The interactions of χ-conopeptide MrIA with the human norepinephrine transporter (hNET) were investigated by determining the effects of hNET point mutations on the inhibitory potency of MrIA. The mutants were produced by site-directed mutagenesis and expressed in COS-7 cells. The potency of MrIA was greater for inhibition of uptake by hNET of [3H]norepinephrine (Ki, 1.89 μM) than [3H]dopamine (Ki, 4.33 μM), and the human dopamine transporter and serotonin transporter were not inhibited by MrIA (to 7 μM). Of 18 mutations where hNET amino acid residues were exchanged with those of the human dopamine transporter, MrIA had increased potency for inhibition of [3H]norepinephrine uptake for three mutations (in predicted extracellular loops 3 and 4 and transmembrane domain (TMD) 8) and decreased potency for one mutation (in TMD6 and intracellular loop (IL) 3). Of the 12 additional mutations in TMDs 2, 4, 5, and 11 and IL1, three mutations (in TMD2 and IL1) had reduced MrIA inhibitory potency. All of the other mutations tested had no influence on MrIA potency. A comparison of the results with previous data for desipramine and cocaine inhibition of norepinephrine uptake for the mutant hNETs reveals that MrIA binding to hNET occurs at a site that is distinct from but overlaps with the binding sites for tricyclic antidepressants and cocaine.

The norepinephrine transporter (NET) terminates neurotransmission by rapid and specific uptake of norepinephrine into presynaptic nerve terminals (for review see Refs. 1 and 2). Tricyclic antidepressants are competitive inhibitors of the NET, and this property contributes to their efficacy in the treatment of conditions such as depression (3). Studies on chimeras between the human NET (hNET) and the highly homologous dopamine transporter (DAT) have provided evidence that the sites involved in the selective inhibition by the tricyclic antidepressants of NET relative to DAT involves the region spanning transmembrane domains (TMDs) 6–8 of the putative 12 TMD topology predicted for these transporters (4, 5). More recently, interactions of tricyclic antidepressants with the NET have been further investigated in a study in which point mutations were introduced into the region spanning TMDs 6–8 of hNET by replacing amino acids in hNET with those found in human DAT (hDAT) (6). The latter study (6) identified that key hNET amino acid residues responsible for the inhibitory effects of tricyclic antidepressants are contained within TMDs 6, 7, and 8 and not in the adjacent intracellular loops (ILs) or extracellular loops (ELs) (6). It was also shown that the identified sites are not important determinants of substrate transport (6). However, TMDs 6–8 are not the only regions important for determining tricyclic antidepressant interactions with the NET. Point mutations of other amino acids in TMDs 2 and 5 and in the first IL of the hNET have also been shown to cause reductions in the affinities for the NET of tricyclic antidepressants, and in some cases, of cocaine (7–9).

Conotoxins are highly structured venom peptides that have proved to be valuable tools for defining the architecture of membrane-bound proteins including sodium channels (10), calcium channels (11), and potassium channels (12). Recently, the χ-conopeptide MrIA, isolated from Conus marmoreus, has been identified to be a non-competitive inhibitor of [3H]norepinephrine uptake by the NET (13) but a competitive inhibitor of [3H]mazindol and [3H]nisoxetine binding to both rat and human NET (26). This activity contrasts with the competitive inhibition of NET transport of norepinephrine by tricyclic antidepressants. In the present study, we investigate MrIA binding to hNET by examining the influence on the inhibitory potency of MrIA of point mutations in regions that have been proposed to be involved in determining the affinity of the tricyclic antidepressants for the hNET (see Fig. 1) (6). The results of this study have implications for NET structure/function relationships, which are presently poorly defined.

** Materials and Methods **

Chemicals—(−) [ring-2,5,6-3H]Norepinephrine (NET-678, 1917 Bq/pmol), [ring-2,5,6,3H]dopamine (NET-673, 2190 Bq/pmol), and [1,2,3H(N)]-5-hydroxytryptamine creatinine sulfate ([1H]5-HT, NET-498, 888 Bq/pmol) were obtained from PerkinElmer Life Sciences. The χ-conopeptide MrIA (supplied by Xenome Ltd, Brisbane, Queensland, Australia) was obtained by solid phase peptide synthesis and purified by reversed-phase high pressure liquid chromatography to >95% purity as previously described (13). The drugs used in the study were as follows: 1-2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine; h, human (with transporter abbreviation); 5-HT, 5-hydroxytryptamine; IL, intracellular loop; SERT, serotonin transporter; TMD, transmembrane domain; U-0521, 3',4'-dihydroxy-2-methylpropiophenone; ANOVA, analysis of variance.

* This research was supported by grants from the National Health and Medical Research Council of Australia. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: NET, norepinephrine transporter; DAT, dopamine transporter; DMI, desipramine; EL, extracellular loop; GBR12909, 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine; h, human (with transporter abbreviation); 5-HT, 5-hydroxytryptamine; IL, intracellular loop; SERT, serotonin transporter; TMD, transmembrane domain; U-0521, 3',4'-dihydroxy-2-methylpropiophenone; ANOVA, analysis of variance.
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FIG. 1. Topological map of the hNET and the mutants used in this study. Extracellular amino acid residues are above the horizontal lines representing the cell membrane, and those below are intracellular residues with TMDs 1–12 between the lines. The letters in the circles show the amino acid sequence (by one-letter code) of the hNET (25). The N and C termini are shown as NH\(_2\) and COOH, respectively. The numbers shown (where possible) are the predicted amino acid positions of the first and last residues of each IL and EL based on hydrophobicity plots and sequence alignment of the monoamine transporters (14, 25). Mutants investigated in this study are indicated by filled symbols and the accompanying labels. Mutations that resulted in an increase (▲) or decrease (▼) in MrIA potency for inhibition of hNET are indicated (see also Table I).

**Uptake Assays**—Culture medium was removed from the transiently transfected COS-7 cells, and they were then washed twice with 1 ml of Krebs/HEPES buffer containing 0.1% bovine serum albumin at 37 °C. The cells were incubated for 60 min at 37 °C with 0.5 ml of Krebs/HEPES buffer containing 0.1% bovine serum albumin in the absence or presence of 0.07–21.3 \( \mu \)M MrIA and inhibitors to determine nonspecific uptake (1 or 10 \( \mu \)M nisoxetine for wild type and mutant hNETs, 1 \( \mu \)M GBR12909 for hDAT, or 1 \( \mu \)M paroxetine for hSERT). \[^{[3H]}\]Norepinephrine, \[^{[3H]}\]dopamine, or \[^{[3H]}\]5-HT (10 nM) was then added for 2 min. The cells were washed three times with Krebs/HEPES buffer at 0 °C and lysed (750 \( \mu \)l of 0.1% Triton X-100 in 10 ml Tris-HCl, pH 7.5, at 37 °C for 60 min). Determination of the protein content by the Lowry method (17) was carried out on duplicate samples of 75 \( \mu \)l of the lysate was determined by the addition of 1.5 ml of Scintiscint scintillation medium (Packard, Melbourne, Victoria, Australia) and liquid scintillation counting. Samples (50 \( \mu \)l) of the \[^{[3H]}\] solutions were taken to determine the specific activity of the incubation solutions.

**Data Analysis**—\[^{[3H]}\]Amine uptake was calculated using the results of the Lowry protein determination and the liquid scintillation counting. Specific uptake of \[^{[3H]}\]amine was calculated as the difference between uptake in the absence (total uptake) and presence (nonspecific uptake) of 500 \( \mu \)l of the lysate was determined by the addition of 1.5 ml of Scintiscint scintillation medium (Packard, Melbourne, Victoria, Australia) and liquid scintillation counting. Samples (50 \( \mu \)l) of the \[^{[3H]}\] solutions were taken to determine the specific activity of the incubation solutions.

**Cell Culture and Transfection**—COS-7 (SV40-transformed African Green monkey kidney cells, American Type Culture Collection, Manassas, VA) were grown at 37 °C in a 5% \( \text{CO}_2 \) humidified atmosphere on standard plastic cultureware in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and 100 \( \mu \)g/ml streptomycin (Invitrogen) to obtain complete medium. COS-7 cells were subcultured onto 12-well culture plates. The cells were transiently transfected with the cDNA of hNET (donated by Professor S. Amara, Vollum Institute, Portland, OR), hDAT (donated by Professor M. Caron, Duke University, Durham, NC), human serotonin transporter (hSERT, donated by Professor R. Blakely, Vanderbilt University School of Medicine, Nashville, TN), or mutant hNET using LipofectAMINE (Invitrogen) as described previously (15). The mutant hNETs were obtained by site-directed mutagenesis in previous studies in our laboratories (6–9, 16, 18). The experiments were performed 48 h following the transfection.

**Uptake Assays**—Culture medium was removed from the transiently transfected COS-7 cells, and they were then washed twice with 1 ml of Krebs/HEPES buffer containing 0.1% bovine serum albumin at 37 °C. The cells were incubated for 60 min at 37 °C with 0.5 ml of Krebs/HEPES buffer containing 0.1% bovine serum albumin in the absence or presence of 0.07–21.3 \( \mu \)M MrIA and inhibitors to determine nonspecific uptake (1 or 10 \( \mu \)M nisoxetine for wild type and mutant hNETs, 1 \( \mu \)M GBR12909 for hDAT, or 1 \( \mu \)M paroxetine for hSERT). \[^{[3H]}\]Norepinephrine, \[^{[3H]}\]dopamine, or \[^{[3H]}\]5-HT (10 nM) was then added for 2 min. The cells were washed three times with Krebs/HEPES buffer at 0 °C and lysed (750 \( \mu \)l of 0.1% Triton X-100 in 10 ml Tris-HCl, pH 7.5, at 37 °C for 60 min). Determination of the protein content by the Lowry method (17) was carried out on duplicate samples of 75 \( \mu \)l of the lysate was determined by the addition of 1.5 ml of Scintiscint scintillation medium (Packard, Melbourne, Victoria, Australia) and liquid scintillation counting. Samples (50 \( \mu \)l) of the \[^{[3H]}\] solutions were taken to determine the specific activity of the incubation solutions.
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where appropriate) followed by Dunnett’s multiple comparison tests (Prism 3) on absolute or log data or by Student’s t test as indicated under “Results.”

**RESULTS**

**Selective Inhibition of hNET by MrIA—**The uptake of \([^{3}H]\)norepinephrine and \([^{3}H]\)dopamine by hNET was determined in the presence of increasing concentrations of MrIA (Fig. 2). The inhibition curves had Hill slopes that were not significantly different from 1. The \( K_{i} \) value of MrIA obtained from these data with norepinephrine as substrate (1.89 \( \mu \)M, 95% confidence limits: 1.76, 2.02 \( \mu \)M, \( n \) = 5) was significantly less (\( p < 0.001 \)) than that with dopamine as substrate (4.33 \( \mu \)M, 95% confidence limits: 3.55, 5.43 \( \mu \)M, \( n \) = 4). MrIA, over the concentration range that caused up to 80% inhibition of \([^{3}H]\)norepinephrine uptake by hNET, had no effect on \([^{3}H]\)dopamine uptake by hDAT or on \([^{3}H]5\)-HT uptake by hSERT (Fig. 2), confirming the lack of effect of MrIA at these related monoamine transporters.

**Influence of hNET Mutants on Inhibition of Norepinephrine Uptake by MrIA—**COS-7 cells transiently expressing wild type hNET or hNET mutants were screened for inhibition of \([^{3}H]\)norepinephrine uptake by MrIA in three series of experiments. In view of the highly selective inhibition of hNET compared with hDAT by MrIA (Ref. 13 and this study), the first two series of experiments investigated the effects of mutations that resulted in amino acid exchanges of residues of the hNET to those of the hDAT in ELs 3 and 4, IL 3, and TMDs 5–8 (Fig. 3, A and B). Inhibition by MrIA was significantly greater than that of hNET for K303C (EL 3), E377G-E382D-A384P (EL 4), and S399P (TMD 8), less than that of hNET for F316C-D336T (mutations in TMD 6 and IL 3), and was the same as hNET for the other 13 mutations in these regions including the F316C and D336T point mutants and the double mutant S399P-G400L (Fig. 3). In the third series of experiments using a different batch of MrIA, the effects of point mutations to alanine or other amino acids in TMDs 2, 4, 5, and 11 and in IL 1 on the inhibition of \([^{3}H]\)norepinephrine uptake by MrIA were investigated. These studies indicated that there was less inhibition of the mutant than that of wild type hNET by MrIA for L114A and L114S in TMD 2 and L114S in TMD 2 and G123A in IL 1 (Fig. 4). No significant differences from hNET were observed for the remaining 10 mutants examined (Fig. 4).

**Determination of \( K_{i} \), Values of MrIA for hNET Mutants—**The effects of a range of MrIA concentrations on \([^{3}H]\)norepinephrine uptake were determined for hNET and five of the mutants, which showed significantly different inhibition by MrIA from that of hNET (Figs. 3A and 4). \( K_{i} \) values of MrIA were determined from the results shown in Fig. 5 for hNET and the five mutants (Table I), assuming that MrIA is a non-competitive inhibitor of norepinephrine uptake by hNET (13). The Hill slopes of the inhibition curves were not significantly different from 1 except for K303C, which showed a small increase. \( K_{i} \) values of MrIA are also shown in Table I for the two mutants that showed significantly different inhibition by MrIA compared with hNET in Fig. 3B, and these values were calculated from the data in Fig. 3B, assuming a Hill slope of 1. The \( K_{i} \) values of MrIA for inhibition of the seven mutant NETs all

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**Fig. 2. Inhibition of hNET, hDAT, and hSERT by MrIA in COS-7 cells transiently expressing the transporters.** Specific uptake of \([^{3}H]\)amine (10 nM, 37°C, 2 min) was determined as the difference between uptakes in the absence and presence of 10 \( \mu \)M nisoxetine for hNET, 1 \( \mu \)M GBR12909 for hDAT, or 1 \( \mu \)M paroxetine for hSERT in the absence or presence of the appropriate concentration of MrIA. The values of percentage inhibition of specific \([^{3}H]\)amine uptake by MrIA are shown as arithmetic means ± S.E. for hNET with \([^{3}H]\)norepinephrine (\( \bullet \), \( n \) = 5) or \([^{3}H]\)dopamine (○, \( n \) = 4) as substrate, hDAT with \([^{3}H]\)dopamine as substrate (■, \( n \) = 2), and hSERT with \([^{3}H]5\)-HT as substrate (▲, \( n \) = 3) versus concentration of MrIA (on a log scale). Curves for hNET were obtained by non-linear regression analysis using a sigmoidal model.

**Fig. 3. Inhibition of \([^{3}H]\)norepinephrine uptake by MrIA in COS-7 cells transiently expressing hNET or hNET with point mutations in ELs 3 or 4, IL 3, or TMDs 5–8.** Specific uptake of \([^{3}H]\)norepinephrine (10 nM, 37°C, 2 min) was determined as the difference between uptakes in the absence and presence of 10 \( \mu \)M nisoxetine. The values for percentage inhibition of specific norepinephrine uptake by MrIA (1.78 \( \mu \)M for panel A and 2.13 \( \mu \)M for panel B) are shown as arithmetic means ± S.E. (\( n \) = 3). The horizontal lines show the inhibition of hNET by MrIA. The data were analyzed by repeated measures one factor ANOVA (\( p < 0.001 \) in A and \( p < 0.01 \) for B) followed by Dunnett’s multiple comparison tests compared with hNET (*, \( p < 0.05 \); ***, \( p < 0.001 \)).
differed significantly from those for the inhibition of hNET with three mutations in ELs 3 and 4 and TMD 8, causing an increase in MrIA potency, and four mutations in IL 1, TMD 2, and TMD 6–IL 3, causing a decrease in MrIA potency (Table I). In Table II, the effects of the point mutations of hNET on the inhibitory potency of MrIA are compared with their effects on the inhibitory potencies of desipramine (DMI) and cocaine and the apparent affinity of dopamine determined from previous studies in our laboratories.

**DISCUSSION**

The χ-conopeptide MrIA is a non-competitive inhibitor of norepinephrine uptake by NET with greater than 1000-fold selectivity for inhibition of NET compared with DAT and SERT (this study and Ref. 13). In contrast to cocaine and tricyclic antidepressants such as DMI (6, 15), MrIA was more potent as an inhibitor of norepinephrine uptake than as an inhibitor of dopamine uptake by hNET. It appears that the potency of MrIA inhibition of hNET is influenced by the nature of the amine being transported. One possible explanation of these results could be that the binding of MrIA is linked to NET gating, which is more efficiently modulated by norepinephrine than dopamine as indicated, for example, by the higher maximal rate of transport of norepinephrine than dopamine (7, 19, 20). However, the data from the current study do not provide conclusive evidence for this proposal.

To identify where MrIA acts on NET, we examined the effect of exchanging residues that differ between NET and DAT on the potencies of MrIA, DMI, and cocaine but these mutations resulted in differing effects on the apparent affinities of the substrate, dopamine (Table II).

| hNET mutant | n | $K_i$ of MrIA ($\mu M$) | Hill slope |
|-------------|---|-----------------------|-----------|
| hNET        | 8 | 1.72 (1.51, 1.95)      | 0.99 ± 0.04 |
| E377G-E382D-A384P | 3 | 0.77 (0.29, 1.52) | 1.00 ± 0.01 |
| K303C       | 3 | 0.90 (0.72, 1.11)      | 1.21 ± 0.03 |
| S399P       | 3 | 0.96 (0.87, 1.06)      | 1.01 ± 0.13 |
| F316C-D336T | 3 | 2.62 (1.15, 6.92)      | 1.00 ± 0.13 |
| G123A       | 3 | 3.47 (2.23, 5.40)      | 1.30 ± 0.19 |
| L114A       | 3 | 5.05 (3.11, 8.21)      | 0.96 ± 0.08 |
| L114S       | 3 | 16.7 (16.0, 17.5)      | 1.24 ± 0.10 |

$p < 0.001$, for comparison of log $K_i$ values with the hNET control (one factor ANOVA, $p < 0.001$ followed by Dunnett’s multiple comparison tests).

$p < 0.05$, for comparison of Hill slope with 1 (Student’s $t$ test).

A comparison of the influence of 30 mutations of the hNET on MrIA, DMI, and cocaine potencies and on the apparent affinity of dopamine is given in Table II. Of the mutants examined, 23 had no effect on MrIA potency. 11 of these mutants also had no influence on DMI or cocaine potency, and 16 had no influence on the apparent affinity of dopamine, including 12 mutants that did not change the potencies of any of the tested compounds. Of the remaining mutants, only the L114A and L114S mutants in TMD 2 concomitantly reduced (3–10-fold) the potencies of MrIA, DMI, and cocaine but these mutations caused increases in the apparent affinity of dopamine. In con-
E377G-E382D-A384P in EL 4) enhanced MrIA potency by three other mutations (K303C in EL 3, S399P in TMD 8, and cocaine or on the apparent affinity of dopamine. Surprisingly, marked reduction in DMI potency and no effect on that of cocaine or on the apparent affinity of dopamine. The double mutant F316C-D336T in TMD 6/IL 3 in MrIA and cocaine potencies but had no effect on DMI potency, reduced both DMI and cocaine potencies, and in particular interest in that they had no influence on MrIA dopamine apparent affinity. The five TMD 5 mutations were of particular interest in that they had no influence on MrIA potency, reduced both DMI and cocaine potencies, and increased the apparent affinity of dopamine compared with hNET. These results confirm previous findings that the binding sites for substrates, DMI, and cocaine are overlapping but not identical (6, 7, 16) and support the idea that the MrIA binding sites are also not identical to but show some overlap with those for NET substrates and inhibitors.

The non-competitive inhibition of norepinephrine transport by MrIA, which is a polar and positively charged peptide, indicates that MrIA binds to an allosteric binding site located in the extracellular region of the NET and that this binding does not change the affinity of norepinephrine for the NET but reduces its rate of translocation from outside to inside. Two mutants in EL 3 and EL 4 (K303C and E377G-E382D-A384P, respectively) enhanced MrIA binding, suggesting that MrIA binding spans these loops which are within the region between TMD 6 and TMD 8 proposed to be involved in the substrate translocation process (20). Two models have been proposed to explain substrate transport by Na+-dependent monoamine transporters such as the NET. In the “alternating model”, binding of norepinephrine (and the cosubstrates Na+ and Cl−) leads to a conformational change of the NET enabling release of norepinephrine (and the cosubstrates) from the transporter on the inside of the membrane. The empty carrier then turns outward to complete the transport cycle (see Refs. 22 and 23). This model implies that the NET forms a type of channel that is closed on one side and is open on the other side. Recently, an open-channel model for coupled substrate and Na+ transport by the Na+-dependent monoamine neurotransmitter transporters, such as NET and SERT, has been proposed based on the assumption that the transporters form a long narrow pore enabling translocation analogous to single-file diffusion (24). If EL 3 and EL 4 form part of the extracellular section of such a pore, non-competitive inhibition of norepinephrine transport by MrIA could be explained if MrIA occludes the pore of the channel. Leucine 114 in TMD 2, close to its junction with IL 1, also influences MrIA (and DMI and cocaine) potency for inhibition of hNET. If pore occlusion is indeed the mechanism by which MrIA inhibits substrate transport by hNET, the pore needs to be of sufficient size to allow MrIA to enter the pore at a sufficient distance to be influenced by amino acid residues in the intracellular part of this TMD.

The results of this study show that MrIA interacts with hNET mostly at residues that differ from those where DMI and

| Mutant Location | ΔMrIA potency | ΔDMI potency | ΔCocaine potency | ΔDopamine apparent affinity |
|-----------------|---------------|--------------|-----------------|----------------------------|
| E113Q TMD2      | 0             | 0            | 0               | 0                          |
| L114A TMD2      | 2.9          | 2.8          | 5.1             | 1.9                        |
| L114S TMD2      | 9.7          | 5.9          | 8.9             | 2.8                        |
| G117A TMD2      | 0            | 5.5          | 0               | 1.5                        |
| R121G IL1       | 0            | 0            | 0               | 1.3                        |
| G123A IL1       | 2.0          | 2.7          | 3.2             |                            |
| Y248A TMD4      | 0            | 0            | 0               |                            |
| T268A TMD5      | 0            | 2.1          | 2.2             | 6.8                        |
| T268H TMD5      | 0            | 2.8          | 1.9             | 7.8                        |
| Y271A TMD5      | 0            | 1.7          | 3.3             | 1.9                        |
| Y271F TMD5      | 0            | 2.4          | 2.1             | 3.1                        |
| Y271H TMD5      | 0            | 1.7          | 2.5             | 1.7                        |
| H280R TMD5      | 0            | 0            | 0               |                            |
| K303C EL3       | 2.0          | 0            | 0               |                            |
| F316C TMD6      | 0            | 6.5          | 2.2             | 0                          |
| A330S IL3       | 0            | 0            | 0               |                            |
| D336T IL3       | 0            | 0            | 0               |                            |
| I364F TMD7      | 0            | 0            | 0               |                            |
| N375P EL4       | 0            | 0            | 0               |                            |
| S399P TMD8      | 1.8          | 0            | 0               |                            |
| G400L TMD8      | 0            | 3.2          | 0               |                            |
| F403A TMD8      | 0            | 0            | 0               |                            |
| A414T TMD8      | 0            | 0            | 0               |                            |
| F311Y TMD11     | 0            | 0            | 0               |                            |
| F316C-D336T TMD6/IL3 | 1.6      | 28           | Small            |                            |
| S399P-G400L TMD8 | 0            | 2400         | 0               |                            |
| S2881-N288D-N292R EL3 | 0       | 0            | 0               |                            |
| F316C-V356S-G400L TMD6/78 | 0    | 36           | 0               |                            |
| E377G-E382D-A384P EL4 | 2.2      | 0            | 0               |                            |
| H370Q-E371K-K373S-E377G EL4 | 0   | 0            | 1.9             |                            |

Ref. 8.
Ref. 6.
Ref. 16.
Ref. 7.
Ref. 9.
Ref. 6.
cocaine bind. Thus, the competitive inhibition of tricyclic antidepressant binding by MrIA (26) most probably occurs via steric overlap with DMI and MrIA interacting at distinct sites on the NET, but the binding of DMI precludes MrIA access, and vice versa. That MrIA and tricyclic antidepressants have separate binding sites is not unexpected given the differences in the mode of action of these two classes of inhibitor for inhibition of noradrenaline uptake (non-competitive and competitive, respectively). Additional interacting residues may reveal additional regions of overlap among MrIA, DMI, cocaine, and substrate binding sites and perhaps reveal additional structural features that are important for the function of the NET.

Acknowledgments—We thank Marisa Papacostas and Sonja Sucic for excellent technical assistance in this study. MrIA-NH₂ was provided by Xenome Ltd. (Brisbane, Queensland, Australia).

REFERENCES
1. Kitayama, S., and Dohi, T. (1996) Jpn. J. Pharmacol. 72, 195–208
2. Hoffman, B. J., Hansson, S. B., Mezey, E., and Palkovits, M. (1998) Front. Neuroendocrinol. 19, 187–231
3. Bönisch, H., and Bruess, M. (1994) Adv. N. Y. Acad. Sci. 733, 193–202
4. Buck, K. J., and Amara, S. G. (1995) Mol. Pharmacol. 48, 1030–1037
5. Giros, B., Wang, Y.-M., Suter, S., McLeskey, S. B., Pfitz, C., and Caron, M. G. (1994) J. Biol. Chem. 269, 15985–15988
6. Roubert, C., Cox, P. J., Bruess, M., Hamon, M., Bönisch, H., and Giros, B. (2001) J. Biol. Chem. 276, 8254–8260
7. Paczkowski, F. A., and Bryan-Lluaka, L. J. (2001) Brain Res. Mol. Brain Res. 97, 32–42
8. Sucic, S., Paczkowski, F. A., Runkel, F., Bönisch, H., and Bryan-Lluaka, L. J. (2002) J. Neurochem. 81, 344–354
9. Paczkowski, F. A., Sucic, S., and Bryan-Lluaka, L. J. (2001) Proceedings of the Ninth International Catecholamine Symposium, Kyoto, Japan, March 31–April 5, 2001
10. Nielsen, K. J., Watson, M., Adams, D. J., Hammarström, A. K., Gage, P. W., Hill, J. M., Craik, D. J., Thomas, L., Adams, D., Alewood, P. F., and Lewis, R. J. (2000) J. Biol. Chem. 275, 27257–27265
11. Elinor, P. T., Zhang, J. F., Horne, W. A., and Tsien, R. W. (1994) Nature 372, 272–275
12. Jacobsen, R. B., Koch, E. D., Lange-Malecki, B., Stocker, M., Verhey, J., Van Wagener, R. M., Vrazovkina, A., Olivera, B. M., and Terlau, H. (2000) J. Biol. Chem. 275, 24639–24644
13. Sharpe, I. A., Gehrmann, J., Loughnan, M. L., Thomas, L., Adams, D. A., Atkins, A., Palant, E., Craik, D. J., Adams, D. J., Alewood, P. F., and Lewis, R. J. (2001) Nat. Neurosci. 4, 902–907
14. Roubert, C., Sagné, C., Kapismali, M., Vernier, P., Bourrat, F., and Giros, B. (2001) Mol. Pharmacol. 60, 462–473
15. Paczkowski, F. A., Bryan-Lluaka, L. J., Perzgen, P., Bruess, M., and Bönisch, H. (1999) J. Pharmacol. Exp. Ther. 290, 761–767
16. Sucic, S., and Bryan-Lluaka, L. J. (2002) Brain Res. Mol. Brain Res. 108, 40–50
17. Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951) J. Biol. Chem. 193, 265–275
18. Cheng, Y., and Prusoff, W. (1973) Biochem. Pharmacol. 22, 3099–3108
19. Graef, K.-H., and Bönisch, H. (1988) in Handbook of Experimental Pharmacology (Trendelenburg, U., and Weiner, N., eds) Vol. I, pp. 193–245, Springer-Verlag New York Inc., New York
20. Buck, K. J., and Amara, S. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12584–12588
21. Svirndas, M., Janin, F., Mezghanni, S., Giros, B., Costentin, J., and Bonnet, J.-J. (2000) Mol. Pharmacol. 58, 1404–1411
22. Harder, R., and Bönisch, H. (1985) J. Neurochem. 45, 1154–1162
23. Bönisch, H. (1998) Methods Enzymol. 296, 259–278
24. DeFelice, L. J., Adams, S. V., and Ypey, D. L. (2001) Biosystems 62, 57–66
25. Pacholczyk, T., Blakely, R. D., and Amara, S. G. (1991) Nature 350, 350–353
26. Sharpe, I. A., Palant, E., Schroeder, C. I., Kaye, D. M., Adams, D. J., Alewood, P. F., and Lewis, R. J. (2003) J. Biol. Chem. 278, 40317–40323

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