DESVENLAFAXINE SUCCINATE (DVS) IDENTIFIES NOVEL ANTAGONIST BINDING DETERMINANTS IN THE HUMAN NOREPINEPHRINE TRANSPORTER


Departments of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 327232-8548 (J.N.M, R.L.R, R.D.B.)
Department of Psychiatry, Vanderbilt University School of Medicine, Nashville, TN 327232-8548 (R.D.B.)
Center for Molecular Neuroscience, Vanderbilt University School of Medicine, Nashville, TN 327232-8548 (R.D.B.)
Women’s Health Research, Wyeth Pharmaceuticals, Collegeville, PA 19426 (D.C.D., R.C.W.)
Chemical and Screening Sciences, Wyeth Pharmaceuticals, Collegeville, PA 19426 (G.S., P.E.M., E.T.)
Running Title: Human Norepinephrine Transporter Antagonist Interactions

Corresponding Author: Randy D. Blakely, Ph.D.
Suite 7140 MRBIII
Center for Molecular Neuroscience
Nashville, TN 37232-8548
Tel: 615-936-3705
FAX: 615-936-3040
Email: randy.blakely@vanderbilt.edu

Number of text pages: 21
Number of tables: 5
Number of figures: 7
Number of references: 40
Number of words in Abstract: 182
Number of words in Introduction: 463
Number of words in Discussion: 1325

Nonstandard abbreviations: Desvenlafaxine succinate (DVS), norepinephrine (NE), serotonin (5-HT), human NE transporter (hNET), human 5-HT transporter (SERT), nisoxetine (NIS), selective 5-HT reuptake inhibitor (SSRI), NE reuptake inhibitor (NRI), 5-HT/NE reuptake inhibitor (SNRI)

Recommended section: Neuropharmacology
ABSTRACT:

Desvenlafaxine succinate (DVS) is a recently introduced antagonist of the human norepinephrine (NE) and serotonin (5HT) transporters (hNET and hSERT, respectively), currently in clinical development for use in the treatment of major depressive disorder and vasomotor symptoms associated with menopause. Initial evaluation of the pharmacological properties of DVS (Deecher et al., 2006) revealed significantly reduced potency for the hNET expressed in membranes as compared to whole cells when competing for $[^3\text{H}]$nisoxetine (NIS) binding. Using hNET in transfected HEK-293 cells, this difference in potency for DVS at sites labeled by $[^3\text{H}]$NIS was found to distinguish DVS, the DVS analog WY-46824, methylphenidate and the cocaine analog RTI-55 from other hNET antagonists such as NIS, mazindol, tricyclic antidepressants and cocaine. These differences appear not to arise from preparation-specific perturbations of ligand intrinsic affinity or antagonist-specific surface trafficking but rather from protein conformational alterations that perturb the relationships between distinct hNET binding sites. In an initial search for molecular features that differentially define antagonist binding determinants, we document that Val148 in hNET transmembrane domain (TM) 3, selectively disrupts NIS binding but not that of DVS.
The norepinephrine (NE) transporter (NET) is a major determinant of central and peripheral noradrenergic signaling, mediating the inactivation of released NE via reuptake of the biogenic amine back into presynaptic neurons (Lorang et al., 1994). The human NET (hNET) is an important target for psychostimulants including cocaine and amphetamines and is a clinical target for the treatment of depression and attention-deficit hyperactivity disorder (ADHD) (Bonisch and Bruss, 2006). Genetic loss of NET in animal models (Xu et al., 2000; Keller et al., 2004; Keller et al., 2006) and humans (Keller and Robertson, 2006) (Usera et al., 2004) precipitates cardiovascular and stress phenotypes and alters behavioral responses to NE-modulatory psychoactive agents (Schroeder et al., 2004; Bonisch and Bruss, 2006). The hNET is a member of the SLC6 family of Na+-coupled solute transporters (Pacholczyk et al., 1991) (Gether et al., 2006). The SLC6 members bear 12 transmembrane domains (TMs) with cytosolic NH2 and COOH termini. This structure model was recently validated by an X-ray crystal structure of an analogous bacterial transporter protein, LeuT\textsubscript{Aa} (Yamashita et al., 2005) (Henry et al., 2006a). The hNET is most closely related in sequence to two other biogenic amine transporters in the SLC6 family, the serotonin (5-HT) and dopamine (DA) transporters (SERT and DAT, respectively).

Although more than a decade has elapsed since the initial cloning of the biogenic amine transporters, only recently have specific determinants of antagonist recognition been elucidated. Progress on the interaction of selective 5-HT reuptake inhibitors (SSRIs) with hSERT proteins has localized high-affinity binding determinants of these agents to TMs 1 and 3 (Barker et al., 1999) (Henry...
et al., 2006b), regions that also support substrate interactions as identified in structure-function studies (Adkins et al., 2001) and crystallographic resolution of leucine binding in LeuT$_{Aa}$ (Yamashita et al., 2005) (Henry et al., 2006a). Less is understood concerning the interactions of hNET with its antagonists or with the class of 5-HT/NE reuptake inhibitors (SNRIs), typified by agents such as venlafaxine (Vis et al., 2005) (Dell’Osso et al., 2006) (Stahl et al., 2005) and the recently described analog desvenlafaxine succinate (DVS) (Deecher et al., 2006) (Alfinito et al., 2006). Previously, we showed that DVS binds to and inhibits both hNET and hSERT using in vitro bioassays. Additionally, using in vivo microdialysis studies, we demonstrated that DVS elevates extracellular levels of both NE and 5HT in rat hypothalamus and frontal cortex. In our initial characterization of DVS, we noted differences in DVS potency for hNET in membrane preparations versus whole cells for displacement of [$^3$H]NIS binding. In this report, we further explored this finding to understand whether loss of potency is a general feature of NET antagonists, whether it is supported by ligand-induced hNET trafficking, and/or whether it reflects unique properties of hNET antagonist binding sites that can be further defined using site-directed mutagenesis.
MATERIALS AND METHODS

Compounds. Desvenlafaxine succinate (DVS) and the DVS analog, WY-46824 (rac-(1-[1-(3-chloro-phenyl)-2-(4-methyl-piperazin-1-yl)-ethyl] cyclohexanol); Patent US 4,826,844; EP 310268), (Fig. 1) were synthesized by the Chemical and Screening Sciences (CSS) group of Wyeth Research. Desipramine (catalog number D-3900) mazindol (catalog number M-2017) imipramine (catalog number I7379) and methylphenidate (catalog number M2892) and cocaine hydrochloride (catalog C5776) were purchased from Sigma-Aldrich (St. Louis, MO). RTI-55 was a gift from F. Ivy Carrol, RTI International.

Radioligands. $[^3\text{H}]$NE, (catalog number NET-048, 5-15 Ci/mmol), $[^3\text{H}]$nisoxetine (catalog number NET-1084, 85.5 Ci/mmol), $[^{125}\text{I}]$ RTI-55 (catalog number NEX-272, 2200 Ci/mmol) and scintillation cocktail (Ultima Gold, catalog number 6013329) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). The $[^3\text{H}]$WY-46824 (Fig. 1, inset) was radiolabeled by the Chemical and Screening Science group of Wyeth Research. 1-[1-(3-Chlorophenyl)-2-(4-methylpiperazin-1-yl)-2-oxoethyl]cyclohexanol was reduced with B$[^3\text{H}]$3 (Than et al., 1995) (3 equiv.) in tetrahydrofuran at 70 °C to produce $[^3\text{H}]$WY-46824 (specific activity = 22 Ci/mmol, as determined by UV @ 249 nm).

Cell Culture, Transfection and Reagents. The hNET-HEK-293 cells, stably transfected with hNET (Pacholczyk et al., 1991) (Galli et al., 1995) or hNET mutants were cultured in growth medium containing high glucose Dulbecco’s Modified Eagel Medium (DMEM; Gibco, Cat. No. 11995), 10% fetal bovine serum.
(FBS; dialyzed, heat-inactivated, US Bio-Technologies, Lot FBD1129HI) and 500 µg/mL G418 (Gibco, Cat. No.10131). Cells were routinely plated at 300,000 cells/T75 flask and split twice weekly. For evaluation of the impact of mutation on antagonist binding, HEK-293 cells were plated at a density of 10,000 cells per well in a 96-well culture plate. Cells were transfected with hNET or hNET mutant constructs with TransIT transfection reagent (Mirus Inc.; 4ml/mg of DNA). Mutation of hNET in pcDNA3.1 to generate mutants V148M and F72Y has been previously described (Henry et al., 2006b). Following transfection (24-48 h), cells were washed with KRH buffer (120mM NaCl, 4.7mM KCl, 1.2 mM KH$_2$PO$_4$, 2.2mM CaCl$_2$, and 10 mM HEPES, pH 7.4) and assayed either as whole cells or used to make membrane preparations in the radioligand binding assays described below.

Membrane Preparation and Radioligand Binding Assays. The preparation of membranes for binding assays were done by dispensing $3\times10^6$ hNET-HEK-293 cells into 80 cm$^2$ NUNC tissue culture flasks (Fisher) containing growth medium and maintained for 2 days prior to harvest. Cells were harvested using cell scrapers and the cell debris collected from 1 flask using phosphate buffered saline (PBS) solution devoid of calcium and magnesium. The cells were centrifuged at 3000xg for 10 min (4°C) to remove residual cell media and to pellet the cells. The supernatant was discarded, and the cell pellet was resuspended in 5 mL of binding buffer (50 mM Tris-HCl, 300 mM NaCl, 5 mM KCl, pH 7.4) and homogenized with a tissue tearer (Biospec Products, Inc.) at a setting of 2. Cell
membranes were centrifuged at 4°C for 10 min at 19,000 xg in a Biofuge Stratus centrifuge (Sorvall). The resulting pellet was resuspended in 5 mL of binding buffer and the above steps repeated twice more. Fresh membranes were suspended in binding buffer (4°C) and prepared to approximately 3 ± 1 µg protein per 200 µl aliquot. Protein assays were performed (Bio Rad) using bovine serum albumin as standard. Binding reactions were performed in borosilicate glass 12X75mm tubes (Fisherbrand Cat# 14-961-26). Cell membranes (200 µL) were added to each reaction tube, followed by 50 µL of compound solution. Desipramine (200 nM final) was added to duplicate tubes, to assess nonspecific binding. Total radioligand bound was defined using [³H]NIS ([N-methyl-3H]nsoxetine), (Amersham Corp.) at 5 nM, [³H]WY-46824 (Wyeth Research) at 200 nM, or [¹²⁵I]RTI-55 (Amersham Corp.) at 8 nM. Stock solutions of WY-46824 and mazindol were prepared in DMSO: H₂O (1:1) at concentrations from 100 nM to 10 mM. RTI-55, nisoxetine, imipramine, desipramine, cocaine, and methylphenedate were prepared in binding buffer. On day of assay, compounds were diluted in assay buffer according to test range (10⁻⁹ to 10⁻² nM). Compounds and membranes were pre-incubated at 4°C for 15 min prior to addition of radioligand, incubating for 2 h at 4°C. Assays were terminated by rapid filtration on a Brandel (Gaithersburg, MD) cell harvester through glass fiber filters pre-equilibrated in 0.3% polyethylenimine. Filters containing [³H] transporter were counted in a Tri-Carb 2900TR liquid scintillation analyzer (Packard) whereas the [¹²⁵I] labeled transporters were analyzed on a Beckman Gamma 4000 gamma counter.
Whole Cell Radioligand Binding Assays: Twenty-four hours prior to assay, cells were plated in 96-well plates at 3,000-5,000 cells/well in growth medium and maintained in a cell incubator (37°C, 5% CO_2). On day 2, growth medium was replaced with 80 µL of assay buffer (25 mM HEPES; 120 mM NaCl; 5 mM KCl; 2.5 mM CaCl_2; 1.2 mM MgSO_4; 2 mg/ml glucose (pH 7.4, 37°C)) containing 0.2 mg/mL ascorbic acid and 1 µM pargyline. A 10 µL aliquot of compound was added to each assay well. Competition radioligand binding assays were conducted with [^3H]NIS or [^125I]RTI-55 (5 nM and 8 nM final concentration, respectively). Saturation binding studies with [^3H]NIS and [^125I]RTI-55 utilized increasing concentrations of radioligand with nonspecific binding defined at each point with 200 nM desipramine. The DVS analog, [^3H]WY-46824, displayed too low an affinity in membranes to be used in saturation analyses and thus use of this label was restricted to competition studies performed at 200 nM final radioligand concentration. Compounds and membranes were pre-incubated at 37°C for 15 min prior to initiating the binding reaction by addition of radioligand. The cells in the assay buffer with test compound and radioligand were incubated for 2 h at 37°C. The cells were washed twice with 200 µL assay buffer at room temperature to remove unbound radioligand. After binding, cells were incubated for 1hr in Microscint20 scintillation fluid (PerkinElmer Life and Analytical Sciences, Boston, MA) and radiolabeled ligand binding quantified using a TopCount plate scintillation counter (PerkinElmer).
**[3H]NE Uptake Assays.** Cells were plated at 3,000 cells/well in a 96-well plate in growth medium and maintained at 37°C, 5% CO₂. On day 2, growth medium was replaced with 80 µL of assay buffer (25 mM HEPES; 120 mM NaCl; 5 mM KCl; 2.5 mM CaCl₂; 1.2 mM MgSO₄; 2 mg/ml glucose (pH 7.4, 37°C) containing 0.2 mg/mL ascorbic acid and 10 µM pargyline. Cells were equilibrated in 90 mL of assay buffer for 10 min at 37°C prior to addition of compounds. Desipramine was delivered to triplicate wells (final concentration 200 nM) to define non-specific NE uptake. A 10 µL aliquot of vehicle or various concentrations of antagonist were added directly to triplicate wells containing cells in 80 µL of assay buffer. The cells were preincubated with test compound for 15 minutes at 37°C. To initiate transport, [3H]NE (1-[7,8³H]norepinephrine, ~30 Ci/mmol from Amersham Biosciences) was diluted in assay buffer (50 nM final assay concentration) and delivered in 10 µL aliquots to each well and the plates were incubated for 10 min at 37°C. Medium was then removed from wells and cells were washed twice with 200 µL assay buffer to remove unincorporated [3H]NE label. The wells containing cells were then incubated for 1 hr in 80 µL Microscint20 scintillation fluid (PerkinElmer Life and Analytical Sciences, Boston, MA) and the [3H]NE accumulation quantified using a Topcount scintillation counter (PerkinElmer).

**Cell Surface Biotinylation Assays.** To examine possible antagonist-induced alterations in transporter surface expression, surface proteins were labeled with the lysine directed, membrane-impermeant biotinylating reagent sulfo-NHS-SS-
biotin (Pierce) as previously described (Apparsundaram et al., 1998). The hNET-HEK-293 cells were plated into 6-well dishes (500,000/well) and the following day cells were washed 4 times with PBS/CM (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, 0.1 mM CaCl2, and 1.0 mM MgCl2) at 4°C and incubated with 1.5 mg/mL of sulfo-NHS-SS-biotin in PBS/CM for 30 min at 4°C. To terminate biotinylation reactions and quench unreacted biotinylating reagent, cells were washed twice with 100 µM glycine in PBS/CM and incubated for 20 min at 4°C in glycine/PBS/CM containing buffer. The buffer was removed and solubilized in RIPA buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) containing protease inhibitors (1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µM pepstatin, and 250 µM phenylmethylsulfonyl fluoride), by shaking for 30 min at 4°C. The supernatants were removed and cleared of insoluble material by pelleting at 20,000 xg for 20 min at 4°C. ImmunoPure immobilized streptavidin beads (Pierce) were washed 3 times with 100 mM glycine /PBS/CM and then 4 times with RIPA buffer, finally resuspending the beads in RIPA buffer. A portion of the supernatant that constituted total solubilized protein was removed for subsequent analysis. A 210 µL aliquot of bead/RIPA slurry was added to 750 µL of supernatant and gently mixed 1 h at room temperature. The streptavidin beads were then washed 4 times with RIPA buffer, and biotinylated proteins were eluted from the beads with 50 µL of Laemmli sample buffer (Laemmli, 1970) and analyzed in parallel with total samples on a 10% SDS-PAGE gel. Proteins were transferred electrophoretically to Immobilon-P membrane (Millipore Corporation, Billerica,
MA) and the membranes were incubated with a monoclonal antibody directed against hNET (NET17-1; Mab Technologies, Inc., Stone Mountain, GA) at a dilution of 1:400 followed by incubation with a goat anti-mouse peroxidase-conjugated secondary antibody (Jackson ImmunoResearch laboratories Inc., West Grove, PA) at a dilution of 1:5000. Visualization of immunoreactivity was achieved using Western Lightning Chemiluminescent Reagent (PerkinElmer). Films were scanned at multiple exposures to insure linearity of exposure. The values were averaged from 3 experiments to obtain mean values with statistical tests comparing vehicle and antagonist treated preparations.

**Statistical Analysis and Data Presentation.** Competition and saturation isotherms were generated using Prizm software (Prizm Graphpad Software Inc., San Diego, CA). Calculation of the dissociation constant \( K_i \) values was based on the \( K_D \) values of the radioligand for each bioassay (Cheng and Prusoff, 1973). Data analyses of the equilibrium radioligand binding assays were done using nonlinear regression curve fitting based on a multisite equation for saturation binding. As Hill terms proved non-significantly different from 1, the Hill term was set to 1 and the analysis repeated to estimate the dissociation constant \( K_D \) and maximal binding capacity \( B_{\text{max}} \) values. Similarly, fits to competition data utilized a single site model to determine IC\(_{50}\) values prior to \( K_i \) conversion. Values reported are the mean of at least 3 independent experiments ± SEM. Potency comparisons and levels of total/surface hNET proteins were made using an
unpaired Student’s t-test. A P<0.05 was used as a threshold for statistical significance.
RESULTS

Affinity Differences of NET Antagonists in Membranes Compared with Whole Cells Containing the hNET.

Radioligand Binding Competition Assays using [³H]NIS. Our previous report demonstrated a reduction in DVS potency when competing for [³H]NIS binding at hNET expressed in MDCK cells in membrane preparations versus whole cells. To extend these studies, we examined the hNET expressed in stably transfected HEK-293 cells. The affinities of DVS and other NET antagonists were compared in [³H]NIS radioligand binding competition assays using either whole cells or membrane preparations. The structures of the primary NET antagonists studied in these experiments and were selected due to their structural diversity (Fig. 1). As initially reported with hNET-MDCK cells, we observed that DVS exhibited lower potency (6-fold) when competing for [³H]NIS labeling of hNET in membrane preparations compared to whole cells expressing the hNET (Table 1). In contrast, NIS, mazindol and the tricyclic antidepressants, imipramine and desipramine, exhibited no change in potency (Table 1). The potency of the ADHD medication methylphenylidate (Ritalin™) was also negatively impacted by membrane preparation (8-fold shift). Although cocaine displayed no shift in potency, the cocaine analog (RTI-55) demonstrated a 23-fold loss in potency when competing [³H]NIS for hNET in whole cells versus membrane preparations (Table 1). The potency shift for the DVS analog, WY-46824, was the greatest among compounds tested (275-fold), and bracketed
potency shifts of other structural related analogs of DVS and WY-46824 (data not shown).

Affinity Comparisons from Competition Assays using either \([^{3}H]\text{NIS}\) Binding or \([^{3}H]\text{NE}\) Uptake Assays. Competition curves depicting DVS, WY-46824 and desipramine for \([^{3}H]\text{NIS}\) labeling in either whole cells or membrane preparations are compared to \([^{3}H]\text{NE}\) uptake inhibition performed in whole cells containing hNET (Fig. 2). Antagonist potencies determined using the \([^{3}H]\text{NE}\) uptake bioassay closely correlate to those affinities reported when \([^{3}H]\text{NIS}\) is used to label hNET in whole cells (Table 1). These data suggest that the membrane preparation introduces a specific change in the hNET that can be revealed in \([^{3}H]\text{NIS}\) radioligand competition assays when a subset of hNET antagonists are employed as competitors.

Radioligand Binding Competition Assays using \([^{125}I]\text{RTI-55}\) Ligand. We next sought to evaluate whether the loss of potency of DVS and WY-46824 was specific to the site labeled by \([^{3}H]\text{NIS}\) or might be indicative of an altered relationship between different classes of hNET antagonists. For these studies, we assessed the inhibitory potency of DVS, WY-46824 and desipramine at the hNET labeled in whole cells or membranes using the \([^{125}I]\text{RTI-55}\) ligand. These \([^{125}I]\text{RTI-55}\) competition studies revealed no statistically significant differences in affinities of DVS, WY-46824 regardless of the hNET source used (Fig. 3). Interestingly, the affinities of DVS and WY-46824 were lower than those reported when competing \([^{3}H]\text{NIS}\) whereas the affinity of desipramine remained unchanged regardless of radioligand (Table 2). This radioligand \((^{[^{125}I]}\text{RTI-55})\)
has been used to specifically label hNET and demonstrates no specific binding in non-transfected cells (data not shown). Even more important is the failure of DVS and WY-46824 to show differences in affinities when competing for $[^{125}\text{I}]$RTI-55 labeling in membrane preparations as it does when $[^3\text{H}]$NIS is the radioligand. Additionally, the affinities noted for DVS and WY-45824 are similar to the affinities reported when $[^3\text{H}]$NIS is competed for the hNET in membrane preparations. These data provide evidence that the membrane preparation is not introducing an artifact in the ability for various radioligands to compete for specific binding sites on the hNET. Thus, the loss of antagonist potency in this configuration is not an intrinsic feature of the antagonist binding site but radioligand-dependent and also the ability of specific NE reuptake inhibitors (NRIs) to interact at the specific site labeled by the radioligand selected.

**Equilibrium Radioligand Binding Assays using $[^{125}\text{I}]$RTI-55 and $[^3\text{H}]$Nisoxetine Reveal Different Binding Densities in Membrane Preparations.**

**Determination of the Dissociation Constant ($K_D$) for $[^{125}\text{I}]$RTI-55 and $[^3\text{H}]$NIS.** The observations of differences in antagonist potencies in membranes depending on the radioligand used led us to assess the intrinsic affinities of hNET sites labeled by $[^3\text{H}]$NIS or $[^{125}\text{I}]$RTI-55. Thus, we generated saturation binding isotherms for $[^3\text{H}]$NIS (Fig. 4A,C) and $[^{125}\text{I}]$RTI-55 (Fig. 4B,D) at hNET in both whole cell and membrane preparations. Our studies revealed no significant differences in the dissociation constant ($K_D$) values of $[^3\text{H}]$NIS or $[^{125}\text{I}]$RTI-55 at
hNET comparing the affinities obtained using whole cells versus membrane preparations (Table 3).

**Determination of the Maximal Binding Capacity (B_{max}) for[^{125}I]RTI-55 and[^{3}H]Nisoxetine.** Interestingly, the maximal binding capacity (B_{max}) values for[^{3}H]NIS and[^{125}I]RTI-55 in whole cells were equivalent, whereas, the number of hNET binding sites labeled by[^{125}I]RTI-55 in membrane preparations is approximately 3.5 times that established for[^{3}H]NIS, suggesting a preparation-specific exposure of sites for the cocaine analog (Table 3).

**A DVS Analog Identifies Affinity Differences of NET Antagonists in Membranes Compared with Whole Cells Containing hNET.**

**Radioligand Binding Competition Assays using[^{3}H]WY-46824.** As membrane preparation does not appear to alter the intrinsic affinity of sites labeled by[^{3}H]NIS or[^{125}I]RTI-55, we sought evidence as to whether membrane preparation alters the intrinsic affinity of DVS-like compounds. To address this objective, the DVS analog, WY-46824, was radiolabeled (Fig. 1, inset). Preliminary evaluation was completed to determine the specific binding of[^{3}H]WY-46824 for the hNET in whole cells and membrane preparations. Although we could establish the presence of hNET specific sites using comparisons to non-transfected cells (data not shown), the specific binding in hNET transfected membranes across a concentration range was too low to generate adequate saturation plots due to increasing nonspecific binding. However, we were able to obtain competition binding data with[^{3}H]WY-46824 in both preparations (Fig. 5).
A loss of potency for both WY-46824 (50-fold) and NIS (256-fold) was observed when competing for [³H]WY-46824 (200 nM), in membrane preparations compared with whole cells (Fig. 5B, C). Interestingly, both NIS and WY-46824 showed similar affinities when competed for [³H]WY-46824 using whole cells (Table 4). We performed these assays in parallel with [³H]NIS competition assays and found, as noted above in both competition and saturation, NIS loses no potency comparing these two preparations (Fig. 5A). These striking data underscore a specific sensitivity of the site labeled by [³H]WY-46824 to membrane preparations which 1) reduces intrinsic WY-46824 affinity and 2) occludes interactions of NIS with the [³H]WY-46824 site.

**Lack of a Role of Antagonist-Modulated NET Trafficking in Loss of Antagonist Potency in Membranes.**

**Western Blot Analyses of hNET.** We considered the possibility that DVS-like compounds trigger a specific loss of hNET surface expression in the whole cell context, leading to assays that reflect only a subset of NET binding sites (with possibly distinct properties) as compared to those monitored in membranes. However, treatments of intact cells with 5 µM WY-46824 or DMI for 30 min and 2 h, followed by surface protein biotinylation (Fig. 6A), purification, and Western blot analysis revealed no changes in either NET total (Fig. 6B) or surface density (Fig. 6C). These studies reinforce the idea that conformational changes in hNET that target distinct antagonist binding sites accounts for the potency changes observed between whole cell and membrane preparations.
Evaluation of Structural Determinants for Selective Antagonist Recognition in hNET.

Identification of V148M as a NIS-Specific Contact. Our studies point to the existence of specific physical differences in determinants of hNET antagonist binding sites, one or more of which can be impacted by membrane preparation. To initiate an investigation of structural determinants that define NIS and DVS-type binding sites, we explored hNETs modified by site-directed mutagenesis to search for potential antagonist potency determinants. In this regard, our lab has recently shown that sites in hSERT TM1 and TM3 (specifically Tyr95 and Ile172) differentially impact the binding of SSRIs when mutated to the positions held by their Drosophila orthologs (i.e. Y95F and I172M) (Barker et al., 1999) (Henry et al., 2006b). The homologs of these sites in hNET are Phe72 and Val148. As DVS binds both to hNET and hSERT, we tested the hNET mutations F72Y and V148M for their effects on NIS and WY-46824 potency using [3H]NIS and [3H]WY-46824 whole cell competition assays (Table 5). The mutation of hNET to F72Y had no effect on either NIS or WY-46824 potency in these assays (data not shown). In contrast, the TM3 substitution V148M resulted in an antagonist-specific loss of potency when competing NIS. The NIS loss in potency for hNET V148M sites labeled by either [3H]NIS or [3H]WY-46824 ~ 14 and 18 fold, respectively (Fig. 7A,B; Table 5), whereas competition with WY-46824 displayed no significant shifts in affinities comparing the mutant against hNET regardless of radioligand (Fig. 7C,D; Table 5). These data reveal a specific, molecular...
determinant of NIS binding that appears to make no important contribution to WY-46824 binding, a requisite feature of the multi-site hypothesis to explain the distinct properties of different classes of hNET antagonists.
DISCUSSION

Modifying extracellular levels of NE by the blockade of NET is an important clinical target for the treatment of mood disorders (Blier, 2001), ADHD (Corman et al., 2004) and menopausal symptoms (Deecher et al., 2006). It is important to understand whether all NET antagonists attenuate NE transport via common or distinct mechanisms. Although the crystal structure of the NET bacterial transporter protein LeuT_Aa, co-crystallized with the substrate leucine in the binding pocket is known (Yamashita et al., 2005). Little information can be used to define the binding sites for many transporter antagonists, and in particular those for hNET. Past studies have indicated that NET antagonists may interact allosterically, observable as a change in antagonist dissociation rates in the presence of a distinct antagonist (Plenge and Mellerup, 1997), findings also observed with certain SERT antagonists (Plenge et al., 1991) (Chen et al., 2005). These findings may be explained through the interdependent interactions of individual antagonists at distinct binding sites on a single protein or may reflect the simultaneous and cooperative occupancy of a common antagonist binding site on a transporter multimer. Although physical evidence for multimers exists among members of the SLC6 family (Kilic and Rudnick, 2000) (Hastrup et al., 2001) including the hNET (Hahn et al., 2003), no evidence as yet relates multimer assembly to the formation or interactions of antagonist binding sites.

We have followed up on observations that a novel class of NET antagonists displays altered potency when cells are disrupted to produce membranes for binding assays (Deecher et al., 2006), supporting the existence
of distinct binding sites for typical NRIs such as NIS and the tricyclic desipramine as compared to DVS series compounds. Our studies here utilized DVS and a DVS analog, WY-46824, but we have observed similar behavior with a large number of other DVS-related analogs (D. Deecher, unpublished findings). The use of multiple radiolabeled hNET antagonists was instrumental in our identification of physical distinctions between binding sites. Thus, whereas DVS and the DVS analog WY-46824, as well as RTI-55 and methylphenidate, shift potency in membranes when \([^3\text{H}]\text{NIS}\) is used to label hNET, the same is not true when \([^{125}\text{I}]\text{RTI-55}\) is used to label hNET. As the \(K_D\) value of NIS is are not altered when assessed directly in whole cell versus membrane environments, these findings suggest that membrane preparation is benign with respect to the integrity of the latter antagonist binding site and either specifically disrupts the other sites or their spatial relationship with the NIS site. The most striking evidence for this idea arose when we examined the ability of NIS to inhibit \([^3\text{H}]\text{WY-46824}\) binding. Whereas NIS potency against \([^3\text{H}]\text{NIS}\) is unaltered by membrane preparation, NIS loses potency against \([^3\text{H}]\text{WY-46824}\) much in the same way that unlabeled WY-46824 loses potency against \([^3\text{H}]\text{NIS}\) labeling. Although we could not obtain reliable saturation isotherms for \([^3\text{H}]\text{WY-46824}\), unlabeled WY-46824 exhibits much lower potency against \([^3\text{H}]\text{WY-46824}\) in competition studies, suggesting that a critical determinant for binding potency of WY-46824 is altered by membrane preparation. One caveat to our interpretations is that the low affinity of \([^3\text{H}]\text{WY-46824}\) results in relatively low signal to noise ratios for whole cell binding assays (~50%) which is even lower in membrane
preparations (~20%). As our whole cell potency values for unlabeled NIS and WY46824 match those obtained with $[^3\text{H}]$NIS, we believe that $[^3\text{H}]$WY-46824 labels a partially overlapping site to that interacting with classical NET antagonists. Interestingly, a potency shift is not evident for DVS or WY-46824 if $[^{125}\text{I}]$RTI-55 is used to label hNET. We note however that in these assays the potency for DVS and WY-46824 is much lower that when these antagonists compete for $[^3\text{H}]$NIS or whole cell $[^3\text{H}]$WY-46824 binding. Since the potency of desipramine is the same when using either ligand, these data suggest that $[^3\text{H}]$NIS and $[^{125}\text{I}]$RTI-55 labeling define distinct but overlapping sites, both of which can be accessed by desipramine but nonequivalently by DVS and WY-46824. These data seem most explainable in terms of three mutually interacting and physically distinct sites, each of which has the capacity to immobilize the transporter or physically occlude the substrate binding pocket.

With respect to the presence of distinct hNET antagonist binding sites, we provide evidence that at least one residue, V148, can differentiate the binding of NIS and WY-46824. This is a particularly important finding as our saturation studies gave little evidence for multiple sites except when we compare between ligands or binding preparations. The V148 residue is the homolog of I172 in hSERT that supports the high-affinity binding of the SSRI, citalopram and fluoxetine (Henry et al., 2006b). Interestingly, while the I172M mutation shifts the potency of SERT for citalopram by two orders of magnitude, it does not impact interactions with 5-HT nor the SSRI, paroxetine (Henry et al., 2006b). In the crystal structure of LeuT $\alpha$ (Yamashita et al., 2005), the residue homologous to
this site lies in TM3 adjacent to the substrate binding pocket. Whereas the I172M substitution in hSERT does not affect 5-HT transport rates, in hNET, the V148M mutation eliminates transport of NE, although the transporter is expressed and reaches the cell surface at wildtype levels (Henry et al., 2006b). This finding suggests a somewhat distinct environment or contribution of this site to transport as compared to hSERT. What appears most relevant is the fact that this site in both hNET and hSERT supports antagonist-selective interactions (citalopram vs paroxetine in hSERT (Henry et al., 2006b), NIS vs WY-46824 in hNET (this study)). Although we provide evidence of a NIS-selective contact at V148, we do not believe that this residue is itself responsible for the differential effects of membrane preparation on the potency of NIS and DVS-type compounds, but rather that the differential impact of membrane preparation compelled us to seek a physical basis for distinct antagonist binding sites, one of which appears to include V148. A specific site sensitive to membrane preparation awaits further investigations. Regardless, we suggest that the tricyclic antidepressants and NIS may bind at or near the substrate binding pocket whereas the DVS class compounds bind to more distal sites, though of course more complex scenarios that take into account multimer-based allosterism can be envisaged.

One important issue not addressed by these studies relates to whether the differences in the modes of binding of NIS and DVS in whole cell versus membrane environments or as distinguished by V148M have important lessons for the development of NET antagonists with differential clinical utility. One possibility that we are excited to consider is that the binding differences observed
in the membrane and whole cell preparations could mirror conformational states achieved through normal modes of transporter regulation or through interactions with transporter accessory proteins. NETs have been found to exhibit changes in surface distribution with chronic stress (Miner et al., 2006) and possibly conformations of internal and surface pools could differentially detect or be stabilized by the binding of NIS and DVS analogs. Studies are now underway to examine this possibility. The hNET is also known to associate with a number of scaffolding or regulatory proteins including PICK1 (Torres et al., 2001), syntaxin 1A (Sung et al., 2003), PP2A (Bauman et al., 2000) and 14-3-3 proteins (Sung et al., 2005). Possibly, these transporter-protein associations, believed to support the trafficking and/or intrinsic activity of hNET, may be sensitive to conformational states stabilized by some transporter antagonists but not others. Support for this concept has recently arisen with evidence that different DAT antagonists influence the transporter’s capacity for phosphorylation (Gorentla and Vaughan, 2005). This concept is all the more plausible given that the initial observations of differences in reduced potency for our antagonists were noted in the membrane preparations. Loss of key protein associations during the process of membrane harvest or normalization of membrane potential could bias conformations selectively for one class of antagonists over another. Having access to reagents that bind to distinct sites and that exhibiting context specific reuptake blockade could greatly extend the therapeutic range of biogenic transporter-based therapeutics.
ACKNOWLEDGEMENTS:

We thank Tammy Jessen, Qiao Han, Jane Wright and Angela Steele for general lab maintenance and expert technical assistance. We are grateful to L. Keith Henry for assistance and insights with properties and binding assays related to hNET F72Y and hNET V148M.
REFERENCES


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099-3108.


and 3 of human serotonin transporters interact to establish high affinity

Keller NR, Diedrich A, Appalsamy M, Miller LC, Caron MG, McDonald MP,
transporter-deficient mice respond to anxiety producing and fearful
environments with bradycardia and hypotension. Neuroscience 139:931-
946.

Keller NR, Diedrich A, Appalsamy M, Tuntrakool S, Lonce S, Finney C, Caron
exhibit excessive tachycardia and elevated blood pressure with

Cardiol 21:173-179.


Laemmli UK (1970) Cleavage of structural proteins during the assembly of the

Lorang D, Amara SG and Simerly RB (1994) Cell-type-specific expression of

Chronic stress increases the plasmalemmal distribution of the
norepinephrine transporter and the coexpression of tyrosine hydroxylase


FOOTNOTES

*J.N.M. and D.C.D. contributed equally to these studies.
LEGENDS FOR FIGURES

Fig. 1. Structural illustrations of norepinephrine reuptake inhibitors A) Desvenlafaxine Succinate (DVS) B) DVS Analog (WY-46824) C) Cocaine Analog (RTI-55) and D) nisoxetine (NIS). Inset box notes the position of tritium (T) labeling sites of the DVS analog, [3H]WY-46824.

Fig. 2. Competition assays using desvenlafaxine succinate (DVS) (A), WY-46824 (DVS analog) (B) and desipramine (C) for [3H]NIS binding reveal differences in affinities dependent on the source of hNET. Intact HEK-293 cells (open circle) or membranes (solid circles) containing the hNET were incubated with 5 nM [3H]NIS and increasing concentrations of desvenlafaxine succinate, WY-46824 or desipramine for 2.5 h at 37°C (whole cells) or 4°C (membranes) as described in Methods. Non-specific binding was defined by 200 nM desipramine. Norepinephrine uptake (open squares) was performed with HEK-293 cells expressing hNET incubated with 50 nM [3H]NE for 5 min as described in Methods. Non-specific uptake was defined with 1 µM desipramine. Results are the average of 3 separate experiments, with individual points assayed in triplicate. The K_i values for these experiments are reported in Table 1.

Fig. 3. Lack of affinity differences when using [125I]RTI-55 in hNET competition assays with either isolated membranes or intact cells. DVS (A), WY-46824 (B), and desipramine (C) competition for [125I]RTI-55 binding at hNET expressed in HEK-293 intact cells and membranes. The HEK-293 intact cells (open symbols)
or membranes (solid circles) stably expressing hNET were incubated with 5 nM $[^{125}\text{I}]$RTI-55 and increasing concentrations of desvenlafaxine succinate, WY-46824 or desipramine for 2.5 h at $37^\circ\text{C}$ (intact cells) or $4^\circ\text{C}$ (membranes) as described in Methods. Nonspecific binding was defined using 200 nM desipramine. Results are the average of 3 separate experiments, with individual points assayed in triplicate. The $K_i$ values for these depicted competition curves are reported in Table 2.

**Fig. 4.** The number of hNET binding sites differ in membranes compared with whole cells, dependent on radioligand used, with no apparent affinity differences using equilibrium binding assays. Saturation analyses of $[^3\text{H}]$NIS (open circles) and $[^{125}\text{I}]$RTI-55 binding (closed circles) to hNET in transfected HEK-293 whole cells and membranes. Whole cell and membranes from HEK-293 cells stably expressing the hNET were incubated with increasing concentrations of either $[^3\text{H}]$NIS or $[^{125}\text{I}]$RTI-55 for 2.5 h as described in Methods. Nonspecific binding was defined 200 nM desipramine. Results are the average of 3 separate experiments, with individual points assayed in triplicate. Graphs are representative plots of single experiments with average values for $K_D$ and $B_{\text{max}}$ values derived from single-site hyperbolic fits (Prizm). The $K_D$ and $B_{\text{max}}$ values for these depicted competition curves are reported in Table 3.

**Fig. 5.** A decrease in affinity was noted for Nisoxetine when competing for $[^3\text{H}]$WY-46824 binding to hNET in membranes. NIS (A and B) and WY-46824 (C)
were competed for \[^3\text{H}\]NIS (A) and \[^3\text{H}\]WY-46824 binding using either whole cells or membranes containing the human norepinephrine transporter. The HEK-293 whole cells (open circles) or membranes (closed circles) stably expressing hNET were incubated with 5 nM \[^3\text{H}\]NIS or 200 nM \[^3\text{H}\]WY-46824 and increasing concentrations of unlabeled NIS or WY-46824 2.5 h at 37°C (whole cells) or 4°C (membranes) as described in Methods. Non-specific binding was defined by 200 nM desipramine. Results are the average of 3 separate experiments, with individual points assayed in triplicate. The \(K_i\) values for these depicted competition curves are reported in Table 4.

**Fig. 6.** The total and cell surface protein levels of hNET do not change after antagonist incubations. HEK-293 cells stably expressing hNET were cultured in the presence of desipramine (DMI) or WY-46824 (WY) or vehicle (control) and biotinylated as described in Methods. Aliquots containing equal amounts of protein were taken from each sample prior to streptavidin extraction of surface protein for total hNET protein. Blots were probed with monoclonal antibody to hNET(NET-17-1) followed by a goat anti-mouse and HRP-conjugated secondary antibody and chemiluminescent detection. Results from a representative blot are shown (A). Molecular mass (kDa) indicated is from prestained standards run in parallel. Quantitative analyses from 3 replicate experiments are shown for total (A) and biotinylated (B) samples.
Fig. 7. The mutation V148M impacts the affinity of nisoxetine binding without altering the binding affinity of WY-46824 in competition studies. Evaluation of mutations in the human norepinephrine transporter were completed in radioligand competition assays using [3H]nisoxetine and [3H]WY-46824. The hNET(wild type) (closed symbols) and hNET(V148M) (open symbols) were evaluated using [3H]NIS or [3H]WY-46824 in HEK-293 transfected cells as described in Methods. Cells were incubated with increasing concentrations of [3H]WY-46824 and [3H]NIS and unlabeled compounds for 2.5 h as described in Methods. Nonspecific binding was defined using 200 nM desipramine. Results are the average of 3 separate experiments, with individual points assayed in triplicate. The Ki values for these depicted competition curves are reported in Table 5.
TABLE 1
Potencies of norepinephrine reuptake inhibitors at the human norepinephrine transporter (hNET)

<table>
<thead>
<tr>
<th></th>
<th>[³H]NIS Competition Binding</th>
<th>[³H]NE Uptake</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kᵢ values</td>
<td>Kᵢ values</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(nM ± S.E.M.)</td>
<td>(nM ± S.E.M.)</td>
<td></td>
</tr>
<tr>
<td>hNET Membranes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nisoxetine 5 +</td>
<td>5 ± 2</td>
<td>14 ± 5</td>
<td>0.4</td>
</tr>
<tr>
<td>Mazindol 1 +</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td>1.0</td>
</tr>
<tr>
<td>Imipramine 30 ± 9</td>
<td>24 ± 9</td>
<td>249 ± 109</td>
<td>0.1</td>
</tr>
<tr>
<td>Desipramine 2 +</td>
<td>1 ± 1</td>
<td>6 ± 2</td>
<td>0.2</td>
</tr>
<tr>
<td>Cocaine 1533 ± 688</td>
<td>550 ± 400</td>
<td>1818 ± 808</td>
<td>0.3</td>
</tr>
<tr>
<td>Desvenlafaxine 4760 +</td>
<td>776 ± 160</td>
<td>255 ± 35</td>
<td>3.0</td>
</tr>
<tr>
<td>Methylphenidate 452 ±</td>
<td>56 ± 9</td>
<td>726 ± 255</td>
<td>0.1</td>
</tr>
<tr>
<td>RTI-55 35 ± 6</td>
<td>1.5 ± 1</td>
<td>17 ± 14</td>
<td>0.1</td>
</tr>
<tr>
<td>WY-46824 5500 ± 200</td>
<td>20 ± 3</td>
<td>141 ± 30</td>
<td>0.1</td>
</tr>
</tbody>
</table>

WC, whole cell; UT, uptake; M, membranes; NIS, nisoxetine; NE, norepinephrine

Kᵢ value = IC₅₀/(1 + [L]/Kₒ) where [L] equals concentration of radioligand added.

Data presented were generated in a minimum of three separate assays conducted on different test days. Each test concentration was run in triplicate.
TABLE 2
Lack of potency differences for hNET inhibitors using $^{125}$I-RTI-55 in competition assays

<table>
<thead>
<tr>
<th>[125I]RTI-55 Radioligand Binding $K_i$ values (nM ± S.E.M.)</th>
<th>Ratio</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>hNET Membranes</td>
<td>hNET Whole Cells</td>
<td>$K_i/K_i$ WC/UT</td>
</tr>
<tr>
<td>Desvenlafaxine</td>
<td>3500 ± 300</td>
<td>3200 ± 900</td>
</tr>
<tr>
<td>WY-46824</td>
<td>120 ± 23</td>
<td>143 ± 34</td>
</tr>
<tr>
<td>Desipramine</td>
<td>2 ± 1</td>
<td>3 ± 2</td>
</tr>
</tbody>
</table>

WC, whole cell; M, membranes; RTI-55, cocaine analog

$K_i$ value = $IC_{50}/(1 + [L]/K_D)$ where [L] equals concentration of radioligand added.

Data presented were generated in three separate assays conducted on different test days. Each test concentration was run in triplicate.
TABLE 3

Differences in hNET binding density in equilibrium membrane binding is radioligand dependent but is not accompanied by affinity changes.

<table>
<thead>
<tr>
<th></th>
<th>hNET Membranes</th>
<th>hNET Whole Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_D (nM ± S.E.M.)</td>
<td>B_max (fmol/mg ± S.E.M.)</td>
</tr>
<tr>
<td>[3H]NIS</td>
<td>11 ± 22</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>[125I]RTI-55</td>
<td>9 ± 8</td>
<td>47 ± 6</td>
</tr>
</tbody>
</table>

NIS, Nisoxetine; RTI-55, cocaine analog

K_D, dissociation constant; B_max, maximal binding capacity

Data presented were generated in three separate assays conducted on different test days. Each test concentration was run in triplicate.
TABLE 4

A decrease in affinity is noted for NIS and WY-46824 when competing for [³H]WY-46824 at hNET in membrane binding assays.

<table>
<thead>
<tr>
<th>Radioligand Binding</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hNET Membranes</td>
<td>hNET Whole Cells</td>
</tr>
<tr>
<td>[³H]NIS (NIS)</td>
<td>16 ± 2</td>
<td>25 ± 7</td>
</tr>
<tr>
<td>[³H]WY-46824 (NIS)</td>
<td>4500 ± 1200</td>
<td>37 ± 10</td>
</tr>
<tr>
<td>[³H]WY-46824 (WY-46824)</td>
<td>2300 ± 500</td>
<td>47 ± 20</td>
</tr>
</tbody>
</table>

NIS, nisoxetine; WY-46824, DVS analog

Radioligand concentration used was 5 nM [³H]NIS and 200 nM [³H]WY-46824

$K_i$ value = $IC_{50}/(1 + [L]/K_D)$ where [L] equals concentration of radioligand added.

Data presented were generated in three separate assays conducted on different test days. Each test concentration was run in triplicate.
TABLE 5

Differential impact of V148M mutation on NIS and WY46824 whole cell hNET binding

<table>
<thead>
<tr>
<th>Radiolabeled Antagonist</th>
<th>[3H]NIS</th>
<th>[3H]WY46824</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA</td>
<td>Wt</td>
<td>V148M</td>
</tr>
<tr>
<td>NIS (Ki, nM)</td>
<td>10 ± 2</td>
<td>141 ± 11</td>
</tr>
<tr>
<td>WY-46824 (Ki, nM)</td>
<td>23 ± 3</td>
<td>31 ± 4</td>
</tr>
</tbody>
</table>

NIS, nisoxetine; WY-46824, DVS analog

$K_i$ value = $IC_{50}/(1 + [L]/K_D)$ where [L] equals concentration of radioligand added.

Data presented were generated in three separate assays conducted on different test days. Each test concentration was run in triplicate.
Fig. 1

A. DVS

\[
\text{N} - \text{OH} \\
\text{Cl} \\
\text{WY-46824, 3 H}
\]

B. WY-46824

\[
\text{N} - \text{OH} \\
\text{Cl}
\]

C. RTI-55

D. Nisoxetine

\[
\text{N} - \text{OH} \\
\text{Cl}
\]
Fig. 2

A. % Specific Binding ([3H]NIS) vs. Desvenlafaxine [M]

B. % Specific Binding ([3H]NIS) vs. WY46824 [M]

C. % Specific Binding ([3H]NIS) vs. Desipramine [M]
Fig. 3

A. % Specific Binding ([125I]RTI-55) vs. Desvenlafaxine [M]

B. % Specific Binding ([125I]RTI-55) vs. WY-46824 [M]

C. % Specific Binding ([125I]RTI-55) vs. Desipramine [M]
Fig. 4

(A) (whole cell)  
(B) (whole cell)  
(C) (membrane)  
(D) (membrane)
Fig. 5

A. % Specific Binding (3H)NMS

B. % Specific Binding (3H)WY46824

C. % Specific Binding (3H)WY46824
Fig. 6

A. DMI  WY  Control

B. Total

C. Surface

107kDa

30min

2hrs

Surface

30min  n=4  2hrs  n=3

% of control

Control  DMI  WY-46902  Control  DMI  WY-46902

% of control

Control  DMI  WY-46902  Control  DMI  WY-46902