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The localization of [^{3}H]-desipramine in central nerve terminals studied with electron microscope autoradiography and subcellular fractionation

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Summary. The cellular and subcellular distribution of [^{3}H]-desipramine (DMI) in rat brain was studied by electron microscope (EM) autoradiography and by subcellular fractionation. A considerable proportion of label was found to be bound to the membranes of presynaptic nerve terminals, as well as to sites inside those terminals.

We have recently shown that, following the intracerebral administration of 6-hydroxydopamine (6-OH-DA), a marked reduction in the binding of [^{3}H]-desipramine (DMI) was found in all the rat brain areas investigated[2]. Furthermore, using light microscope autoradiography, we have demonstrated a preferential binding of [^{3}H]-DMI in the dopamine-rich caudate nucleus[2]. Since 6-OH-DA is known to be a relatively selective toxic agent for dopaminergic neurons of the caudate-putamen[3], this nucleus was selected for a more detailed electron microscope (EM) autoradiography. Investigation of binding of psychotropic drugs to particulate fractions from brain has been already established as a means of examining their subcellular distribution[4,5]. We now report on the localization of [^{3}H]-DMI in the rat brain, both by EM autoradiography and subcellular fractionation, following i.p. injection of the radiolabelled drug.

Materials and methods. Male Wistar rats were injected i.p. with 200 µCi/kg of [^{3}H]-desipramine-hydrochloride (44 Ci/mmole, IAEC-Negev, Beer-Sheva, Israel), diluted with unlabeled DMI (20 mg/kg), in a final volume of 0.5 ml saline.

30 min after injecting [^{3}H]-DMI, 8 rats were sacrified by decapitation and the pooled cerebral hemispheres homogenized in a solution containing 320 mM sucrose, 1 mM EDTA and 1 mM potassium phosphate buffer at pH 7.4. The homogenate was fractionated according to the method of Morgan et al.[6], with slight modifications. Aliquots (0.2 ml) of each fraction were taken for counting and protein determination[7].

Then 30 min after injecting the labelled drug, 2 rats were sacrified by perfusion with 4% paraformaldehyde, 0.5% glutaraldehyde and 0.54% glucose in 0.1 M phosphate buffer (pH 7.4), under nembutal anasthesia (50 mg/kg). The partially fixed caudate nuclei were carefully dissected into small cubes which were kept in the same fixative overnight and then post-fixed for 2 h in 2% buffered OsO_4, dehydrated and embedded in epon-araldite. Sections of 400–600 nm thickness were prepared for autoradiography by applying Illford L4 emulsion. Following exposure of 6–8 weeks, the sections were developed and examined by a Phillips 300 EM, after staining with methanolic uranyl acetate and with lead citrate.

Results and discussion. The subcellular distribution of [^{3}H]-DMI, expressed as cpm/mg protein, is summarized in the table 1. There was no loss of radioactivity (100% recovery) during the 1st centrifugation, 40% of the total radioactivity was lost during the Ficoll gradient step and an additional

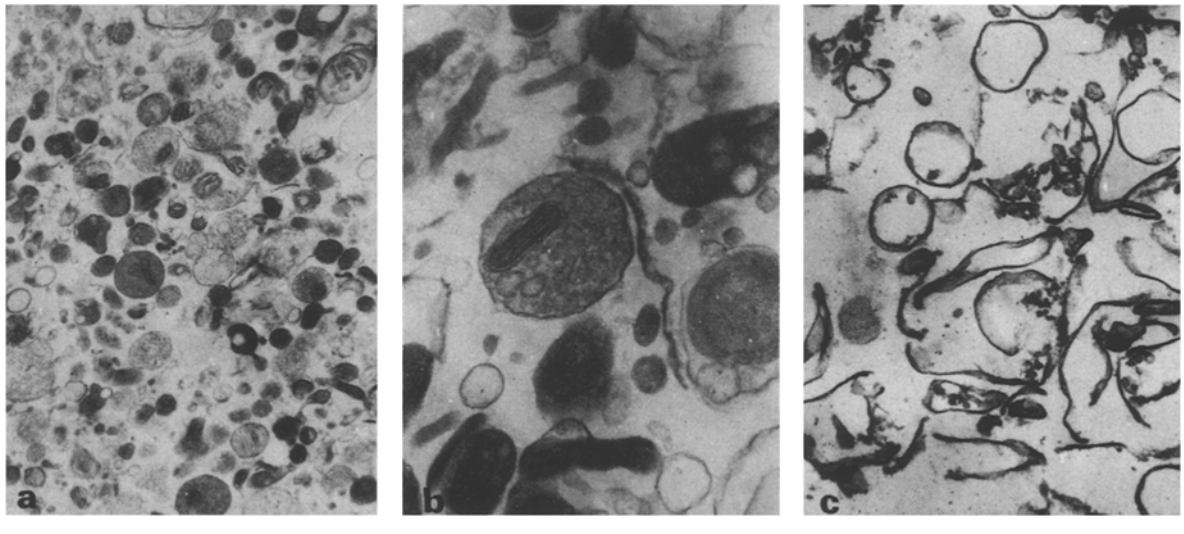

Fig. 1. EM micrographs of synaptosomal and SPM preparations: *a* Typical field of synaptosomes, prepared from pooled 12 and 16% discontinuous Ficoll gradients. × 15,000. *b* Synaptosomes as in figure 1a, at a higher magnification. × 35,000. *c* Typical field of the synaptosomal plasma membrane (SPM) fraction. × 30,000.

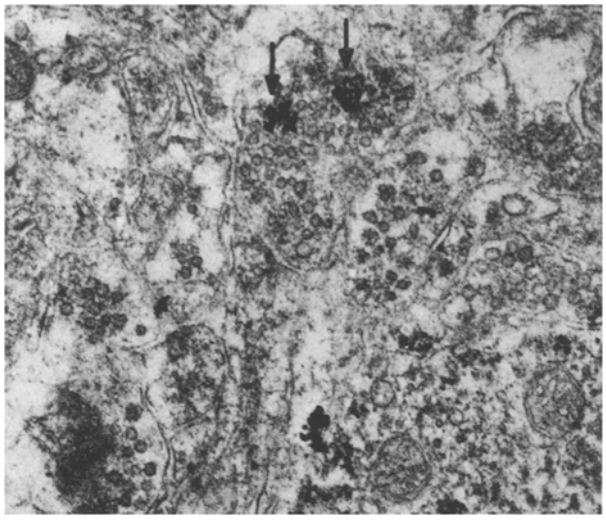

Fig. 2. Autoradiogram of a butonic area from the caudate nucleus after i.p. injection of [^{3}H]-DMI. Autoradiographed grains located over the vesicles (arrows), inside the nerve terminal. × 21,800.

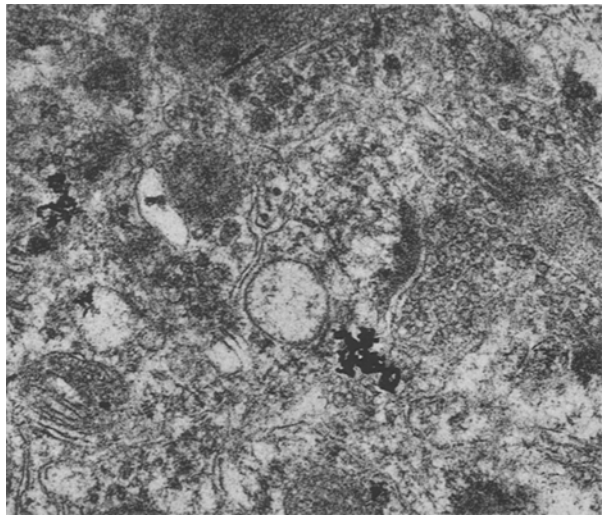

Fig. 3. Autoradiogram of a synaptic area from the caudate nucleus after i.p. injection of [^{3}H]-DMI. A cluster of grains seen adjacent to the synaptic cleft. × 24,500.

40% lost during the sucrose gradient step (from lysed synaptosomes to synaptic plasma membranes (SPM). The purity of the fractions was checked by electron microscopy (figure 1).

A net increase in radioactivity was found in the course of the purification steps, from homogenate, crude mitochondrial pellet to 'purified' synaptosomes. Our results also demonstrate a preferential binding of [^{3}H]-DMI to the whole synaptosomal fraction, which contained double the radioactivity found in the mitochondrial and myelin fractions. Lysis of the synaptosomes and the release of their content into the supernatant caused a decrease in the amount of bound [^{3}H]-DMI. A further loss of radioactivity occurred during the preparation of purified SPM. Nevertheless, the SPM fraction was highly enriched with the label, as compared to the accompanying mitochondrial and intrasynaptosomal debris.

The distribution pattern of the autoradiographed grains is expressed as a percentage of total grains counted over specific cellular areas. Counts were taken in random grid squares and on scanning a large number of autoradiograms; about 40% of the radioactive grains were found over nerve endings, 20% over axons, 25% over dendrites or dendritic spines and the remainder distributed over cytoplasm and nuclei of neuronal and glial cells. This suggests a preferential association of silver grains with nerve terminals containing many small vesicles (figure 2). Also, at the same time, grains associated with synaptic locations (figure 3), and with unmyelinated axons were commonly observed.

The autoradiograms presented here support our previous suggestion that [^{3}H]-DMI is bound mainly to presynaptic nerve terminals[3]. This is in accord with the distribution pattern of radioactivity in subcellular fractions obtained from rat brain homogenates, where a substantial proportion of the label was found in synaptosomal fractions.

The cellular and subcellular localization of 2 other tricyclic antidepressants, i.e. imipramine and dimetacrine, have been reported[5,8,9]. A considerable (40%) proportion of the autoradiographic grains of [^{3}H]-dimetacrine was found to be located over synaptic areas of the rat cerebral cortex and [^{3}H]-imipramine was found to be bound specifically, though with low affinity, to purified synaptosomes in vitro. Hunt et al.[5] have found that the maximal binding capacity of the SPM fraction for [^{3}H]-imipramine, in vitro, was roughly one-fourth of that of whole synaptosomes. In our experiment, following lysis and the release of the soluble content of the synaptosomes, the amount of radioactivity associated with the membrane (SPM) fraction was less from that bound to undisrupted synaptosomes. This may indicate that, in vivo, the drug first interacts with the synaptic membranes, then diffuses into the interior of the terminals where it remains entrapped. We assume that diffusion occurs, since the binding of [^{3}H]-imipramine[3] and of [^{3}H]-DMI (our results, unpublished) to synaptosomes does not involve an active transport process. Another possible reason for the decline in the radioactivity bound to the SPM fraction, could be the dissociation of the tritiated drug from the membrane-binding sites during the gradient and resuspension phases, since this binding does not appear to be of a very high affinity (unpublished).

Recent hypotheses on the action of antidepressant drugs suggest an affinity for various receptor binding sites in brain tissue[10-13]. In addition, the effect of those drugs in inhibiting norepinephrine and dopamine re-uptake into central catecholaminergic nerve endings is well documented[4]. The finding that DMI binds to the synaptic membranes

Subcellular distribution of [^{3}H]-DMI binding in rat cerebral hemispheres

Fraction	[^{3}H]-DMI cpm/mg protein
Whole homogenate	450 ± 45
S_1 (supernatant, 10,000 × g, 10 min)	407 ± 40
P_1 (crude nuclear pellet)	460 ± 15
P_2 (crude mitochondrial pellet, 12,000 × g, 30 min)	578 ± 13
Subfractionation of P_2	
Myelin (9% Ficoll gradient layer)	390 ± 8
Synaptosomes (pooled 12 and 16% Ficoll gradient layers)	711 ± 4
Mitochondria (pellet of Ficoll gradients)	343 ± 2
Lysed synaptosomes (osmolysis followed by 20,000 × g, 12 h)	400 ± 4
Subfractionation of lysate	
Synaptosomal plasma membranes (SPM) (0.6 + 0.8 + 1.0 M sucrose gradient layers, 25,000 × g, 2 h)	255 ± 13
Mitochondria (1.2 M sucrose gradient layer, 25,000 × g, 2 h)	32 ± 10

Values represent averages from 2 separate counts.

and also enters the intact nerve terminals provides support for a dual mode of action, involving both binding to receptor sites and re-uptake inhibition of different neurotransmitter systems.

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Dissociation constants of 4-acetoxy-piperidines and -thiacyclohexanes at the muscarinic receptor[1]

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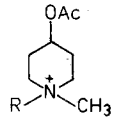
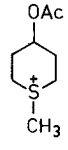
Summary. The dissociation constants (K_A) and the relative intrinsic efficacies (e) of 4-acetoxy-piperidines and -thiacyclohexanes were determined on the guinea-pig isolated left atrium. The differences in the muscarinic potencies are associated with differences in affinities and efficacies, respectively.

In previous papers from our laboratory, it has been shown that compounds **I–IV** are muscarinic agonists[3–6]. Stereochemical and thermodynamic parameters seem to be responsible for great differences in the muscarinic activity of **I–IV**.





R **I** H
II CH_3

III = CIS
IV = TRANS

As the compounds **I–IV** are agonists, 2 parameters are necessary to characterize their pharmacological action: efficacy (ability to activate the muscarinic receptor) as well as affinity. Using an ED_{50} value as an approximation of a K_A value for an agonist, a potential error can arise when there is a large receptor reserve for the agonist with respect to the response being measured. If the assumption of a receptor reserve is correct, then it is impossible to obtain truly reliable values for the dissociation constant K_A directly from agonist dose-response curves. To obtain these 'true' K_A values together with values for efficacies for compounds **I–IV**, a method has been used in this work originated by Stephenson[7] and Furchgott[8,9]. The validity of this approach has been questioned by Triggle and Triggle[10], but recent radioligand-binding studies lead to the suggestion that the K_A-values determined with such a pharmacological procedure are directly coupled to the response being measured and are true dissociation constants of the agonist-receptor complexes[11].

Methods. The experiments were performed on isolated left atria of guinea-pigs electrically driven with a frequency of 2 Hz and 3 msec duration by means of rectangular impulses of 4–6 V. Only atria from reserpinized (5 mg/kg) guinea-pigs were used[9]. The acetylcholinesterase was blocked by DFP (5×10^{-5} M), since all the agonists are substrates for this enzyme[12,15]. To block nicotinic receptors, hexamethonium (7×10^{-5} M) was used.

A fraction of the total concentration of the muscarinic receptors in the atria were irreversibly inactivated by pretreatment with dibenamine (1×10^{-4} M, 45 min). According to occupation theory, the following equation applies[8,9]:

$$\frac{1}{[A]}=\frac{1-q}{q\times K_A}+\frac{1}{q\times[A']}$$

q is the fraction of muscarinic receptors still active, [A] and [A'] are the respective concentrations of the agonists giving equal negative inotropic responses before and after inactivation with dibenamine, and K_A is the dissociation constant

Muscarinic activities of acetylcholine (Ach) and **I–IV** on the guinea-pig isolated left atrium (mean ± SE), negative effects on the force of contraction

	n*	$ED_{50}\times10^{-8}$ M**	Relative activities	$K_A\times10^{-6}$ M	Relative affinities	Relative intrinsic efficacies***	K_A/ED_{50}
Ach	12	3.08 ± 0.38	1.00	3.86 ± 0.63	1.00	1.00	125
I	8	563 ± 60	0.0055	329 ± 131	0.012	0.47 ± 0.095	58
II	8	690 ± 50	0.0045	256 ± 96	0.015	0.35 ± 0.10	37
III	8	1.60 ± 0.10	1.93	3.17 ± 0.40	1.22	1.50 ± 0.25	198
IV	8	146 ± 23	0.021	339 ± 60	0.012	1.76 ± 0.27	232

* Number of observations. ** According to Lambrecht[6]. *** The relative intrinsic efficacies were calculated from paired values between Ach and **I–IV**.