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CHARACTERIZATION AND ONTOGENESIS OF [^{3}H]-DESIPRAMINE BINDING SITES IN DEVELOPING FETAL RAT CEREBRAL CELLS IN CULTURE

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Abstract—The tricyclic antidepressant drug desipramine (DMI) binds specifically to cultured cells from the fetal rat brain tissue in a saturable, displacable manner. The number of the binding sites is small during the first 48 hr in culture and increases markedly from day 4 and on, in a time-dependent course which parallels early synaptogenesis. DMI binding is effectively inhibited following chronic treatment of the cultures with 6-hydroxydopamine (6-OH-DA). In morphologically mature cultures, the IC_{50} value of [^{3}H]-DMI is 4×10^{-6} M and the maximal number of binding sites is 160–200 pmoles/mg protein. The binding characteristics and the pharmacological profile of desipramine in developing cerebral cells are in excellent agreement with those found in adult rat brain.

The binding of tricyclic antidepressants in rat brain has been recently shown to be both specific and saturable [1, 2]. In a previous study [3], we have found that binding of [^{3}H]-desipramine (DMI), characterized by low affinity and high capacity, is associated mainly with nerve terminals. The ontogenetic appearance of tricyclics binding sites in the developing nervous system has, so far, not been investigated. In the present study we have studied this problem using cells derived from the fetal rat brain which, under culture conditions, express several of the properties characteristic of mature nervous tissue [4]. These cultures provide a useful tool for developmental studies, since it is possible to correlate neurochemical events with morphology and ultrastructure, under controlled conditions. We wish to report on the rise of [^{3}H]-DMI binding sites as a function of the appearance of synapses in cerebral cells in culture. The pharmacological profile of the drug was also compared to that found for adult rat brain homogenates [3]. Further confirmation of the possible localization of the binding sites for DMI was obtained using chronic treatment with 6-hydroxydopamine (6-OH-DA) to produce degeneration of presynaptic catecholaminergic nerve terminals [5].

METHODS

Cell culture. The experiments were performed on cultures obtained from fetal rat brain tissue at 16 days gestation, as described elsewhere [4]. In brief, dissociated cerebral cells were seeded onto polylysine precoated petri dishes in Basal Medium Eagle (BME) augmented with glucose (0.6 g%), containing 20% fetal calf serum and 10% egg ultrafiltrate. The initial plating medium was replaced after 48 hr by BME containing 10% serum and the medium changed every 10–12 days thereafter.

Drugs and pharmacology. [^{3}H]-Desipramine hydrochloride (44 Ci/mmole) was purchased from IAEC, Beer-Sheva, Israel. Unlabeled DMI (Ciba-Geigy), fluxethine (Eli Lilly), mianserine (Organon) and amitriptyline (Teva) were gifts from the respective companies. The other drugs used were obtained from commercial sources. The affinities of the various antidepressants and antagonists for the [^{3}H]-DMI binding sites were determined by measuring their IC_{50} values.

Binding assay. On the appropriate day, monolayers from 2–3 culture dishes were scraped off with a rubber policeman in Ca^{2+}, Mg^{2+} free phosphate buffered saline (PBS) (pH 7.4) and centrifuged at 500 *g* for 2 min. The pellet was homogenized in 10 vol of ice cold 0.32 M sucrose, first in a glass–glass and then in a glass–teflon homogenizer. Aliquots (50 μl) of the homogenate were incubated with [^{3}H]-DMI in a final volume of 2 ml modified Krebs–Hensleit buffer (pH 7.4). After 20 min at 25° the reaction was terminated by filtering through GF/C filters. The filters were washed with 4×3 ml portions of the ice-cold incubation buffer, transfered into vials containing 10 ml of 33% (v/v) Triton X-100, 0.8% PPO and 0.01% POPOP in toluene and counted by liquid scintillation spectrometry.

The specific binding of [^{3}H]-DMI was defined as total binding minus the binding in the presence of 2×10^{-4} M of unlabeled DMI or amitriptyline. Protein was determined by the method of Lowry *et al.* [6] and the DNA content was measured according to Burton [7].

RESULTS

The specific binding of [^{3}H]-DMI to the cultured fetal brain cells was saturable over a wide concentration range (Fig. 1a). In comparison to adult rat brain homogenates [3], the non-specific binding (per

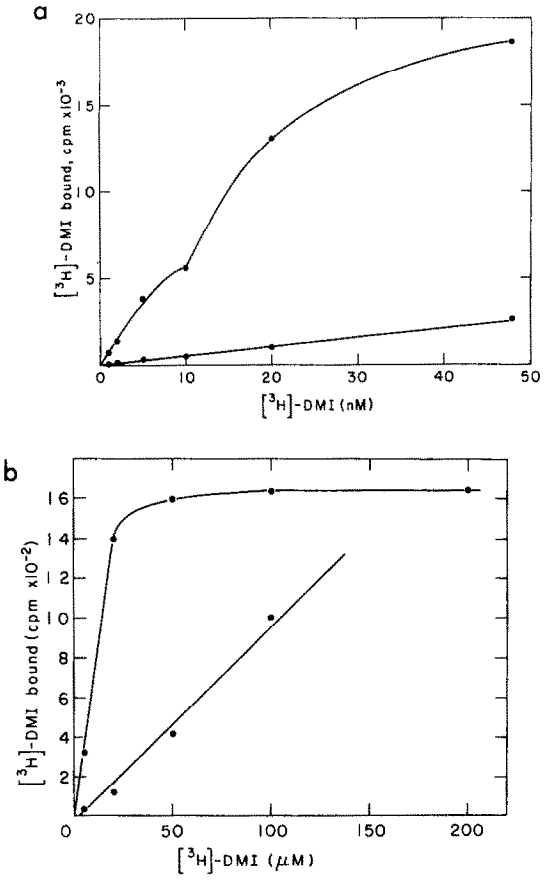

Fig. 1. Binding of [^{3}H]-DMI to homogenates from 12-day-old cultures at low (panel a) and high (panel b) drug concentrations. The lower line in both panels represents non-specific binding in the presence of an excess of unlabeled DMI. The upper line is the specific binding, defined as total binding minus the non-specific binding.

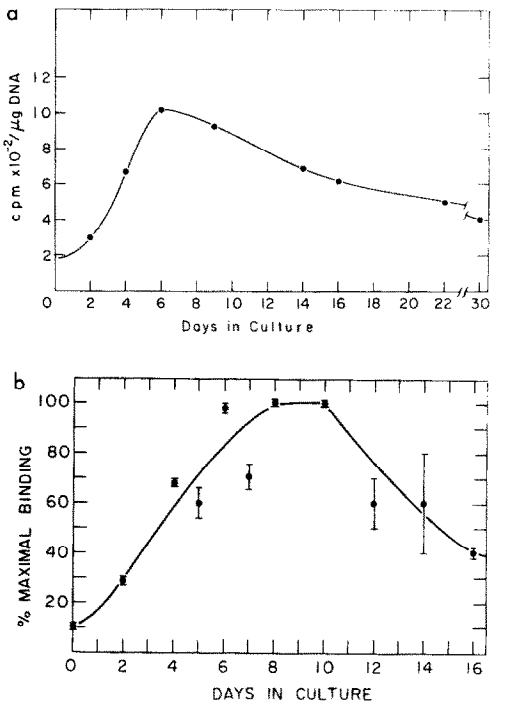

Fig. 2. Ontogenesis of specific [^{3}H]-DMI binding to cultured brain cells homogenates, determined in the presence of 5 nM[^{3}H]-DMI. Panel a: specific binding expressed per μg DNA; panel b: specific binding per mg protein, normalized as percentage of the maximal attainable binding. Each point represents the mean value ± S.D. of 3 different experiments.

mg protein) was found to be considerably lower. The specific binding was linearly proportional to the amount of homogenate in the assay and very similar to that in the adult brain homogenates. The binding process reached equilibrium within 1 min, and had an ED_{50} value of 10 μM and a B_{max} of 160–200 pmoles/mg protein (Fig. 1b), which is comparable with the value in the adult rat brain homogenate [3]. The pharmacological profile of [^{3}H]-DMI binding sites is shown in Table 1. It is evident that the IC_{50} values of various antidepressants and a number of antagonists are all in the μM range.

Table 1. Displacement of [^{3}H]-DMI binding (20 μM) by various ligands

Ligand	IC_{50}(M)
DMI	2.5
Amitriptyline	2
Fluxethine	12
Mianserine	4.5
Dopamine	>100
Haloperidol	3
Norepinephrine	>100
Serotonin	>100
Methiothepin	0.6
Atropine	>100
GABA	>100
Picrotoxin	>100

The ontogenesis of the [^{3}H]-DMI binding sites is depicted in Fig. 2. Only little specific [^{3}H]-DMI binding was detected in the cells during the first 48 hr in culture. Binding activity increased markedly thereafter and reached a maximal level between 6 and 10 days *in vitro*. From day 10 to 16, a decrease in binding activity, as normalized per cellular protein, was evident (Fig. 2b). However, when normalized per cellular DNA, this decrease was less pronounced (Fig. 2a), since in contrast to cellular proteins, the DNA content after 1 week in culture is practically unchanged [8]. Cultures maintained for prolonged periods *in vitro* and consisting mainly of glial and ependymal cells had a low binding activity, when normalized either per DNA or per protein. This value is similar to that of 3-day-old cultures.

Chronic treatment with 7μM 6-OH-DA for 48 hr caused a 43 per cent inhibition of the binding activity, as shown in Table 2. When the concentration of 6-OH-DA was doubled, a 78 per cent inhibition was observed. Morphologically, the cytotoxic effect of 6-OH-DA was pronounced mainly on the neuritic processes which showed an abundance of varicosities.

Table 2. The effect of chronic treatment of cerebral cells by 6-OH-DA on binding of [^{3}H]-DMI

6-OH-DA (M)	Time (hr)	Residual specific binding (%)
	–	100
10^{-6}–10^{-4}	–	99–95
7×10^{-6}	48	56
7×10^{-6}	72	57
1.4×10^{-5}	48	22

* Cerebral cells after 14 days in culture were incubated for 48 or 72 hr in the presence of varying concentrations of 6-OH-DA. The medium was rinsed off and the monolayer washed 3 times with PBS. The [^{3}H]-DMI binding was performed on homogenates, as described in Methods. Homogenates of untreated cultures incubated in the presence of 10^{-4} or 10^{-6}M 6-OH-DA served as controls. Each experiment was performed on duplicate cultures.

DISCUSSION

The present study demonstrates that [^{3}H]-DMI binds to primary fetal rat brain cultures in a specific and saturable fashion. Other antidepressants and some dopaminergic, serotonergic and α and β adrenergic antagonists, but not agonists (see Table 1), are capable of displacing [^{3}H]-DMI specific binding. These binding characteristics are almost identical to those found in adult rat brain homogenates [3]. This indicates that many properties of antidepressant interactions with the central nervous tissue may be investigated in this *in vitro* system.

On studying the appearance of the [^{3}H]-DMI binding sites at various stages of differentiation, we have found little specific binding in the cells during the first 2 days in culture. Morphologically, this period is characterized by cell migration, establishment of a neuritic network and by the absence of synapses [9]. Following the second day in culture, binding activity increases markedly and reaches its maximal value between days 6 and 10. At this period numerous growth cones or axonal swellings containing clear vesicles are seen and the first immature synapses appear [9]. These first synapses are characterized by very few synaptic vesicles and by a slight, symmetrical membrane thickening. Synapses are more frequently observed after 10 days in culture, demonstrating further structural development, with asymmetrical membrane thickening and increased numbers of clear synaptic vesicles. From day 10 to 20 *in vitro*, when the cultures become enriched with ultrastructurally mature synapses, the binding activity of DMI decreases slowly (see Figs. 2a and b). This is in contrast to the increase in muscarinic receptors recently demonstrated in similar cultures [10]. It appears, therefore, that the peak in [^{3}H]-DMI binding sites precedes the period of synapse maturation. The slow decrease in the number of binding sites might be due to a selective loss in their population with time in culture.

The marked reduction in [^{3}H]-DMI binding sites in 30 to 40-day-old cultures can be attributed to the loss of neuronal cell bodies and neurites. This loss can be enhanced by repeated changes of the growth medium (unpublished observations), resulting in an enrichment of ependymal and glial cells, the latter containing large amounts of astrocyte-specific glial fibrillary acidic protein [11]. The reduced binding of [^{3}H]-DMI provides further evidence of its association with neuronal elements in the nervous system.

The preferential association of [^{3}H]-DMI with presynaptic areas was demonstrated by electron microscopic autoradiography [12], and in an *in vivo* study [13] using 6-OH-DA lesioned rats. Additional evidence for the localization of the DMI binding was obtained by chronic treatment of the cells in culture with 6-OH-DA. The marked loss in the number of [^{3}H]-DMI binding sites suggests that *in vitro*, as *in vivo*, the presynaptic structures of catecholaminergic nerve endings account for the major part of [^{3}H]-DMI binding.

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