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Structural properties of phenylethylamine derivatives which inhibit transport-P in peptidergic neurones

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1 Transport-P is an antidepressant-sensitive, proton-dependent, V-ATPase-linked uptake process for amines in peptidergic neurones of the hypothalamus. It is unusual in its anatomical location in postsynaptic neurones and in that it is activated by its substrate (prazosin). This study examined the structural properties of phenylethylamine derivatives which are substrates for transport-P, as judged by competitive inhibition of the uptake of prazosin 10^{-6} M in immortalized hypothalamic peptidergic neurones.

2 A basic amine was essential for activity; absence of the amine or neutralization with a carboxyl group abolished activity. Primary, secondary and tertiary amines were active but quaternary and guanyl amines were inactive.

3 A phenyl group was essential for activity at transport-P. Potency at transport-P was reduced by phenolic hydroxyl groups and enhanced by phenolic halogens. Thus, for maximal potency, the phenyl group should be hydrophobic. Phenolic methoxyl groups had no effect on potency at transport-P.

4 A side chain was necessary for activity at transport-P. Potency at transport-P was reduced by β-hydroxyl and enhanced by α-methyl groups.

5 These findings further distinguish transport-P from other amine uptake processes in the brain.

Keywords: Biological transport; structure-activity relationship; hypothalamus; prazosin; α-adrenoceptors; LHRH

Introduction

Presynaptic nerve terminals possess two sequential uptake processes for amines. Transporter molecules which are located in the plasma membranes of presynaptic nerve terminals utilize the electrochemical gradient of sodium ions which is generated by the Na^+/K^+ ATPase (sodium pump) to accumulate amines from the extracellular synaptic space into the cytoplasm. These plasma membrane transporters can be blocked by tricyclic antidepressants (for recent reviews see Amara & Kuhar, 1993; Lester *et al*., 1994). The second set of transporter molecules in presynaptic nerve terminals are located in the membranes of neurosecretory vesicles. These vesicular transporters utilize the electrochemical gradient of protons which is generated by vacuolar-type ATPase (V-ATPase) to accumulate amines from the cytoplasm to the interior of the neurosecretory vesicles. Vesicular transporters can be blocked by reserpine (for reviews see Liu & Edwards, 1997; Schuldiner *et al*., 1995). Amine uptake processes also exist in non-neuronal cells; they are independent of protons and sodium ions but they can be blocked by steroid hormones (Hendley *et al*., 1970; Salt, 1972; Kimelberg, 1986; Schomig *et al*., 1992). These uptake processes are believed to terminate the actions of the neurotransmitter amines by reducing their concentrations in the extracellular synaptic space.

In addition to the uptake processes in presynaptic nerve terminals and in glial cells, the postsynaptic, peptidergic neurones of the hypothalamus possess an unusual uptake process (transport-P; Al-Damluji & Krsmanovic, 1992; Al-Damluji *et al*., 1993). This uptake process became evident while hypothalamic neurones were being examined for the presence of α_1 adrenoceptors. The α_1-adrenoceptor ligand [^{3}H]-prazosin bound to the cells and was displaced by unlabelled prazosin in concentrations up to 10^{-7} M. However, at concentrations of unlabelled prazosin greater than 10^{-7} M, there was a paradoxical increase in the accumulation of [^{3}H]-prazosin which could be abolished by desipramine; in the presence of desipramine, only displacement of [^{3}H]-prazosin by unlabelled prazosin was seen. These findings were interpreted as indicating the presence in peptidergic neurones of α_1-adrenoceptors and an unusual uptake process. As the concentration of unlabelled prazosin is increased, [^{3}H]-prazosin is displaced from the receptors; we subsequently cloned α_1-adrenoceptor cDNA from these neurones, providing further evidence for this part of the hypothesis (White & Al-Damluji, 1997). The uptake process is evident at nanomolar concentrations of prazosin, but when the concentration of unlabelled prazosin is increased, there is a further activation of the uptake process, manifested by the paradoxical increase in accumulation of the radioligand (Al-Damluji & Krsmanovic, 1992; Al-Damluji *et al*., 1993). The paradoxical increase requires the electrochemical gradient of protons and is linked to V-ATPase (Al-Damluji & Kopin, 1996a).

The functional properties of transport-P are distinguishable from presynaptic amine uptake processes. Thus, the paradoxical increase of the radioligand was not seen when presynaptic neurones were studied in an identical manner (Al-Damluji & Kopin, 1996a). Transport-P differs further from presynaptic plasma membrane transporters in that it is not absolutely dependent on sodium ions or the sodium pump (Al-Damluji & Kopin, 1996a). Although transport-P is dependent on protons and is linked to V-ATPase, it is distinguishable from presynaptic vesicular amine transporters in that it is resistant to reserpine (Al-Damluji & Kopin, 1996a). Transport-P differs from Uptake$_2$ in non-neuronal cells in that it is insensitive to steroid hormones (Al-Damluji & Kopin,

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1996a). Further, microscopic studies using a fluorescent analogue of prazosin demonstrated that in the hypothalamus, transport-P exists predominantly in neurones rather than in glial cells (Al-Damluji *et al*., 1997).

The present study aimed to define some of the structural properties of compounds which interact with transport-P. The α_1-adrenoceptor agonist methoxamine is a phenylethylamine derivative which has prominent effects on hypothalamic neuroendocrine function (for review, see Al-Damluji, 1993). The structural similarity between methoxamine and prazosin prompted an examination of the effects of phenylethylamine derivatives. The study examined phenylethylamine analogues for their ability to antagonize competitively the uptake of prazosin 10^{-6} M in immortalized hypothalamic gonadotrophin-releasing hormone (GnRH; LHRH) neurones. These cells retain many characteristics of differentiated hypothalamic neurones (for review, see Weiner *et al*., 1992). In the presence of unlabelled prazosin 10^{-6} M, [^{3}H]-prazosin is completely displaced from α_1-adrenoceptors (Al-Damluji & Kopin, 1996a). Nevertheless, as prazosin and methoxamine are ligands for α_1-adrenoceptors, the findings were compared to the structural properties of phenylethylamine derivatives which are active at α-adrenoceptors and at other amine uptake processes. The findings indicate that activity at transport-P requires structural properties which are different from those required for binding to α-adrenoceptors and other amine uptake processes in the brain.

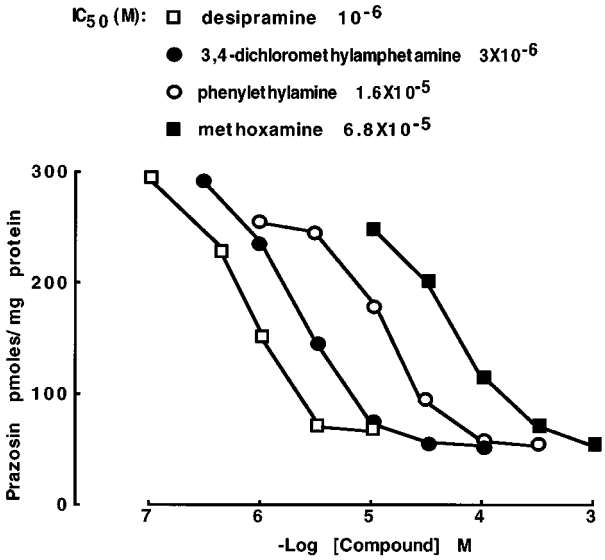


Figure 1 Effects of phenylethylamine and two of its derivatives (3,4-dichloromethylamphetamine and methoxamine) on the uptake of prazosin 10^{-6} M in immortalized peptidergic neurones (GT1-1 GnRH cells). The effect of desipramine is also shown for comparison. The vertical axis shows the total amount of prazosin accumulated in the cells by correcting for specific activity, ie, [^{3}H]-prazosin + unlabelled prazosin mg^{-1} protein.

Methods

Cell culture

Immortalized GT1-1 GnRH neuronal cells were cultured as previously described in detail (Al-Damluji *et al*., 1993). Briefly, the cells were grown in Corning 75 cm^2 or 150 cm^2 flasks in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 (ratio 1 : 1) containing 10% FBS, sodium bicarbonate 3.7 g l^{-1} and gentamicin 100 mg l^{-1}, in a humidified atmosphere containing 5% CO_2 in air. Culture media were changed at 48 h intervals. When the cells reached confluence, they were dispersed in the presence of trypsin, DNAseI and EDTA and incubated in Costar or Corning 12-well plates (2×10^6 cells/well). Culture media were changed at 48 h intervals and the experiments were carried out four days following culture.

Uptake studies

Uptake studies were performed on intact cells, as previously described in detail (Al-Damluji *et al*., 1993). Drugs were dissolved in uptake buffer consisting of DMEM with 25 mM HEPES and 0.5×10^{-3} M sodium ascorbate, pH 7.4. The cells were washed twice with 1 ml of buffer and then incubated for 60 min at 37°C in buffer containing [^{3}H]-prazosin 2×10^{-9} M and unlabelled prazosin 10^{-6} M, with or without phenylethylamine derivatives in concentrations of 10^{-6} M to 10^{-3} M. Accumulation of prazosin and antidepressants reaches equilibrium within 60 min (Al-Damluji & Kopin, 1996b). At the end of the incubation period, the wells were placed on ice and the cells were washed twice with 1 ml volumes of ice-cold buffer. The cells were then solubilized with two ml of a warm solution of 0.1% sodium dodecyl sulphate and 0.1 M NaOH. Fifty microlitre aliquots were removed for protein assay and 10 ml of scintillation liquid was then added to the cell extract, mixed and radioactivity was measured in a scintillation spectrometer with an efficiency of 50%. Protein content was measured by the bicinchoninic acid modification of the biuret reaction (Smith *et al*., 1985) using albumin standards and reagents supplied by Pierce (Rockford, Illinois or Chester, Cheshire).

A representative member of each group of compounds was tested for its ability to inhibit the uptake of prazosin competitively. This was done by examining the effects of different concentrations of the phenylethylamine derivative in the presence of different concentrations of unlabelled prazosin, while the concentration of [^{3}H]-prazosin was kept constant at 2×10^{-9} M.

Efficacy was defined as % inhibition of the uptake of prazosin 10^{-6} M when the test compound was used in a maximal concentration (10^{-4} M or 10^{-3} M). Efficacy was expressed as % of the effect of a maximal inhibitory concentration of desipramine (10^{-5} M). Typically, desipramine 10^{-5} M inhibited the accumulation of prazosin 10^{-6} M by 80% (Figure 1; Al-Damluji & Kopin, 1996b); the remaining 20% was regarded as non-specific uptake for the purposes of this study. Half-maximal inhibitory concentrations (IC_{50} values) were calculated from the concentration-response curves. IC_{50} values were calculated only for compounds which achieved a maximal inhibitory response, defined as the inhibitory effect of desipramine 10^{-5} M (see Figure 1). When a compound did not achieve the maximal inhibitory response, IC_{50} values were not calculated and the data were expressed only as efficacy (% inhibition relative to desipramine 10^{-5} M); for these compounds, the concentrations from which the efficacy values were derived are indicated in Table 1 or in the relevant figures. Relative potencies of compounds were calculated as follows: relative potency = (IC_{50} drug A/IC_{50} drug B) − 1

It is necessary to subtract '1' from the quotient to take account of the fact that equipotent compounds have equal IC_{50} values. IC_{50} values were used to examine the effects of structural modifications on the ability of phenylethylamine analogues to inhibit the accumulation of prazosin 10^{-6} M in

Table 1 Effects of selected substitutions on the efficacy of phenylethylamine derivatives and related compounds in inhibiting the uptake of prazosin in GT1-1 GnRH cells

Compound	*Efficacy %*
Absence of alkyl amine	
2,5-Dimethoxyacetophenone 10^{-3} M	0
3,4-Dimethoxyacetophenone 10^{-3} M	5
2,5-Dimethoxybenzaldehyde 10^{-3} M	0
1,4-Dimethoxybenzene 10^{-3} M	0
3,4-Dimethoxyphenylacetic acid 10^{-3} M	3
Presence of α-carboxyl group	
L-Phenylalanine 10^{-3} M	2
L-Tyrosine 10^{-3} M	0
L-DOPA 10^{-3} M	0
L-Serine 10^{-4} M	0
Secondary, tertiary, quaternary and guanyl amines	
HEAT (4-hydroxyphenylethylaminomethyltetralone; secondary) 10^{-3} M	100
Fluoxetine (secondary) 10^{-4} M	100
Verapamil (tertiary) 10^{-4} M	100
MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; tertiary) 10^{-4} M	100
MPP^+ (1-methyl-4-phenylpyridinium; quaternary) 10^{-3} M	14
Bretylium (quaternary) 10^{-4} M	7
Meta-iodo-benzyl-guanidine 10^{-3} M	25
N-guanyltyramine 10^{-3} M	18
Guanethidine 10^{-4} M	8
Phenolic hydroxyl and methoxyl groups	
(−)-Metaraminol (1-[3-hydroxyphenyl]-2-amino-1-propanol) 10^{-3} M	13
(−)-Adrenaline (1-[3-,4-dihydroxyphenyl]-2-methylamino-ethanol 10^{-4} M	14
(−)-Phenylephrine (1-[3-hydroxyphenyl]-2-methylamino-ethanol 10^{-3} M	37
(±)-Normetanephrine (1-[3-methoxy-4-hydroxyphenyl]-ethanolamine 10^{-3} M	14
(−)-Isoprenaline (1-[3,4-dihydroxyphenyl]-2-isopropylamino-ethanol 10^{-4} M	18
Miscellaneous amines and neurotransmitters	
5-Hydroxytryptamine 10^{-4} M	0
L-Tryptophan 10^{-4} M	3
Histamine 10^{-4} M	4
L-Histidine 10^{-4} M	8
Acetylcholine 10^{-4} M	5
Choline 10^{-4} M	9
DL-Vesamicol 10^{-5} M	29
L-Glutamate 10^{-4} M	0
L-Aspartate 10^{-4} M	0
Glycine 10^{-4} M	0
GABA 10^{-4} M	0
ATP 10^{-4} M	20
Adenosine 10^{-3} M	17
Cocaine 10^{-4} M	100
Phenoxybenzamine 10^{-4} M	22

GT1-1 GnRH cells. The mathematical basis for using IC_{50} values for such purposes has been described by De Lean *et al.* (1978).

Each experimental point was carried out in triplicate and each experiment was replicated at least once; the minimum number of estimations for each experimental point was therefore six. The data are expressed as the means ± s.e.mean. S.e.mean are not shown where they are smaller than the sizes of the symbols.

Materials

Immortalized GnRH neuronal cells (GT1-1 cells; Mellon *et al.*, 1990) were generously provided by Dr R.I. Weiner. Heat-inactivated foetal bovine serum (FBS) was from Life Technologies (Gaithersburg, Maryland or Paisley, Scotland). Culture media were from Sigma-Aldrich (St. Louis, Missouri or Poole, Dorset). [^{3}H]-prazosin was from Amersham (TRK.843; specific activity 74 – 83 Ci mmol^{-1}). Unlabelled compounds were from Sigma-Aldrich, Research Biochemicals International (Natick, Massachusetts or St Albans, Hertfordshire) or Tocris-Cookson (Bristol, Avon).

Results

The amine group

Absence of an alkyl amine abolished the ability of phenylethylamines to inhibit the uptake of prazosin 10^{-6} M. Thus, phenylethanolamine was fully active in inhibiting the uptake of prazosin (efficacy 99%; IC_{50} 54×10^{-6} M) whereas its analogue phenylethylalcohol was inactive (efficacy 4%; Figure 2). Table 1 lists a series of phenylalkyl compounds which lack an alkyl amine, all of which were essentially inactive in inhibiting the accumulation of prazosin 10^{-6} M.

Presence of a carboxyl group on the α carbon abolished the ability of phenylethylamines to inhibit the uptake of prazosin; phenylethylamine (efficacy 100%; IC_{50} 16×10^{-6} M) and tyramine (efficacy 100%; IC_{50} 800×10^{-6} M) were fully active whereas their respective carboxylated analogues phenylalanine (efficacy 2%) and tyrosine (efficacy 0%) were inactive (Figure 2 and Table 1). Table 1 lists other carboxylated compounds which were inactive at inhibiting the accumulation of prazosin.

Presence of an aminomethyl group slightly reduced potency (Figure 3). Thus, amphetamine (IC_{50} 6×10^{-6} M) was 1.5 fold

more potent than methylamphetamine (IC_{50} 15×10^{-6} M), and norephedrine (IC_{50} 37×10^{-6} M) was 0.2 fold more potent than ephedrine (IC_{50} 43×10^{-6} M). However, these secondary amines retained full efficacy in inhibiting the uptake of prazosin, as did tertiary amines (Table 1). In contrast, quaternary amines and guanidines were inactive. Thus, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a tertiary amine, was fully active (efficacy 100%; IC_{50} 16×10^{-6} M), in contrast to its quaternary amine analogue 1-methyl-4-phenylpyridinium (MPP^+; efficacy 14%; Figure 2). Similarly, tyramine (efficacy 100%; IC_{50} 800×10^{-6} M) was fully active in contrast to N-guanyl-tyramine (efficacy 18%; Figure 2). Table 1 lists further quaternary and guanyl-amines which were inactive in inhibiting the uptake of prazosin 10^{-6} M.

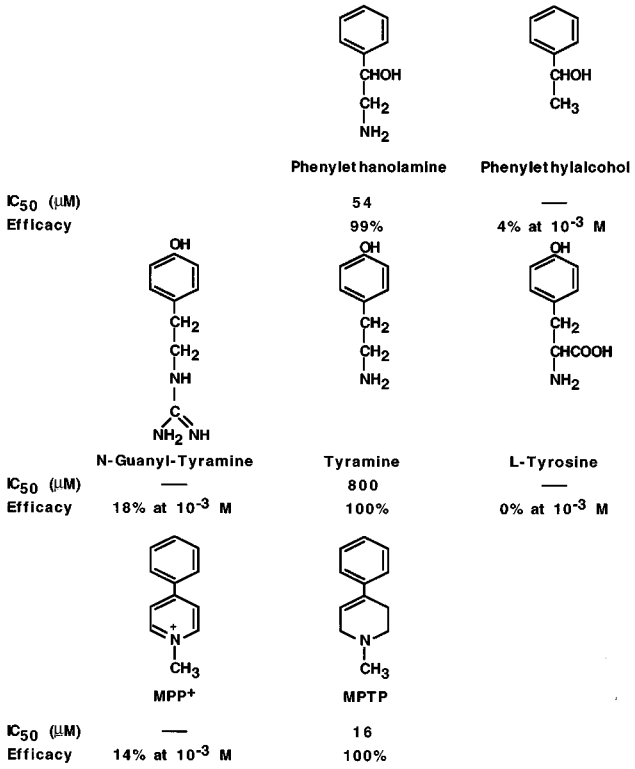


Figure 2 Effects of substitutions of the alkyl amine on the potency and efficacy of phenylethylamine derivatives at inhibition of the uptake of prazosin 10^{-6} M in immortalized peptidergic neurones (GT1-1 GnRH cells). In this and in subsequent Figures, potency was expressed as IC_{50} and efficacy was defined as % inhibition of the uptake of prazosin 10^{-6} M, relative to desipramine 10^{-5} M. IC_{50} values were only calculated for compounds which achieved a maximal inhibitory effect, equal to the effect of desipramine 10^{-5} M.

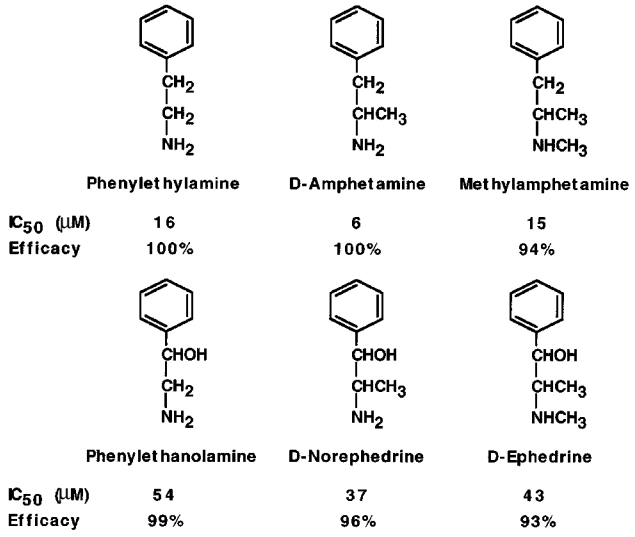


Figure 3 Effects of amino-methyl, α-methyl and β-hydroxyl groups on the potency and efficacy of phenylethylamine derivatives at inhibition of the uptake of prazosin 10^{-6} M in immortalized peptidergic neurones.

The alkyl side chain, α methyl and β hydroxyl groups

Aniline was essentially inactive in inhibiting the uptake of prazosin 10^{-6} M but lengthening the alkyl side chain progressively increased potency (Figure 4).

Presence of a methyl group on the α carbon enhanced potency at transport-P (Figure 3). This effect was observed in the following series of compounds: amphetamine (IC_{50} 6×10^{-6} M) was 1.7 fold more potent than phenylethylamine (IC_{50} 16×10^{-6} M); norephedrine (IC_{50} 37×10^{-6} M) was 0.5 fold more potent than phenylethanolamine (IC_{50} 54×10^{-6} M).

Presence of a hydroxyl group on the β carbon reduced potency at transport-P (Figure 3). This effect was observed in the following series of compounds: phenylethylamine (IC_{50} 16×10^{-6} M) was 2.4 fold more potent than phenylethanolamine (IC_{50} 54×10^{-6} M); amphetamine (IC_{50} 6×10^{-6} M) was

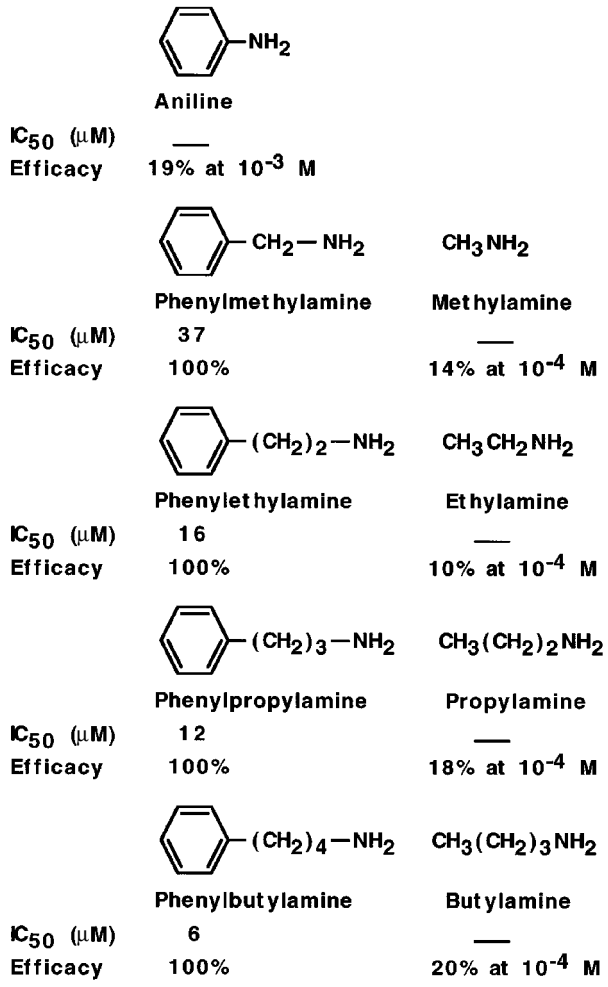


Figure 4 Effects of the phenyl group and length of the alkyl side chain on the potency and efficacy of phenylethylamine derivatives at inhibition of the uptake of prazosin 10^{-6} M in immortalized peptidergic neurones.

5.2 fold more potent than norephedrine (IC_{50} 37×10^{-6} M); and methylamphetamine (IC_{50} 15×10^{-6} M) was 1.9 fold more potent than ephedrine (IC_{50} 43×10^{-6} M). Further, tyramine was more potent than octopamine (efficacy 100% vs 21%) and dopamine was more potent than noradrenaline (efficacy 41% vs 28%; Figure 5). The combined effect of an α methyl group and absence of a β-hydroxyl group increased potency 8 fold (amphetamine IC_{50} 6×10^{-6} M vs phenylethanolamine IC_{50} 54×10^{-6} M; Figure 3).

R-(−)-Amphetamine was equipotent to **S**-(+)-amphetamine (IC_{50} 6.7×10^{-6} M and 6×10^{-6} M, respectively). 1**R**,2**S**-(−)-Ephedrine was equipotent with 1**S**,2**R**-(+)-ephedrine (IC_{50} 3.4×10^{-5} M and 4.3×10^{-5} M, respectively). 1**R**,2**S**-(−)-Norephedrine was equipotent with 1**S**,2**R**-(+)-norephedrine (IC_{50} 4.0×10^{-5} M and 3.7×10^{-5} M, respectively).

The phenyl group

Absence of the phenyl group abolished the ability of phenylalkylamines to inhibit the uptake of prazosin (Figure 4). Thus, methylamine, ethylamine, propylamine and butylamine (efficacy 14%, 10%, 18% and 20%, respectively) were essentially inactive in comparison to their respective phenylalkyl analogues (phenylmethylamine efficacy 100%, IC_{50} 37×10^{-6} M; phenylethylamine efficacy 100%, IC_{50} 16×10^{-6} M; phenylpropylamine efficacy 100%, IC_{50} 12×10^{-6} M; phenylbutylamine efficacy 100%, IC_{50} 6×10^{-6} M).

Phenolic hydroxyl, halogen and methoxyl groups

Presence of a single phenolic hydroxyl group in the *para* position strongly reduced potency at transport-P (Figure 5). Thus, phenylethylamine (IC_{50} 16×10^{-6} M) was 49 fold more potent than tyramine (IC_{50} 800×10^{-6} M) in inhibiting the uptake of prazosin. Presence of a second phenolic hydroxyl group in the *meta* position further reduced potency at transport-P (dopamine efficacy 41%). The effect of the phenolic *para* hydroxyl group was also seen in the following series of compounds: phenylethanolamine and octopamine; norephedrine and α-methyl-octopamine (Figure 5).

Phenolic chlorine atoms increased potency (Figure 6). Thus, 2,4-dichlorophenylethylamine (IC_{50} 4×10^{-6} M) was 3 fold more potent than phenylethylamine (IC_{50} 16×10^{-6} M); 3,4-dichloro-phenylethanolamine (IC_{50} 4×10^{-6} M was 12.5 fold more potent than phenylethanolamine (IC_{50} 54×10^{-6} M); 3,4-dichloromethylamphetamine (IC_{50} 3×10^{-6} M) was 3.7 fold more potent than methylamphetamine (IC_{50} 14×10^{-6} M). Substitution of chlorine atoms with hydroxyl groups in the same positions reduced potency (3,4-dichloro-phenylethanolamine efficacy 100% vs noradrenaline efficacy 28%).

Analogues of phenylethylamine which possessed one phenolic methoxyl group were equipotent with the parent compound, regardless of whether the methoxyl group was in the *ortho*, *meta* or *para* position (Figure 7). Further, methoxyphenamine was equipotent with methylamphetamine (Figure 7). Of the three compounds which possessed a dimethoxyphenyl group, 2,5-dimethoxyphenylethylamine was equipotent with phenylethylamine but 3,4-dimethoxyphenylethylamine (IC_{50} 69×10^{-6} M) was 3.3 fold less potent than phenylethylamine (IC_{50} 16×10^{-6} M) and methoxamine (IC_{50}

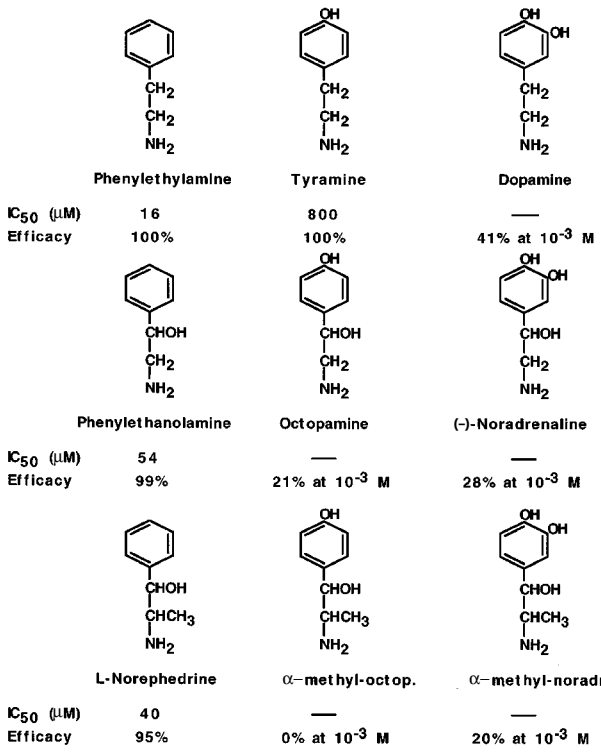


Figure 5 Effects of phenolic-hydroxyl and β-hydroxyl groups on the potency and efficacy of phenylethylamine derivatives at inhibition of the uptake of prazosin 10^{-6} M in immortalized peptidergic neurones.

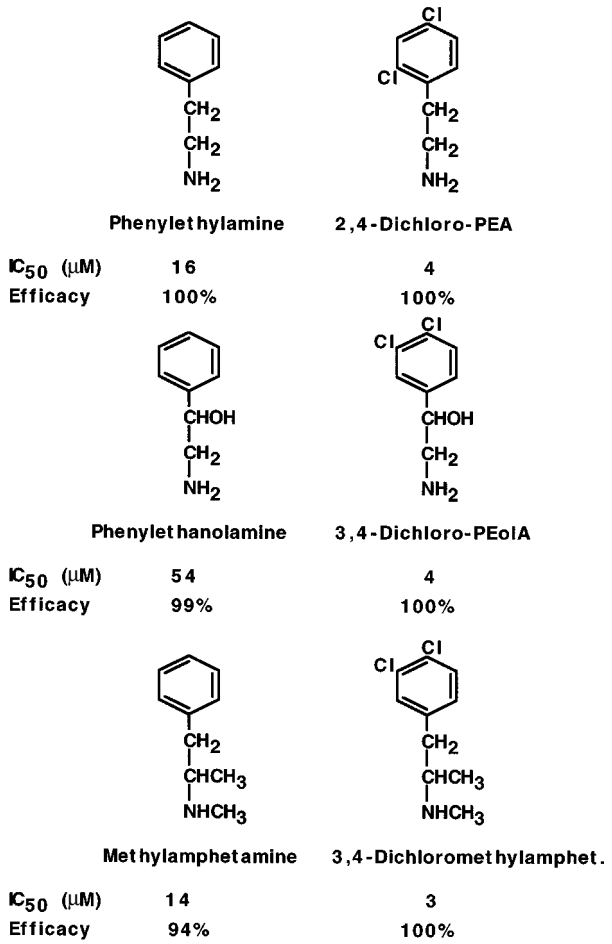


Figure 6 Effects of phenolic chlorine substitutions on the potency and efficacy of phenylethylamine derivatives at inhibition of the uptake of prazosin 10^{-6} M in immortalized peptidergic neurones.

68×10^{-6} M) was 0.7 fold less potent than norephedrine (IC_{50} 40×10^{-6} M; Figure 7).

Figure 8 shows that in four separate experiments, increasing the concentration of unlabelled prazosin in the range 10^{-6} to 3×10^{-6} M caused the paradoxical increase in accumulation of [^{3}H]-prazosin. Phenylethylamine, methylamphetamine, 3,4-dichloromethylamphetamine and 2,5-dimethoxyphenylethylamine represent the different series of compounds which inhibited the uptake of prazosin in this study. The inhibitory effects of these four compounds could be reversed by increasing the concentration of unlabelled prazosin (Figure 8), indicating that they are competitive inhibitors of transport-P.

Discussion

We have provided evidence for a novel uptake process for amines. The anatomical location in postsynaptic (peptidergic) neurones and the functional properties distinguished transport-P from other amine uptake processes. We now define the structural properties of phenylethylamines which interact with transport-P. The essential structure is a hydrophobic phenyl ring and an amine which is separated from the phenyl ring by a carbon chain. These properties are different from those of phenylethylamines which interact with other amine transporters and receptors.

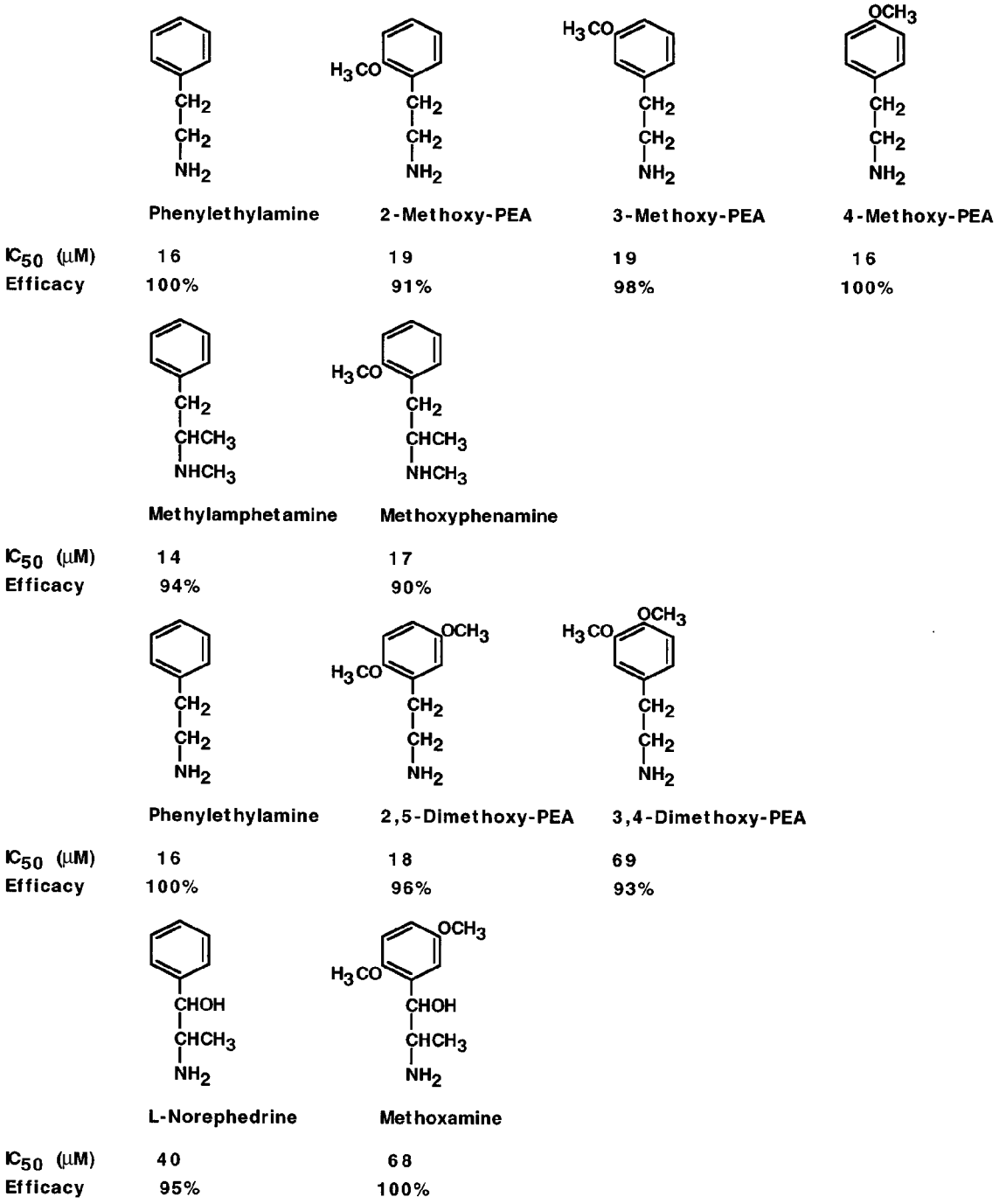


Figure 7 Effects of phenolic methoxyl substitutions on the potency and efficacy of phenylethylamine derivatives at inhibition of the uptake of prazosin 10^{-6} M in immortalized peptidergic neurones.

There is an absolute requirement for an amine in the side chain; absence of the amine or neutralization by a nearby carboxyl group on the α carbon abolished activity (Figure 2 and Table 1). An amino-methyl group slightly reduced potency at transport-P (Figure 3). Despite the lower potency of these secondary amines, it is clear that secondary and tertiary amines are fully active at transport-P (Table 1). However, quaternary and guanyl amines are inactive (Figure 2 and Table 1). At pH 7.4, the amine exists in a protonated form (Maxwell *et al.*, 1970; Lentzen & Philippu, 1981) and this presumably enables interaction with a negatively charged group in the transport-P site, allowing entry into the cells. An amino-methyl group causes steric hindrance which may reduce potency. It is possible that the permanent positive charge in quaternary and guanyl amines may prohibit interaction with a strongly hydrophobic residue in the transport-P site. Amino-methyl groups also reduced the affinity of phenylethylamines for the presynaptic plasma membrane dopamine and noradrenaline $Uptake_1$ sites but they enhanced affinity for noradrenaline $Uptake_2$ and for α_1- and α_2-adrenoceptors (Table 2; Burgen & Iversen, 1965; Grohman & Trendelenburg, 1984; Horn, 1973; Ruffolo *et al.*, 1988; Nichols & Ruffolo, 1991). Amino methyl groups had no effect on affinity for the vesicular uptake process in rat brain or in bovine adrenal medulla (Slotkin & Kirshner, 1971; Slotkin *et al.*, 1979; Peter *et al.*, 1994).

Potency was reduced by a β-hydroxyl group and increased by an α-methyl group (Figures 3 and 5). These substitutions alter the molecular conformation of the alkylamine side chain; the presence of a β-hydroxyl group exerts an electrostatic pull on the positively charged amine, resulting in preponderance of a conformational form in which the amine is folded towards the β-hydroxyl group (Pullman *et al.*, 1972; Ison *et al.*, 1973). Conversely, the presence of an α-methyl group causes steric hindrance which reduces the likelihood of such folding (Ison *et al.*, 1973). Phenolic hydroxyl groups exert no significant electrostatic effect on the rotational conformation of the

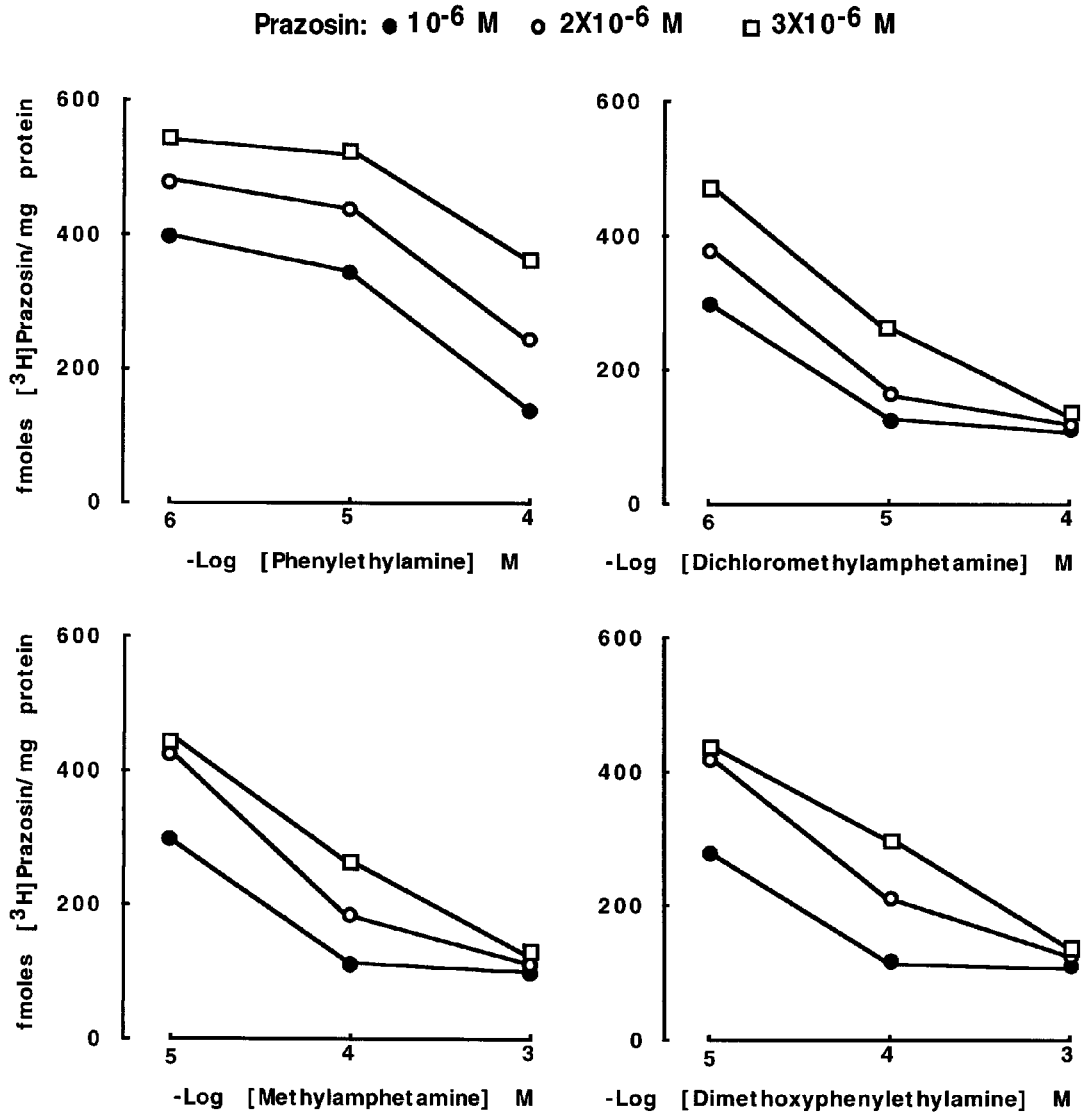
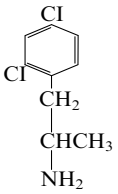
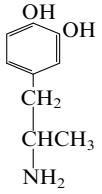
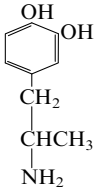
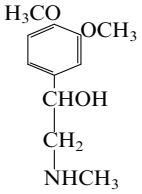
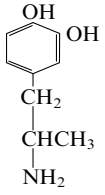
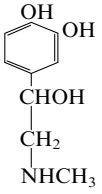
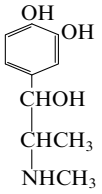


Figure 8 Reversal of the inhibitory effect of phenylethylamine derivatives by increasing concentrations of unlabelled prazosin; indicating that the effects of the phenylethylamines are competitive. The points do not fall on straight lines because they form sigmoidal concentration-response curves in these log-linear plots (see Figure 1). Note that the vertical axis shows only the amount of [^{3}H]-prazosin mg^{-1} protein accumulated in the cells; this demonstrates that increasing concentrations of unlabelled prazosin cause the paradoxical increase in accumulation of [^{3}H]-prazosin in the peptidergic neurones.

Table 2 Comparison of transport-P to other amine transporters and α-adrenoceptors

Substitution	*Transport-P*	*NA Uptake-1*	*Vesicular*	*Uptake-2*	*Dopamine*	*α_1-AR*	*α_2-AR*
Phenolic OH^-	Inhibit	Enhance	Enhance	Inhibit	Enhance	Enhance	Enhance
Phenolic OCH_3	No effect	Inhibit	Inhibit	Enhance	Inhibit	Enhance	No effect
β-OH^-	Inhibit	Inhibit	Inhibit	Enhance	Inhibit	Enhance	Enhance
configuration	Equal	R-(−)	R-(−)	R-(−)	Equal	R-(−)	R-(−)
α-Methyl	Enhance	Enhance	Enhance	Inhibit	Enhance	Inhibit	Enhance
configuration	Equal	Equal	S-(+)		S-(+)	Equal	S-(+)
Amino methyl	Inihbit	Inhibit	No effect	Enhance	Inhibit	Enhance	Enhance
Expected optimum substitutions from available data							

amine (Pullman *et al*., 1972; Ison *et al*., 1973). The enhancement of potency by an α-methyl group and reduction by a β-hydroxyl group suggest that folding of the side chain does not favour interaction with the transport-P site, which presumably favours a conformation in which the side chain is fully extended away from the phenyl group. In support of this suggestion, shortening the side chain progressively reduced potency whereas lengthening the side chain increased potency at transport-P (Figure 4).

β-Hydroxyl and α-methyl groups influence the potencies of phenylethylamines at transport-P in a similar manner to their effects on the affinities of these compounds for the presynaptic plasma membrane transporters for dopamine and noradrenaline $Uptake_1$ and the vesicular transporters in brain and adrenal medulla (Table 2; Burgen & Iversen, 1965; Horn, 1973; Slotkin *et al*., 1975; 1979; Pacholczyk *et al*., 1991; Giros *et al*., 1994). Studies which used rigid analogues confirmed that the side chain of phenylethylamines is in a fully extended conformation when these compounds interact with the presynaptic plasma membrane dopamine and noradrenaline $Uptake_1$ sites (Horn & Snyder, 1972; Miller *et al*., 1973; Horn, 1974). In contrast, a β-hydroxyl group is essential for agonist activity at α_1- and α_2-adrenoceptors (Table 2; Ruffolo *et al*., 1988; Nichols & Ruffolo, 1991). However, an α-methyl group enhances affinity of phenylethylamines for α_2-adrenoceptors but reduces affinity for α_1-adrenoceptors (Table 2; Ruffolo *et al*., 1988; Nichols & Ruffolo, 1991). These receptors presumably require different conformations of the side chain for maximal agonist binding (DeMarinis *et al*., 1981). As in the case of α_1-adrenoceptors, affinity for $Uptake_2$ is enhanced by a β-hydroxyl group and reduced by an α-methyl group (Table 2; Burgen & Iversen, 1965; Grohmann & Trendelenburg, 1984).

Amphetamine possesses a single chiral centre around the α-carbon whereas ephedrine and norephedrine possess two chiral centres around the α- and β-carbons. The equipotent effects of these three sets of enantiomers suggest that transport-P may not distinguish between stereochemical arrangements of a methyl group at the α-carbon or a hydroxyl group at the β-carbon of phenylethylamines. The presynaptic plasma membrane dopamine transporter in rat brain recognizes asymmetry of a methyl group on the α-carbon but does not distinguish asymmetry of a hydroxyl group on the β-carbon (Iversen *et al*., 1971; Ferris *et al*., 1972; Harris & Baldessarini, 1973; Thornburg & Moore, 1973; Koe, 1976; Meiergerd & Schenk, 1994; Giros *et al*., 1994). In contrast, α_1-adrenoceptors, the presynaptic plasma membrane noradrenaline transporter and $Uptake_2$ distinguish asymmetry of a hydroxyl group at the β-carbon but not a methyl group at the α-carbon (Table 2; Iversen *et al*., 1971; Ferris *et al*., 1972; Grohman & Trendelenburg, 1984; Bryan & O'Donnell, 1984; Ruffolo *et al*., 1988). α_2-Adrenoceptors and presynaptic vesicular monoamine transporters distinguish asymmetry of both hydroxyl group at the β-carbon and a methyl group at the α-carbon (Table 2; Slotkin *et al*., 1979; Ferris & Tang, 1979; Ruffolo *et al*., 1988; Peter *et al*., 1994). Hydroxyl and methyl groups are small entities; it is possible that larger substitutions at the α- or β-carbons may be recognized stereospecifically by transport-P.

A phenyl group was essential for activity at transport-P; alkyl amines which lacked a phenyl group were inactive in comparison to their phenyl alkyl analogues (Figure 4). Phenolic hydroxyl groups reduced potency (Figure 5), suggesting that the phenyl group should be hydrophobic for optimum activity at transport-P. This suggestion is strengthened by the finding that hydrophobic chlorine atoms in the phenyl ring increased potency (Figure 6). Substitution of chlorine atoms with hydroxyl groups in the same positions reduced potency, suggesting that the enhancing effect of chlorine is unlikely to be due to an electronegative effect, but is more likely due to the hydrophobic nature of chlorine. These surprising findings are in striking contrast to the structural properties of phenylethylamines which bind α_1- and α_2-adrenoceptors, where phenolic hydroxyl groups strongly increased affinity (Table 2; Ruffolo *et al*., 1988; Nichols & Ruffolo, 1991). Phenolic hydroxyl groups also increased the affinity of phenylethylamines for presynaptic plasma membrane dopamine and noradrenaline $Uptake_1$ transporters, and for vesicular transporters in rat brain and adrenal medulla, although their effects on these transporters were less prominent than on α_1- and α_2-adrenoceptors (Table 2; Burgen & Iversen, 1965; Horn, 1973; Slotkin *et al*., 1975; 1979; Peter *et al*., 1994). These findings explain our previous observation that very small amounts of noradrenaline accumulated in hypothalamic cells (fmol noradrenaline mg^{-1} protein vs pmol prazosin mg^{-1} protein; Al-Damluji *et al*., 1993); potency at transport-P is reduced by the phenolic and β-hydroxyl groups in noradrenaline (Figure 5).

A single phenolic methoxyl group had no effect on potency at transport-P (Figure 7). The lack of effect of these neutral groups is compatible with the suggestion that the phenyl ring of these compounds should be hydrophobic for optimum activity at transport-P. In clear contrast, a single phenolic methoxyl group reduced the affinity of phenylethylamines for the presynaptic plasma membrane dopamine and noradrenaline $Uptake_1$ sites (Table 2; Burgen & Iversen, 1965; Horn,

1973). Phenolic methoxyl groups enhanced the affinity of phenylethylamines for noradrenaline $Uptake_2$ (Burgen & Iversen, 1965; Grohman & Trendelenburg, 1984) and for α_1-adrenoceptors (DeMarinis *et al*., 1981), but they had no effect on the affinity of these compounds for α_2-adrenoceptors (Table 2; Ruffolo *et al*., 1988).

The conclusions of this study are based on the potencies of compounds at transport-P, as indicated by inhibition of the uptake of prazosin. Inhibition of uptake does not necessarily indicate that these compounds are themselves internalized by the uptake process; that would require direct measurement of the accumulation of radioactively labelled compounds in the cells. We therefore studied the accumulation of [^{3}H]-verapamil, which is a phenylethylamine derivative which possesses the structural properties which enable interaction with transport-P, as identified in this study. [^{3}H]-verapamil was internalized by GnRH cells in a similar manner to prazosin (Al-Damluji, 1996). Further, phenylethylamine and its derivatives inhibited the uptake of prazosin competitively (Figure 8), suggesting that these compounds and prazosin act on the same transport-P carrier molecule in GnRH neurones. It therefore seems likely that the structural properties which were described in this study may define some of the requirements for interaction of phenylethylamines with the transport-P carrier molecule in peptidergic neurones. An alternative explanation is that these amines may have inhibited the uptake of prazosin by dint of their lipophilic nature, which may have enabled them to diffuse across cell membranes, resulting in neutralization of the acidified intracellular compartment in which prazosin is accumulated. However, this seems an unlikely explanation as uptake of prazosin was unaffected by some highly lipophilic amines, including reserpine, phenoxybenzamine and vesamicol (Al-Damluji & Kopin, 1996a; Table 1). At present, the most likely explanation for the findings is that these compounds compete with prazosin for binding to a carrier molecule in peptidergic neurones.

We found previously that transport-P resembled presynaptic vesicular monoamine uptake in that it required an electrochemical proton gradient and was linked to V-ATPase. However, transport-P differed from vesicular transporters in that it was insensitive to reserpine (Al-Damluji & Kopin, 1996a). The present work has identified further differences between transport-P and presynaptic vesicular amine uptake. The most prominent differences are: (1) a phenolic *para* hydroxyl group enhanced affinity for vesicular uptake in rat brain (Slotkin *et al*., 1979; Lentzen & Philippu, 1981), but reduced affinity for transport-P (Figure 5). (2) Transport-P is more selective in its substrate spectrum than vesicular monoamine transporters; transport-P does not accumulate 5-hydroxytryptamine (5-HT) or histamine (Table 1), whereas these two amines are accumulated by the presynaptic vesicular monoamine transporter in rat brain (Slotkin *et al*., 1979; Peter *et al*., 1994; Merickel & Edwards, 1995). (3) Cocaine inhibits transport-P (Table 1) but has no effect on the vesicular monoamine transporter (Liu *et al*., 1992). Transport-P is unaffected by acetylcholine or by vesamicol (Table 1), which inhibits the presynaptic vesicular acetylcholine transporter (Liu & Edwards, 1997).

Prazosin is a substrate for $Uptake_2$ in non-neuronal cells (Grohmann & Trendelenburg, 1984). However, $Uptake_2$ differs from transport-P in that it is independent of the electrochemical gradient of protons (Schomig *et al*., 1992) and is inhibitable by steroid hormones (Salt, 1972). The present study has identified further differences between transport-P and $Uptake_2$; (1) phenolic methoxyl groups increased affinity for $Uptake_2$ (Burgen & Iversen, 1965; Grohmann & Trendelenburg, 1984) but had no consistent effect on potency at transport-P (Figure 7). (2) Affinity for $Uptake_2$ was increased by a β-hydroxyl group and reduced by an α-methyl group (Burgen & Iversen, 1965; Grohmann & Trendelenburg, 1984), whereas these substitutions had the opposite effect on potency at transport-P (Figures 3 and 5). (3) $Uptake_2$ accumulates a wide range of compounds, including 5-HT, histamine and isoprenaline (Grohmann & Trendelenburg, 1984) which do not interact with transport-P (Table 1), indicating that transport-P is more selective in its substrate spectrum than $Uptake_2$. Thus, transport-P differs from $Uptake_2$ in its neuronal location (Al-Damluji *et al*., 1997), functional properties (Al-Damluji & Kopin, 1996a) and in the structure of its ligands.

This work has defined some of the structural properties of molecules which are capable of interacting with transport-P. These properties can be clearly distinguished from those for interaction with other amine transporters and adrenoceptors.

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