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[^{3}H]DOXEPIN INTERACTIONS WITH HISTAMINE H_1-RECEPTORS AND OTHER SITES IN GUINEA PIG AND RAT BRAIN HOMOGENATES

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[^{3}H]Doxepin, a tricyclic antidepressant, binds to brain homogenates with two saturable components. The high affinity component, with a dissociation constant (K_D) of 0.26 nM, is associated with histamine H_1-receptors. This high affinity binding shows stereospecificity in that d-chlorpheniramine is 100 times more potent than the pharmacologically less active l-isomer. Its drug specificity and regional variation closely parallel those exhibited by [^{3}H]mepyramine binding. The drug specificity of the low affinity component is distinct from that of histamine H_1-receptors, with no stereospecificity for chlorpheniramine isomers. Furthermore, all the H_1-histamine antagonists tested display micromolar potency at the low-affinity doxepin sites but nanomolar potency at the high-affinity doxepin sites associated with a physiological histamine H_1-receptor. The drug specificity of the low affinity site does not correspond to that of any known neurotransmitter receptor. Tricyclic antidepressants display IC_{50} values of 30—600 nM for the inhibition of [^{3}H]doxepin binding to the low-affinity component with most values in the 0.1—0.3 μM affinity range.

Tricyclic antidepressant | Histamine H_1-receptor | Mepyramine | [^{3}H]Doxepin | Rat brain | Guinea pig brain | Chlorpheniramine

1. Introduction

Histamine H_1-receptors can be labeled in brain membranes (Tran et al., 1978; Hill et al., 1978) and peripheral tissue (Hill et al., 1977) with [^{3}H]mepyramine. The binding sites display drug specificity characteristic of physiological histamine H_1-receptors with some differences in drug selectivity in various brain regions and species (Chang et al., 1979a, b; Hill and Young, 1980). Some tricyclic antidepressants are more potent in competing at histamine H_1-receptor binding sites than in affecting any other neurotransmitter system (Tran et al., 1978). Doxepin is the most potent of the antidepressants examined, with a K_i value of 0.5 nM (Tran et al., 1978) at H_1-receptors labeled with [^{3}H]mepyramine and similar potency in blocking histamine H_1 mediated enhancement of cyclic GMP levels in neuroblastoma clones (Richelson, 1978). In the present study we have evaluated [^{3}H]-doxepin binding to brain membranes.

2. Materials and methods

[^{3}H]Doxepin (42.6 Ci/mmol) was prepared by New England Nuclear (Boston, Massachusetts). The tritiated material migrated as a single band in thin layer chromatography on silica gel in ethylacetate/octanol, 17/3 (vol/vol). The sources of drugs were the same as described by Tran et al. (1978).

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Frozen guinea pig or rat brains were thawed on ice in cold Na/K phosphate buffer (50 mM, pH 7.4) and homogenized with a Polytron in the same buffer at 60 vol. The homogenate was centrifuged (50 000 $g \times 10$ min). The pellet was resuspended in the same volume of ice-cold Na/K phosphate buffer and centrifuged again. The final pellet was resuspended in the original volume of buffer by Polytron homogenization. For regional distribution studies, fresh guinea pig or rat brains were used and dissected on ice into individual regions.

To measure specific binding of [^{3}H]-doxepin, 100 μl of [^{3}H]doxepin and 100 μl of unlabeled inhibitors were added to 800 μl of final tissue homogenate (10 mg/tube) in a 5 ml glass test tube. Incubation was carried out at 23—25°C for 60 min and terminated by addition of 4 ml of ice-cold buffer followed by rapid filtration onto glass fiber filters (GF/B) positioned over a vacuum and washed with 3 × 4 ml ice-cold buffer. Radioactivity trapped on the filters was counted in 10 ml of Formula 947 (New England Nuclear) at an efficiency of 28% after a 12 h storage at 4°C.

Specific binding was defined as radioactivity bound after subtraction of nonspecific binding determined in the presence of 2 μM doxepin. Binding to the filters in the absence of tissue gave only about 50 cpm. Thin layer chromatographic analysis of tritiated material bound to membranes after incubation with 4 nM [^{3}H]doxepin, or in the supernatant fluid showed no metabolism of [^{3}H]doxepin after incubation. [^{3}H]Mepyramine binding was assyed as described in Tran et al. (1978).

The experimental binding data for saturation of doxepin binding was subjected to a non-linear least-squares fitting algorithm using a model for association of doxepin to two independent receptor sites (Schrager, 1970). The model utilized to determine the number and affinity of the two binding sites is given by the equation.

$$D_R = \frac{(D)\,B_H}{1 + K_H(D)} + \frac{(D)\,B_L}{1 + K_L(D)} \text{ transformed}$$

where D_R = represents total bound doxepin, D represents free doxepin, K_H and K_L are the equilibrium affinity constants of doxepin to each receptor and B_H and B_L are the total concentration of the two receptor sites.

3. Results

3.1. General properties of [^{3}H]doxepin binding

Specific binding of [^{3}H]doxepin to guinea pig and rat cortical membranes is linear over the range of 2—15 mg wet weight tissue. In typical experiments employing 0.5 nM [^{3}H]-doxepin incubated with 10 mg rat or guinea pig brain membranes, total binding is about 2700 cpm and nonspecific binding, assayed in the presence of 2 μM doxepin or 0.2 μM d-chlorpheniramine, is 400 cpm. Thus, specific binding, the difference between total and nonspecific binding is 86% of total binding. By contrast, with [^{3}H]mepyramine employed at 0.5 and 4 nM for studies with guinea pig and rat brain membranes respectively, the specific binding is 88% of total binding for guinea pig brain and only 60% in the case of rat brain membrane (table 1).

3.2. Kinetics of [^{3}H]doxepin binding

At 25°C, [^{3}H]mepyramine associates quite rapidly with guinea pig brain membranes (Chang et al., 1979a). Binding reaches equilibrium values at approximately 10 min, with half maximal binding attained at about 1 min. The dissociation rate of [^{3}H]mepyramine from H_1-receptors is also very rapid with a $t_{1/2}$ of 10 min. Kinetics of both association and dissociation are much slower for [^{3}H]-doxepin. At 25°C and 0.5 nM, [^{3}H]doxepin binding to guinea pig brain membranes is time dependent, reaching equilibrium by 60 min, which is used for routine incubations (fig.

TABLE 1

Equilibrium constants of [^{3}H]mepyramine and [^{3}H]-doxepin binding to histamine H_1-receptors in guinea pig and rat brain membranes.

	K_D (nM)	B_{max} (pmol/g tissue)	Percent specific binding
Rat			
Mepyramine	4.0	10.7	60%
Doxepin	0.25	6.5	86%
Guinea pig			
Mepyramine	0.5	7.2	85%
Doxepin	0.26	6.7	86%

K_D and B_{max} values were derived from saturation analysis of [^{3}H]mepyramine and [^{3}H]doxepin binding to crude membrane preparations from rat and guinea pig whole brain. Percent specific binding of [^{3}H]mepyramine and [^{3}H]doxepin to rat and guinea pig brain was determined using 0.5 nM [^{3}H]doxepin in both tissues and 4.0 nM and 0.5 nM [^{3}H]mepyramine in rat brain and guinea pig brain, respectively. K_D and B_{max} for [^{3}H]doxepin are those of a computer estimate of the high affinity component only.

1A). Half maximal binding is attained by 12 min. The bi-molecular rate constant of association is 0.032 $min^{-1} \cdot nM^{-1}$. Dissociation of [^{3}H]doxepin at 25°C was determined by incubating 0.5 nM [^{3}H]doxepin with guinea pig brain membranes at 25°C for 60 min, whereupon 2 μM unlabeled doxepin or 0.2 μM triprolidine was added and residual binding examined at different time points. Under these conditions dissociation is monophasic when plotted on a semilogarithmic scale with half life for dissociation of 45 min (fig. 1B). The dissociation constant (K_D) calculated from the ratio of K_{-1}/k_1 is 0.48 nM. These parameters of binding kinetics are sensitive to changes in temperature employed for the binding studies. For example, at 37°C and 0.5 nM, [^{3}H]doxepin binding to guinea pig membranes is much faster. Binding begins to plateau at 15 min. Half maximal binding is attained by 1.5 min. The dissociation of specifically bound [^{3}H]doxepin is also very rapid at 37°C. The half life of dissociation is

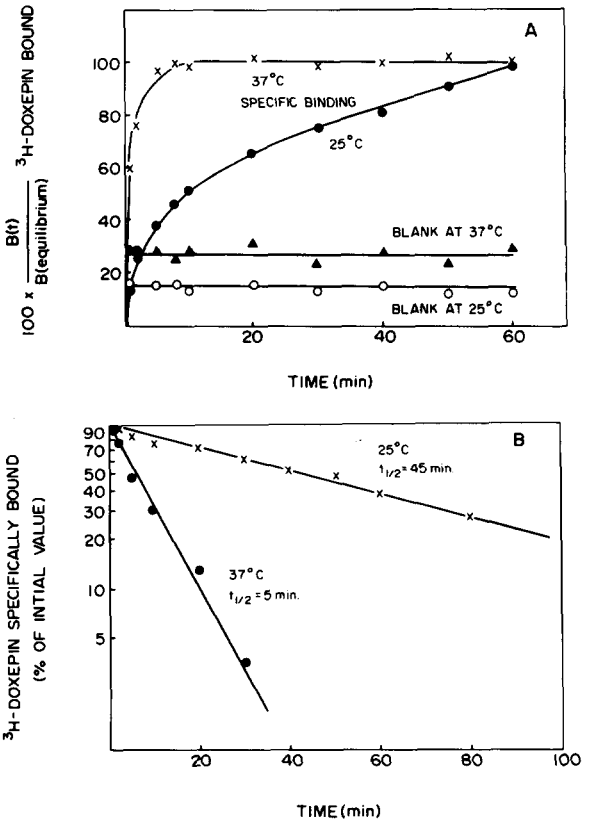

Fig. 1. Kinetics of [^{3}H]doxepin specific binding. (A) Association: specific [^{3}H]doxepin binding to guinea pig brain membranes (10 mg wet weight) at 25 and 37°C was measured at various time intervals after the addition of [^{3}H]doxepin (0.5 nM final concentration). Specific binding was defined as the difference between the binding in the presence (blank) and absence (total) of 0.2 μM triprolidine. Points shown are those from a single experiment performed in triplicate which was replicated three times. The bimolecular rate constant is calculated according to the equation $K_i = [2.203/t(a-b)]\log[b(a-x)/a(b-x)]$, where a is the initial concentration of [^{3}H]doxepin (0.5 nM) and b is the initial receptor concentration (0.18 nM). ×, Specific binding at 37°C; ▲, nonspecific binding, at 37°C; ● and ○ are specific and nonspecific binding, respectively, at 25°C. B(t) is the amount of [^{3}H]doxepin bound at time t. B(equilibrium) = 2700 cpm at 25°C and 1900 cpm at 37°C. (B) Dissociation: specifically bound [^{3}H]doxepin was assayed at 25°C and 37°C at various intervals after incubation with [^{3}H]doxepin (0.5 nM) to equilibrium (60 min) under standard assay conditions. At time zero, triprolidine 0.2 μM, was added to the incubation and the reaction was terminated at various intervals t. Data are expressed as the percent of specific binding at zero time.

approximately 8 times more rapid than that at 25°C with a $t_{1/2}$ of 5.5 min.

3.3. *Saturation analysis of [^{3}H]doxepin binding*

Saturation of specific [^{3}H]doxepin binding was examined by incubating brain membranes with varying concentrations of [^{3}H]doxepin. Computer fit plots of specific [^{3}H]doxepin binding versus concentration of tritiated ligand indicated that the binding is biphasic with a slight inflection at 4—5 nM. The binding does not plateau until the concentration of [^{3}H]doxepin is greater than 40 nM. Specific binding is a curvilinear function of [^{3}H]-doxepin concentration while nonspecific binding, determined in the presence of 2 μM triprolidine increases linearly. Scatchard analysis of binding in either rat or guinea pig brain membranes reveals 2 distinct components using a computer fit model. The high and low

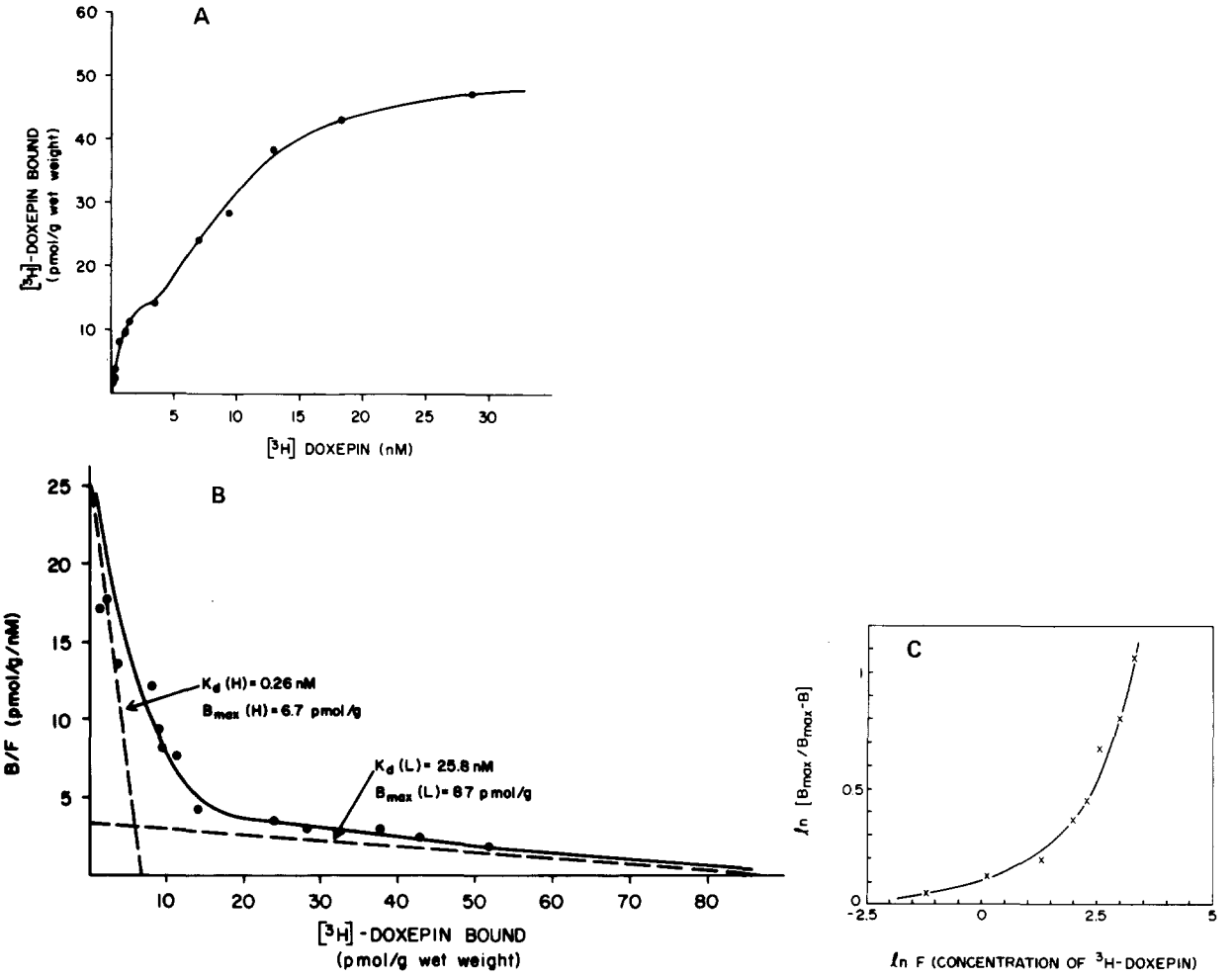

Fig. 2. Specific [^{3}H]doxepin binding to guinea pig brain membranes. (A) The curve represents a computer fit of the experimental data as described in Materials and methods. [^{3}H]doxepin at various concentrations was incubated with guinea pig brain membranes (10 mg) at 25°C for 60 min in 1 ml of 50 mM Na/K phosphate buffer (pH 7.5) in the presence or absence of 2 μM doxepin. The experiment was replicated three times. (B) Scatchard analysis of the data shown in (a). K_D (H) = 0.26 nM and B_{max} (H) = 6.7 pmol/g of tissue; K_D (L) = 25.8 nM and B_{max} (L) = 87 pmol/g of tissue. (C) Hill plot of the same data.

affinity components have maximal numbers of binding sites (B_{max}) of 6.7 and 87 pmol/g wet weight tissue respectively and dissociation constants of 0.26 and 25.8 respectively (fig. 2B). Hill analysis of specific [^{3}H]doxepin binding demonstrates an upward convex plot (fig. 2C) which is again consistent with more than one independent binding site.

3.4. Drug influences on [^{3}H]doxepin binding in brain membranes

Inhibition curves of various antihistamines for binding of [^{3}H]doxepin to guinea pig brain membranes at low (0.5 nM) and high (4 nM) concentrations were compared to the inhibition of 4 nM [^{3}H]mepyramine (fig. 3A). With [^{3}H]mepyramine all antihistamines examined display a single component of displacement with Hill coefficients of about 1.0. The IC_{50} values computed for these drugs at [^{3}H]mepyramine sites (table 1) are closely similar to those observed previously (Tran et al., 1978; Chang et al., 1979a). When the high affinity doxepin component is examined using 0.5 nM [^{3}H]doxepin, the IC_{50} determined values are essentially the same as for [^{3}H]mepyramine (table 2).

Thus, among the ethylenediamine-antihistamines, mepyramine and triprolidine have

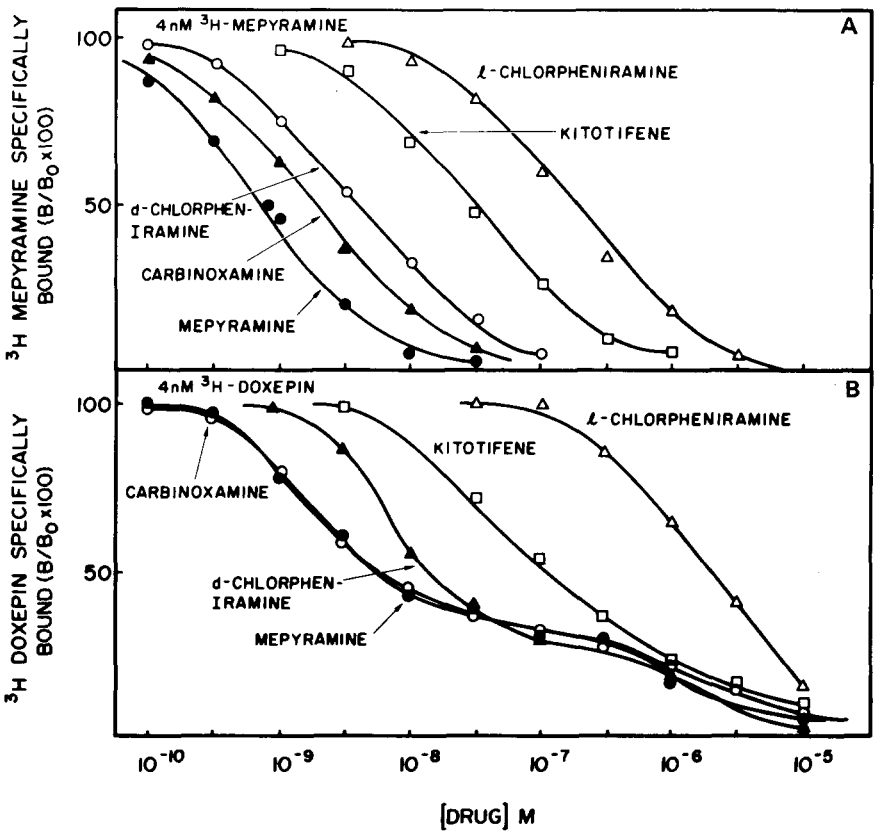

Fig. 3. Displacement of specific [^{3}H]doxepin and [^{3}H]mepyramine binding by various drugs in guinea pig brain. (A) Specific [^{3}H]mepyramine binding was determined as the difference between binding of 4 nM [^{3}H]mepyramine in the presence and absence of 2 μM triprolidine. Results are expressed as percent of specific binding remaining at different concentrations of unlabeled drugs. (B) Specific [^{3}H]doxepin binding was determined with 4 nM [^{3}H]doxepin. Blank values were determined in the presence of 2 μM doxepin. The experiments were replicated three times.

TABLE 2

Drug effects on [^{3}H]doxepin and [^{3}H]mepyramine binding to guinea pig brain membranes.

The inhibition of specific binding of [^{3}H]dopexin (0.5 nM) and [^{3}H]mepyramine (4 nM) to histamine H_1-receptors was determined with 6—9 concentrations of competing drugs assayed in triplicate. The mean inhibitory concentration (IC_{50}) values were determined from log-probit analysis. The experiments were carried out with 10 mg of guinea pig brain membranes in a final volume of 1.0 ml of 50 mM Na/K phosphate buffer (pH 7.4). Low affinity [^{3}H]doxepin sites were labeled with 4 nM [^{3}H]doxepin in the presence of 0.2 μM triprolidine to block off that portion of binding attributable to histamine H_1-receptors. NE = less than 15% displacement observed at 10 mM; ND = not determined.

Drugs	[^{3}H]doxepin (0.5 nM)	IC_{50} (nM)	
		[^{3}H]Mepyramine (4 nM)	[^{3}H]Doxepin (4 nM) + 0.2 μM Triprolidine
H_1-Antihistamines			
d-Chlorpheniramine	4.8	4.0	4000
l-Chlorpheniramine	440	420	5000
Mepyramine	1.0	1.2	3000
Triprolidine	1.2	1.2	5000
Carbinoxamine	7.6	8.0	4400
Promethazine	7.6	7.0	3400
Kitotifene	30.0	56.0	4400
Neuroleptics			
Promazine	7.6	4.0	ND
Clozapine	7.0	8.0	ND
Chlorpromazine	5.2	9.0	ND
Tricyclic antidepressants			
Doxepin	1.0	1.0	30
Amitriptyline	9.2	8.2	150
Nortriptyline	80	92	340
Protriptyline	120	120	400
Imipramine	60	52	500
Chlorimipramine	200	200	300
Desipramine	600	500	640
Other drugs			
Atropine	3800	3600	NE
Cimetidine	NE	NE	NE
d-LSD	3800	3400	NE
Phentolamine	7600	6600	NE
(±)-Propranolol	NE	NE	9600
Histamine [1]	13 800	13 800	ND
Histamine [1] + 100 mM NaCl	106 000	106 000	ND

[1] IC_{50} values for histamine were determined in Tris-HCl buffer (50 mM, pH 7.4 in the presence or absence of 100 mM NaCl.

similar potencies with IC_{50} values of about 1.0 nM, which is about 4 times more potent than the alkylamine chlorpheniramine and 8 times more active than the ethanolamine carbinoxamine and phenothiazine promethazine. d-Chlorpheniramine is 100 times more potent than the l-isomer. Among the neuroleptics, clozapine, chlorpromazine and promazine have IC_{50} values of 4—8 nM. Drugs known to lack H_1 antihistamine activity, such as atropine, d-LSD, phentolamine, propanolol, and cimetidine, are relatively ineffective in com-

peting for [^{3}H]doxepin binding.

By contrast, when inhibition curves are conducted with 4 nM [^{3}H]doxepin, biphasic inhibitions are observed (fig. 3B). For example, complete inhibition by chlorpheniramine takes place over four orders of magnitude of concentration of unlabeled inhibitor. Two distinct phases of inhibition are apparent for chlorpheniramine. The low affinity component of chlorpheniramine inhibition of [^{3}H]doxepin under these conditions displays very little stereospecificity for the d- and l-stereoisomers. To assess the relative potencies of various drugs at the low affinity [^{3}H]-doxepin site, we conducted inhibition experiments with 4 nM [^{3}H]doxepin in the presence of 0.1 μM mepyramine or 0.2 μM triprolidine, which is sufficient to reduce the high affinity component of [^{3}H]doxepin binding maximally with little effect on the low affinity component (fig. 3B). Drug potencies at the low affinity sites differ markedly from their effects at the high affinity sites. For instance, d- and l-chlorpheniramine are equally potent with IC_{50} values of 4—5 μM. Other antihistamines examined are also relatively inactive with IC_{50} values in μM range. Interestingly, propranolol has about 10 μM potency at the low affinity site while it is essentially inactive at the high affinity [^{3}H]-doxepin binding site or at [^{3}H]mepyramine sites (table 1).

The low affinity [^{3}H]doxepin binding sites do not involve histamine H_2, serotonin, muscarinic cholinergic or α-adrenergic receptors. Atropine, cimetidine, d-LSD and phentolamine at 10^{-5} M are ineffective in competing for this site. Raisman et al. (1979) recently described a high affinity [^{3}H]imipramine binding site to which clinically employed tricyclic antidepressants have fairly high affinity. The relative potencies of drugs at the low affinity [^{3}H]doxepin sites differs from their effects at [^{3}H]imipramine sites. For instance, while doxepin is the most potent antidepressant at the low affinity [^{3}H]doxepin sites, with an IC_{50} value of 30 nM, the corresponding IC_{50} for doxepin at the [^{3}H]imipramine site is 300 nM. By contrast, imipramine is the most potent competitive inhibitor at the [^{3}H]imipramine sites. At the [^{3}H]doxepin sites, doxepin is six times more potent than amitriptyline which is in turn 2 to 3 times more active than nortriptyline, protriptyline, imipramine and chlorimipramine (table 1). Desipramine is somewhat less effective with an IC_{50} value of 640 nM. By comparison, at the [^{3}H]imipramine sites, doxepin is 12 times less active than amitriptyline which is in turn equipotent to protriptyline, desipramine and chlorimipramine with IC_{50} values of 18—25 nM.

3.5. Regional distribution of [^{3}H]doxepin binding

The differences between high and low affinity [^{3}H]doxepin sites are further substantiated by the dissimilarities in their regional distributions in the brain. The high affinity binding component was assessed with 0.5 nM of [^{3}H]doxepin and the low affinity component with 4 nM [^{3}H]doxepin in the presence of 0.2 μM triprolidine to block off any binding to the high affinity histamine H_1-receptor. Binding levels in the thalamus-hypothalamus (diencephalon) are taken as 100%. Highest levels of high affinity [^{3}H]doxepin binding in the guinea pig brain occur in the cerebellum (table 3) which is 5 times higher than the diencephalon. Binding levels in the midbrain, hippocampus, cerebral cortex and brain stem are respectively 3, 1.7, 2.7 and 2 times more than the diencephalon. The corpus striatum is the only region with less binding than the combined thalamus and hypothalamus. The regional distribution of this high affinity binding component resembles our previous studies with [^{3}H]mepyramine binding (Chang et al., 1979a). The low affinity component of [^{3}H]-doxepin binding to guinea pig brain is markedly different (table 2). Highest binding occurs in the midbrain which is 3 times higher than the diencephalon, while the brain stem possesses only 3% of binding detected in the diencephalon. Cerebellum, cerebral cortex

and hippocampus each have approximately twice the amount of low affinity binding of diencephalon.

Regional distributions of low and high affinity components also differ in the rat brain. Moreover, relative levels of high and low affinity sites in various regions differ dramatically between rat and guinea pig. Thus, in the rat, the order of receptor density from highest region to the lowest is (table 3): High affinity site: cerebral cortex ⩾ brain stem > midbrain > hippocampus > striatum > thalamus and hypothalamus (diencephalon) > cerebellum; low affinity site: cerebral cortex > midbrain ⩾ striatum > diencephalon > hippocampus. Low affinity binding is not detectable in rat cerebellum and brain stem. The regional distribution of low affinity [^{3}H]doxepin binding is another indication that this binding site is not related to the high affinity [^{3}H]imipramine binding site as determined by Raisman et al. (1979).

TABLE 3

Regional distribution of high and low affinity [^{3}H]-doxepin binding sites in guinea pig and rat brain membranes.

High affinity [^{3}H]doxepin binding was assessed at 0.5 nM final concentration with (nonspecific binding) or without (total binding) 0.2 μM mepyramine or triprolidine. Specific binding was computed as the difference between total and nonspecific binding. Results are expressed as percent of the specific binding of hypothalamus plus thalamus. Low affinity [^{3}H]doxepin binding was determined with 4 nM [^{3}H]doxepin in the presence of 0.1 μM mepyramine or 0.2 μM triprolidine. Blank values were determined with 2 μM doxepin. Data are mean values from 3—5 experiments whose results varied less than 15%. Under these conditions, specific high and low affinity binding levels to guinea pig hypothalamus-thalamus are 975 and 950 cpm/10 mg wet weight respectively. The specific binding values for rat hypothalamus-thalamus are 756 and 520 cpm/10 mg wet weight for high and low affinity sites respectively.

Regions	Percent of hypothalamus-thalamus	
	High affinity	Low affinity
Guinea pig		
Cerebral cortex	210	179
Hippocampus	268	163
Corpus striatum	49	92
Cerebellum	522	223
Brain stem	172	3
Midbrain	312	288
Hypothalamus-thalamus (diencephalon)	100	100
Rat		
Cerebral cortex	215	455
Hippocampus	145	88
Corpus striatum	105	287
Cerebellum	89	0
Brain stem	198	0
Midbrain	176	297
Hypothalamus-thalamus (diencephalon)	100	100

4. Discussion

[^{3}H]Doxepin binds to mammalian brain membranes with two distinct components. Scatchard analysis of the binding isotherm indicates that both components are saturable with B_{max} values of 6.7 and 86 pmol/g wet weight tissue respectively for the high and low affinity components. The high affinity component has a dissociation constant (K_D) of 0.26 nM. This binding appears to be associated with histamine H_1-receptors. The drug specificity at this high affinity doxepin site parallels that demonstrated at H_1-receptors labeled with [^{3}H]mepyramine (Tran et al., 1978; Hill et al., 1978). Regional distributors of high affinity [^{3}H]doxepin binding to both rat and guinea pig brain resemble those demonstrated by [^{3}H]mepyramine binding in both species (Chang et al., 1979a; Hill and Young, 1980). [^{3}H]Doxepin is thus a suitable ligand for labeling the H_1-receptor. [^{3}H]-Doxepin offers considerable advantages over [^{3}H]mepyramine in that the former can be obtained in twice the specific radioactivity of the latter. Furthermore, doxepin binds with similar affinity to both rat and guinea pig brains (table 1) while mepyramine binds with

different affinities in varying species and organs (Chang et al., 1979a, b). The B_{max} value of [^{3}H]doxepin binding to the high affinity site is 6.7 pmol/g wet weight tissue, a value similar to that obtained with [^{3}H]-mepyramine. When employed at 0.5 nM, 90% of [^{3}H]doxepin binding is specific.

The low affinity binding site for [^{3}H]-doxepin is saturable with an affinity of about 26 nM as determined by saturation binding experiments. The drug specificity of this low affinity [^{3}H]doxepin site is distinct from sites labeled by [^{3}H]imipramine (Raisman et al., 1979) and serotonergic (Peroutka and Snyder, 1979), muscarinic (Yamamura et al., 1974) and α- and β-adrenergic (U'Prichard et al., 1977; Bylund and Snyder, 1976) receptor sites. The physiologic role of the low affinity sites is unclear. Circulating levels of tricyclic antidepressants (about 100 nM; Asberg et al., 1973; Braithwaite et al., 1972) correspond to potencies of these drugs at the low affinity [^{3}H]doxepin site. Accordingly, one would anticipate occupancy of these sites in the brains of patients receiving tricyclic antidepressants.

Acknowledgements

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