors of binding (Table 2). This is consistent with the report that tryptamine and 6-hydroxytryptamine exert a weak dopamine-like hyperpolarising action on certain *Helix* neurones[11].

These data favour the hypothesis that ^{3}H-LSD labels the dopamine receptor which mediates hyperpolarisation in these cells. With this in mind, two main groups of psychoactive agents were tested for competition with ^{3}H-LSD binding. Ergot alkaloids and derivatives such as ergotamine, ergometrine and *d*-LSD, which are potent inhibitors of dopamine-induced hyperpolarising responses in invertebrates[3,12,13] (and are much less active against depolarising responses), were very potent inhibitors of dopamine-sensitive ^{3}H-LSD binding (Table 2). A number of neuroleptics which, conversely, are potent blockers at depolarising dopamine synapses but are relatively inactive at hyperpolarising dopamine sites[3,14], were much less effective than the ergot drugs in displacing dopamine-sensitive [^{3}H]-LSD binding. The neuroleptics are more potent dopamine receptor blockers in vertebrates[4,5] than in *Helix* (Table 2), and the order of potency reported for these agents in calf brain does not parallel that found in molluscan ganglia. In addition, although both *d*-butaclamol and the *cis*-thioxanthene drugs, flupenthixol and chlorprothixene, are stereospecific inhibitors of binding at [^{3}H]-haloperidol-labelled vertebrate dopamine receptors[4,5,15], only *d*-butaclamol was a stereospecific inhibitor of [^{3}H]-LSD binding in snail brain (Table 2). While the spectrum of drug action on these [^{3}H]-LSD-labelled dopamine receptors is clearly different from that previously described in vertebrate tissues using neuroleptic ligands, a recent report[7] suggests that approximately 15–20% of specific [^{3}H]-LSD binding to calf caudate membranes involves dopamine receptors. If a pharmacological characterisation of these receptors yields data similar to those reported here, it would be consistent with behavioural experiments[16] which suggest[17] that the vertebrate CNS also contains ergometrine-sensitive dopamine receptors analogous to those in the molluscan CNS.

The results presented above are compatible with the theory that [^{3}H]-LSD labels the dopamine receptor which mediates a hyperpolarising response in molluscan neurones. Electrophysiological studies, based on the pharmacology reported here, are now underway to confirm this hypothesis.

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Tentative identification of β-adrenoreceptor subunits

MANY hormones and neurotransmitters interact with specific receptors which are coupled to the enzyme adenylate cyclase[1–4]. Sutherland *et al.* were the first to suggest that the receptor constitutes the regulatory subunit possessing the allosteric effector site of the adenylate cyclase enzyme. Recently, two lines of experimental evidence have supported the postulate that the β-adrenoreceptor and the membrane bound enzyme adenylate

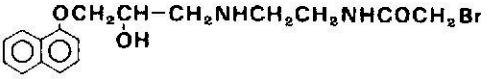

Fig. 1 Structure of the β-adrenoreceptor affinity label NHNP-NBE. The synthesis of *N*-[2-hydroxy-3-(1-naphthyloxy)-propyl]-*N'*-bromoacetyl-ethylenediamine (NHNP-NBE) was described earlier[10,11]. The compound was shown to inhibit irreversibly the catecholamine-dependent adenylate cyclase but not the NaF-dependent adenylate cyclase activity of turkey erythrocytes[9,10]. The affinity label irreversibly blocks the specific binding of [^{3}H]-propranolol[9,10] and of [^{125}I]-hydroxybenzylpindolol[10].

cyclase are situated on separate macromolecules. Lefkowitz *et al.*[5,6] succeeded in separating the β-adrenoreceptor from the adenylate cyclase by molecular sieve chromatography of digitonin solubilised from erythrocyte membranes. Schramm

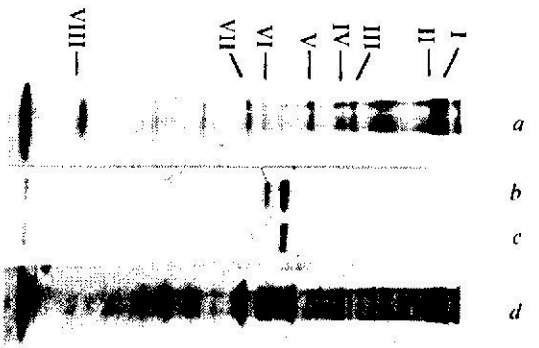

Fig. 2 The identity and the molecular weight of the β-adrenoreceptor submit. Rat skeletal myoblasts (L6P cells) were grown on a collagen matrix as described earlier[12,13]. After removal of the growth medium, the cells were washed with 0.02 M Tris-HCl, *p*H 7.4, containing 0.15 M NaCl. Then the cells were incubated for 10 min at 37 °C with 5.0×10^{-6} M of the tritiated affinity label [^{3}H]-NHNP-NBE (5.5 Ci mmol^{-1}) in the presence or absence of 1.0×10^{-6} M hydroxybenzylpindolol (HYP). Each growth bottle was then washed 20 times with 8.0 ml of 0.02 M Tris-HCl, *p*H 7.4, containing 0.13 M NaCl. The cells were removed from the growth bottles by scraping with a rubber 'policeman' and centrifuged at 400*g* for 5 min. They were resuspended in the same buffer and recentrifuged. The washing procedure was repeated twice. Protein was determined by the Lowry method[13]. The cells were dissolved in the electrophoresis buffer which contained 10% sodium dodecyl sulphate (SDS)[15], and identical amounts of protein (100 μg) were applied for slab gel electrophoresis using the set up of Fairbanks *et al.*[16]. The slab gels were stained with Coomassie blue, photographed, and then subjected to autoradiography for 8 weeks according to the procedure developed by Bonner and Lasky[10]. The molecular weight of the labelled bands was determined by comparing their mobilities with those of proteins from human erythrocyte membranes with known molecular weights. The latter were subjected to electrophoresis in the presence of SDS in parallel to the labelled L6P cells. The molecular weight of the proteins from the human erythrocytes are as follows[17]: Band I, 240,000; II, 215,000; III, 89,000; IV, 72,000; V, 43,000; VI, 35,000; VII, 29,000. The two protein bands from L6P cells labelled with the affinity label have an apparent molecular weight of 37,000 and 41,000. *a*, Human erythrocyte membranes; *b*, L6P cells labelled with the [^{3}H]-affinity label in the absence of HYP; *c*, L6P cells labelled with the [^{125}I]-affinity label in the presence of HYP; *d*, protein stain (Coomassie blue) of L6P proteins.