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Effects of neonatal antithyroid treatment on brain [^{3}H]-imipramine binding sites

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1 The action of the antithyroid, sulphydryl reagent methimazole (MMI) on the specific binding of [^{3}H]-imipramine in the cerebral cortex and corpus striatum of immature and mature rats has been examined.

2 Chronic administration of MMI through the first 30 days of life decreased the number of imipramine binding sites in cortical but not striatal membranes, as assessed 48 h after the last injection of goitrogen.

3 A similar treatment did not affect the binding profile of [^{3}H]-imipramine in mature rats.

4 Acute administration of MMI to 30 day-old rats increased the number of imipramine binding sites shortly after the injection, an effect no longer evident 48 h later.

5 MMI *in vitro* increased the binding of [^{3}H]-imipramine.

6 It is concluded that maturational impairment of the hypothyroid cortex, rather than any alteration of membrane bound thiol groups, was a major cause for the diminished binding of [^{3}H]-imipramine in MMI-treated, immature rats.

Introduction

Experimental hypothyroidism is known to affect the number of several neurotransmitter receptors in the rat brain (see Vaccari, 1983, for references). A diminished binding of the tricyclic antidepressant imipramine to brain and platelet putative 'receptors' has been suggested to represent a biological 'marker' for endogenous depression (Langer *et al.*, 1981). Furthermore, [^{3}H]-imipramine recognition sites are anatomically and functionally associated with the 5-hydroxytryptamine (5-HT) uptake mechanism (Langer *et al.*, 1980).

It was reasonable to expect that [^{3}H]-imipramine binding would be affected in the hypothyroid brain since: (a) several mental and behavioural symptoms occurring in hypothyroidism are also characteristic of endogenous depression, when the number of imipramine binding sites is decreased, and (b) synaptosomal 5-HT uptake, claimed to be associated with the binding of imipramine, is impaired in brain regions of young hypothyroid rats (Schward & Keesey, 1978). Thus, the question arose as to whether brain [^{3}H]-imipramine binding sites can be affected in experimental hypothyroidism.

Methods

Primary-like hypothyroidism was induced in newborn male and female, Charles River CD rats with a daily subcutaneous injection of 20 mg kg^{-1} methimazole (MMI) in 0.9% NaCl from birth to day 30 of age (Vaccari & Timiras, 1981). Pregnant rats had been maintained on an iodine-free diet during the last 6 days of pregnancy until day 4 postpartum; MMI-treated pups were fed iodine-free diet from day 22 up to day 32. Male, 3 month-old rats weighing approx. 300 g were rendered hypothyroid (Hajós *et al.*, 1973) with a daily subcutaneous injection of 50 mg kg^{-1} MMI for 30 days. Both immature and adult controls (euthyroids) received an equal volume of saline alone. All rats were killed 48 h after the last injection, unless specifically indicated; the entire cortex (plus hippocampus) and the corpus striatum were quickly dissected and stored at − 80°C until assayed. The nuclear pellet of one cortical or two striatal homogenates for each experiment was sedimented at 900 *g* for 10 min; partially purified membranes were then obtained, and the binding of [^{3}H]-imipramine

(0.25–10 nM) was measured essentially as described by Raisman *et al.* (1980). Membranes (200 μl of 1:26 w/v suspension) were mixed with [^{3}H]-imipramine in the absence or presence of 100 μM desipramine as the displacer, in 50 mM Tris-HCl buffer, pH 7.5 at 4°C, containing 120 mM NaCl and 5 mM KCl, and incubated in a total volume of 0.5 ml over ice for 60 min. The incubation mixtures were then diluted with 4 ml cold assay buffer, immediately filtered through Whatman GF/B filters, and washed with 2 × 4 ml buffer. Correction for binding of [^{3}H]-imipramine to the filters was made by direct measurement of the radioactivity 'specifically' taken up by the filters in the absence of membranes (chronic experiments with neonatal rats and *in vitro* effects of MMI). Since this procedure was time-consuming and expensive, in further experiments (chronic administration of MMI to adult rats and acute effects of MMI), filters were pre-wetted with 100 μM unlabelled imipramine (Wirz-Justice *et al.*, 1983) in order to saturate binding 'sites' on the glass fibre. The binding profile of [^{3}H]-imipramine was similar after both procedures, though the latter gave consistently smaller values of specific radioactivity as compared to the former procedure. A direct comparison of absolute values for imipramine binding at different ages was, thus, not possible. Problems associated with binding of [^{3}H]-imipramine to the filters have been recently well studied by Phillips *et al.* (1984).

The concentration of accessible sulphydryl (SH) groups was assayed (Ellman, 1959) in 400 μl of membrane suspension mixed with 0.1 M phosphate buffer,

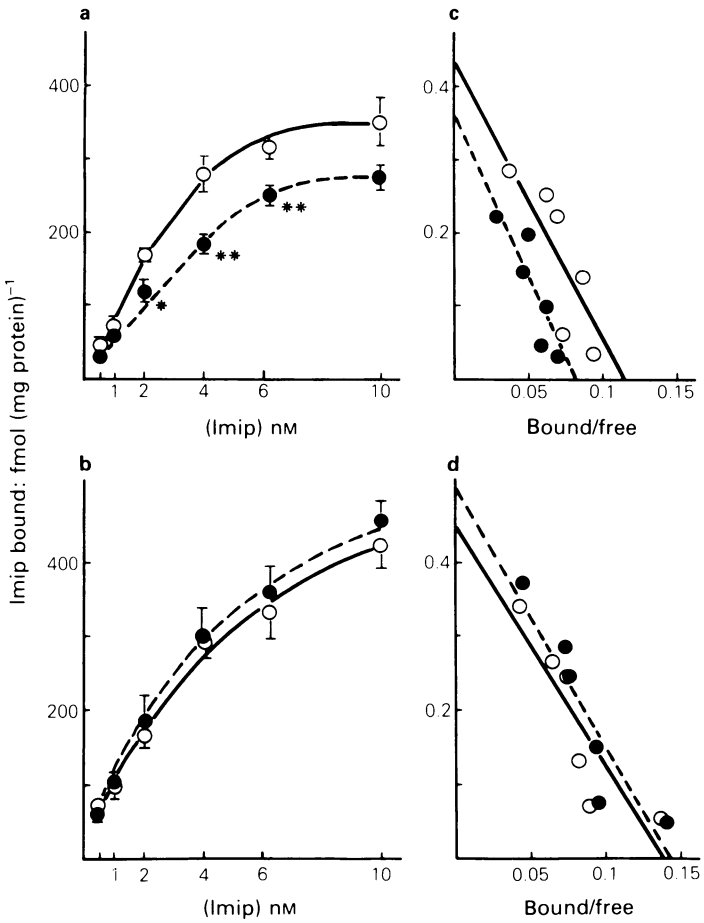

Figure 1 Equilibrium binding of [^{3}H]-imipramine (Imip) to the cerebral cortex (a) and corpus striatum (b) of euthyroid (○) and hypothyroid (●), 32 day-old male and female (1 : 1) rats. The data points are means ± s.e.mean of 6 (○) or 8 (●) experiments done in triplicate with cortical membrane preparations from 6 or 8 rats, or with striatal membranes from 12 or 16 rats. Panels (c) and (d) illustrate a typical Eadie-Hofstee plot. Binding parameters are shown in Table 1. * $P < 0.02$; ** $P < 0.005$ as compared to their respective euthyroid group.

Table 1 Binding characteristics of [^{3}H]-imipramine in the hypothyroid brain

Age of treatment	*Thyroid state*	*Cortex*		*C. striatum*	
		B_{max}	K_D	B_{max}	K_D
Neonatal	Euthyroid	477 ± 50	3.8 ± 0.6	549 ± 44	4.1 ± 0.4
	Hypothyroid	385 ± 16*	4.7 ± 0.3	569 ± 74	4.9 ± 0.6
Adult	Euthyroid	291 ± 44	3.3 ± 0.6	262 ± 21	2.2 ± 0.6
	Hypothyroid	341 ± 39	3.3 ± 0.5	246 ± 23	2.6 ± 0.5

Values shown are means ± s.e.mean from 6–8 experiments using 6 or 7 ligand concentrations, each in triplicate. B_{max}: fmol (mg protein)$^{-1}$, and K_D: nM values were obtained with Eadie-Hofstee analysis. Correction for binding of [^{3}H]-imipramine to the filters was performed by direct measurement in each experiment (neonatal samples), or by saturating the binding 'sites' on the filters with unlabelled 100 μM imipramine (adult samples). For further details see Methods section.

* $P < 0.05$

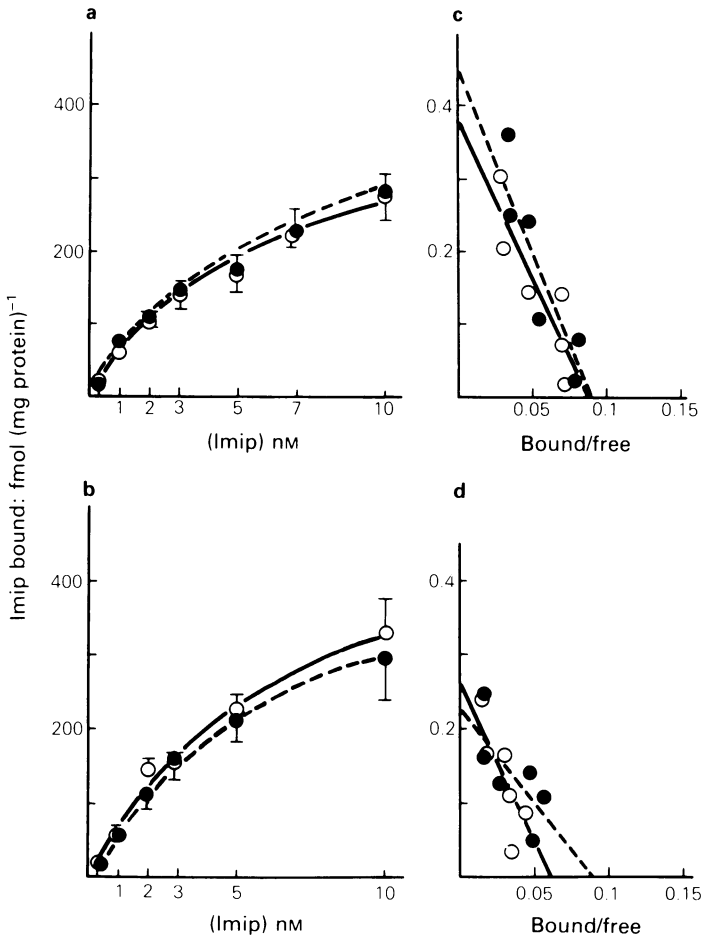

Figure 2 Equilibrium binding of [^{3}H]-imipramine (Imip) to the cerebral cortex (a) and corpus striatum (b) of euthyroid (○) and hypothyroid (●) 4 month-old male rats. The data points are means ± s.e.mean of 8 (a) or 6 (b) experiments done in triplicate with cortical membrane preparations from 8 rats, or with striatal preparations from 12 rats. Panels (c) and (d) illustrate a typical Eadie-Hofstee plot. Binding parameters are shown in Table 1.

pH 8.0, in the presence of 71 μM 5,5′-dithio*bis* (2-nitrobenzoic) acid. The absorbance was read at 413 nm.

Protein samples were analysed with bovine serum albumin as standard (Lowry *et al.*, 1951).

Eadie-Hofstee analysis for saturation curves of [^{3}H]-imipramine binding was performed according to Zivin & Waud (1982). Statistical significance of differences was assessed with Student's *t* test.

Methimazole (MMI) was obtained from Sigma Chemical Co. Ltd.; 5,5′-dithio*bis* (2-nitrobenzoic) acid was from Serva Feinbiochemica. Desipramine and imipramine were kindly donated by Ciba-Geigy Ltd. [^{3}H]-imipramine (55 Ci mmol^{-1}) was purchased from New England Nuclear.

Results

Neonatal thyroid deficiency delayed the eye opening, impaired the body growth, and provoked cardiac hypotrophy. Goitrogen administration to adult rats almost abolished body growth (while controls grew by 32%), and decreased by 35% the heart weight as compared to euthyroids. At both ages, hypothyroid rats were clearly hypoactive and slow reactive.

Effects on [^{3}H]-imipramine binding

The specific binding of [^{3}H]-imipramine to the cerebral cortex from neonatally-rendered hypothyroid rats was consistently (by 35% at 4 nM ligand) decreased as compared to euthyroids (Figure 1), an effect related to a decrease in the B_{max} value (maximum number of binding sites) (Table 1). Thyroid deficiency did not affect imipramine binding either in the neonatal striatum (Figure 1) or in cortical and striatal membranes from adult rats (Figure 2 and Table 1).

In order to assess whether the last injection of MMI 48 h earlier could have directly influenced imipramine binding in the hypothyroid cortex, 30 day-old male rats were given an individual injection of goitrogen (20 mg kg^{-1} s.c.), and then killed 5 h or 48 h later. The binding of [^{3}H]- imipramine to cortical membranes increased by 28% 5 h after the injection, and was similar to that in saline-treated rats at 48 h (Table 2).

The binding reaction of [^{3}H]-imipramine (4 nM) was also performed in the presence or absence of 3.5 μM MMI. This concentration was estimated to correspond roughly to the goitrogen content in each 200 μl aliquot of hypothyroid tissue used for the receptor assay shortly after an individual injection of MMI to 30 day-old pups. The density of imipramine binding sites in whole brain (less cerebellum) membranes in the absence of MMI was 224 + 22 fmol (mg protein)$^{-1}$, and that in the presence of MMI was 373 + 30 fmol (mg protein)$^{-1}$, ($n = 6$; $P < 0.05$).

Table 2 Time course of [^{3}H]-imipramine (4 nM) binding after acute administration of methimazole (MMI)

Treatment	*Time after MMI*	*fmol (mg protein)*$^{-1}$
NaCl	5 h	56.8 ± 5.3
MMI		72.7 ± 4.9*
NaCl	48 h	69.7 ± 5.0
MMI		81.7 ± 9.2

Values are means ± s.e.mean from 6 experiments performed in the brain cortex of 30 day-old rats, at various times after an individual injection of MMI (20 mg kg^{-1} s.c.) or saline.
* $P < 0.05$.

Alterations in membrane bound SH groups

Since MMI is a disulphide bond reducing reagent, and the probable participation of free SH groups in imipramine recognition sites of human platelets has been suggested (Wennogle *et al.*, 1981), we wondered whether alterations of [^{3}H]-imipramine binding were related to impaired SH concentrations in hypothyroid, neonatal cortical membranes. The amount of accessible SH groups was similar in euthyroid and hypothyroid membranes from both immature and mature rats (Table 3). Five hours after an individual injection

Table 3 Estimation of membrane bound protein SH groups in the cerebral cortex after chronic and acute administration of methimazole (MMI)

Treatment	*Age*	*Time after MMI*	*SH* nmol (mg protein)$^{-1}$
Chronic	Neonatal	48 h	
NaCl			71.6 ± 4.3
MMI			62.5 ± 4.0
Chronic	Adult	48 h	
NaCl			56.7 ± 3.1
MMI			57.5 ± 2.9
Acute	30 days	5 h	
NaCl			62.8 ± 7.0
MMI			44.2 ± 2.4**
Acute	30 days	48 h	
NaCl			80.5 ± 6.5
MMI			71.5 ± 5.9

Values shown are means ± s.e.mean from 5–8 experiments performed after a chronic or an individual administration of MMI (20 mg kg^{-1} s.c. to immature rats, or 50 mg kg^{-1} s.c. to mature rats).
** $P < 0.025$.

of MMI (20 mg kg^{-1}), however, there was a decrease (by 30%) in the concentration of SH groups, an effect no longer evident 48 h later (Table 3).

Discussion

The present results show that experimental, primary-like hypothyroidism significantly decreased the number of [^{3}H]-imipramine binding sites in the cerebral cortex of 32 day-old rats, while imipramine binding in the corpus striatum was unaltered. Differential sensitivity of brain areas to dysthyroidism reportedly occurs in other receptor systems such as the muscarinic cholinoceptor and GABA-receptor systems (Patel *et al.*, 1980), and in central adrenoceptor function (Atterwill *et al.*, 1983). It is probably related to specific maturational schedules of brain regions and neurone types and, thus, to their different adaptive responses to thyroid imbalance. Innervation by monoaminergic neurones in the corpus striatum seems to be functionally more competent than that in the neonatal cortex (Coyle, 1977), where early thyroid deprivation markedly decreases the density of axon terminals (Legrand, 1979). More generally, early hypothyroidism permanently decreases the number of synapses to below normal (Legrand, 1984). Thus, this decrease in the number of cortical imipramine binding sites may have been caused by hypothyroid-delayed sprouting of 5-HT terminal and non terminal axons, where [^{3}H]-imipramine putative 'receptors' appear to be located (Fuxe *et al.*, 1983). A maturational origin for receptor alterations was strongly supported from the fact that chronic administration of goitrogen to mature rats did not affect the binding of [^{3}H]-imipramine to cortical and striatal membranes (Figure 2). Furthermore, chronic treatment, and not the last injection of goitrogen 48 h before rats were killed, was responsible for binding alterations, inasmuch as an individual injection of MMI to 30 day-old pups did not affect [^{3}H]-imipramine binding 48 h later (Table 2). This also indicates that the decrease in [^{3}H]-imipramine binding following the neonatal treatment was not a late adaptive response to the early increase in the number of binding sites seen, as a matter of fact, shortly after an individual injection of MMI (Table2). This increase occurring when tissue levels of goitrogen must be high was of particular interest, since a similar effect was obtained when the binding reaction of [^{3}H]-imipramine was run in the presence of MMI, a thiol compound (present results), and additional disulphide bond reducing reagents (Vaccari, 1984).

The content of accessible SH groups was similar in euthyroid and hypothyroid cortical membranes, thus indicating that chronic administration of MMI through the sensitive period of brain development did not cause membrane alterations which could itself have hindered [^{3}H]-imipramine binding at its recognition sites. Nevertheless, since low *in vitro* concentrations, or an individual injection of MMI both increased the binding of [^{3}H]-imipramine, some functional role of membrane SH groups and/or disulphide bridges in central imipramine recognition sites may be suggested. Increasing evidence shows, in fact, that sulphur-containing bonds are close to, or located at the [^{3}H]-imipramine binding site in human platelets (Wennogle *et al.*, 1981; Davis, 1984). Of course, the present decrease in the concentration of membrane bound SH groups occurring shortly after acute administration of MMI, when the binding of [^{3}H]-imipramine was enhanced, may be not relevant for receptor changes. As a matter of fact, the number of thiol groups putatively associated with a receptor must be very small compared to the bulk of SH groups in the membrane. One cannot exclude the possibility that these gross changes may reflect more subtle alterations in the binding subunit microenvironment.

In conclusion, brain immaturity and chronic antithyroid treatment are essential for changes in the binding of [^{3}H]-imipramine to membranes from selected brain regions. Goitrogen-provoked maturational impairment of the matrix for imipramine binding sites may, thus, overcome any direct influence of MMI on thiol homeostasis near to, or within the 'receptor' area.

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