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**Title Page**

**Desvenlafaxine Succinate: A New Serotonin and Norepinephrine Reuptake Inhibitor***

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**Running title:** Desvenlafaxine: A New SNRI

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**Nonstandard abbreviations:** 5-HT, serotonin; DA, dopamine; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; DVS, desvenlafaxine succinate; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; hDAT, human dopamine transporter; hNET, human norepinephrine transporter; HPLC, high performance liquid chromatography; hSERT, human serotonin transporter; NE, norepinephrine; PEI, polyethylenimine; SNRI, serotonin and norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor.

**Recommended section:** Neuropharmacology

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## ABSTRACT

The purpose of this study was to characterize a new chemical entity, desvenlafaxine succinate (DVS). DVS is a novel salt form of the isolated major active metabolite of venlafaxine. Competitive radioligand binding assays were performed using cells expressing either the human serotonin (5-HT) transporter (hSERT) or norepinephrine (NE) transporter (hNET) with $K_i$ values for DVS of 40.2 ± 1.6 and 558.4 ± 121.6 nM, respectively. DVS showed weak binding affinity (62% inhibition at 100 μM) at the human dopamine (DA) transporter. Inhibition of $^{3}$[H]5-HT or $^{3}$[H]NE uptake by DVS for the hSERT or hNET produced $IC_{50}$ values of 47.3 ± 19.4 and 531.3 ± 113.0 nM, respectively. DVS (10 μM), examined at a large number of non-transporter targets, showed no significant activity. DVS (30 mg/kg, orally) rapidly penetrated the male rat brain and hypothalamus. DVS (30 mg/kg, orally) significantly increased extracellular NE levels compared with baseline in the male rat hypothalamus, but had no effect on DA levels using microdialysis. To mimic chronic selective serotonin reuptake inhibitor treatment, and to block the inhibitory 5-$HT_{1A}$ autoreceptors, a 5-$HT_{1A}$ antagonist, WAY-100635 (0.3 mg/kg, subcutaneously), was administered with DVS (30 mg/kg, orally). 5-HT increased 78% compared with baseline with no additional increase in NE or DA levels. In conclusion, DVS is a new 5-HT and NE reuptake inhibitor in vitro and in vivo that demonstrates good brain-to-plasma ratios, suggesting utility in a variety of central nervous system-related disorders.

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## Introduction

Biogenic amines such as serotonin (5-HT), norepinephrine (NE), and dopamine (DA) are neurotransmitters found in areas of the central nervous system (CNS) known to be important for regulation of cognitive function, mood, thermoregulation, pain sensation, sexual function, and various aspects of endocrine function related to homeostasis. Their synthesis is highly regulated, and long-term disturbance of the regulatory pathways for these neurotransmitters may lead to disruptions in overall health and quality of life. As with any specific mechanism that controls a physiological process, regulation of these neurotransmitters is a target of many pharmacological agents. Agents that have been developed to modulate these key neurotransmitters are well characterized and have provided evidence that regulation of these neurotransmitters impacts physiological outcomes specific to each neurotransmitter. There are multiple means of regulating these neurotransmitters. One means is by inhibiting their presynaptic reuptake. Monoamine reuptake inhibitors work by binding to their respective transporter proteins located presynaptically. This binding interaction results in an increase in elimination time of the neurotransmitter cycle; thus, the extracellular concentration of the neurotransmitters increases in the synaptic cleft. This increase in neurotransmitter in the cleft allows for increased downstream cellular signaling (Blakely and Bauman, 2000). Serotonin and norepinephrine reuptake inhibitors (SNRIs) work by blocking the presynaptic reuptake of 5-HT and NE, resulting in an increased, sustained level of both of these neurotransmitters. The 5-HT and NE monoamine transporters are members of the 12 transmembrane-spanning transporter family that moves monoamines across the cell membrane with high affinity in a sodium-dependent manner (Pacholczyk et al., 1991; Amara and Kuhar, 1993; Uhl and Johnson, 1994).

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Currently two drugs approved in the United States, venlafaxine and duloxetine, are selective inhibitors of neuronal 5-HT and NE reuptake (Muth et al., 1986; Owens et al., 1997; Bymaster et al., 2001). Neither has significant affinity for muscarinic, cholinergic, $H_1$-histaminergic, or $\alpha_1$-adrenergic receptors in vitro, supporting the hypothesis that the various anticholinergic, sedative, and cardiovascular effects seen with activity at these receptors will not be present with their clinical use. In essence, the selectivity of these drugs for 5-HT and NE transporters will diminish the potential for adverse effects seen with less selective, centrally active treatments. Both drugs are classified as SNRIs and have been shown to have utility in multiple therapeutic end points. Specifically, venlafaxine is indicated for the treatment of major depressive disorder, generalized anxiety disorder, social anxiety disorder, and panic disorder, whereas duloxetine is indicated for the treatment of major depressive disorder (Stahl et al., 2005) and for the treatment of diabetic peripheral neuropathic pain ( Goldstein et al., 2005; Kirwin and Goren, 2005).

Desvenlafaxine succinate (DVS), the succinate salt of the isolated major active metabolite of venlafaxine, is a pharmacologically distinct molecule currently in clinical development. This compound was evaluated in a series of competitive radioligand binding and transport inhibition bioassays at the three human monoamine transporters (5-HT, NE, DA). Additionally, DVS was also examined for potential affinity at a large number of other non-transporter targets to ascertain its selectivity for the 5-HT and NE monoamine transporters. Furthermore, DVS was evaluated in vivo to determine brain penetrability and effect on neurochemical levels in the brain of male rats. The results of these experiments indicate that DVS is a SNRI both in vitro and in vivo. DVS

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demonstrates good brain-to-plasma ratios, suggesting utility in CNS, peripheral nervous system, and peripheral-related disorders associated with changes in these neurotransmitters.

## Materials and Methods

### Compounds

Desvenlafaxine succinate was synthesized by the Discovery Medicinal Chemistry group of Wyeth Research. Desipramine (Cat. No. D-3900), fluoxetine Cat. No. F-132), paroxetine (Cat. No. P-1372), and mazindol (Cat. No. M-2017) were purchased from Sigma-Aldrich (St. Louis, MO). Radioligands ([$^3$H]NE, Cat. No. NET-048, 5-15 Ci/mmol; [$^3$H]nisoxetine, Cat. No. NET-1084, 85.5 Ci/mmol; [$^3$H]WIN-35,428, Cat. NET-1033, 85.6 Ci/mmol; [$^3$H]hydroxytryptamine creatinine sulfate, Cat. No. NET-498, 25-35 Ci/mmol) and scintillation cocktail (Ultima Gold, Cat. No. 6013329) were purchased from PerkinElmer (Boston, MA).

### Cell Culture

HEK293 cells, stably transfected with the human 5-HT transporter (hSERT), were cultured in growth medium containing high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Cat. No. 11995) with 10% fetal bovine serum (FBS; dialyzed, heat-inactivated, US Bio-Technologies, Lot FBD1129HI) and 500 µg/ml G418 (Gibco, Cat. No. 10131). Cells were grown to 80–90% confluence in T175 flasks. MDCK-Net6 cells, stably transfected with human NE transporter (hNET) (Pacholczyk et al., 1991), were cultured in growth medium containing high-glucose DMEM (Gibco, Cat. No. 11995) with 10% FBS (dialyzed, heat-inactivated, US Bio-Technologies, Lot FBD1129HI) and 500 µg/ml G418 (Gibco, Cat. No. 10131). Cells were plated at 300,000/T75 flask and cells were split twice weekly. The JAR cell line (human placental

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choriocarcinoma) containing the hSERT was purchased from ATCC (Cat. No. HTB-144). The cells were cultured in growth medium containing RPMI 1640 (Gibco, Cat. No. 72400), 10% FBS (Irvine, Cat. No. 3000), 1% sodium pyruvate (Gibco, Cat. No.1136), and 0.25% glucose. Cells were plated at 250,000 cells/T75 flask and split twice weekly. For all assays, cells were plated in Wallac 96-well sterile plates (PerkinElmer, Cat. No. 3983498). Membranes from CHO cells expressing recombinant human DA transporter (hDAT) were purchased from PerkinElmer (Cat. No. RBHDATM) and maintained at –80ºC until assay day.

**5-HT Radioligand Membrane Binding Assay**

Frozen membranes were suspended in binding buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, pH 7.4) and prepared to approximately 5 µg protein per 50 µl aliquot. Binding reactions were run in 96 well OptiPlates (PerkinElmer). Cell membranes (50 µl) were added to each well of the reaction plate, followed by 40 µl binding buffer, 10 µl test compound or buffer, or 50 µl [$^3$H]citalopram (1 nM final concentration). The [$^3$H]citalopram was run at the approximate $K_D$ concentration and 94-96% specific binding was achieved in this assay. Data from wells containing fluoxetine (10 µM) were used to define non-specific hSERT membrane binding (minimum binding in the presence of a 5-HT reuptake inhibitor). All reaction wells received 50 µl Wheatgerm Agglutinin SPA beads (Amersham; containing 0.5 mg) made in 50 mM Tris-HCl, 120 mM NaCl, and 5 mM KCl (pH 7.4). The reaction plates were then incubated for a minimum of 1 h to reach equilibrium. The plates were then counted on a TopCount® scintillation counter.

**NE Radioligand Membrane Binding Assay**



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Fresh or frozen membranes were suspended in binding buffer (50 mM Tris-HCl, 300 mM NaCl, 5 mM KCl, pH 7.4) and prepared to approximately 3 ± 1 µg protein per 150 µl aliquot. Binding reactions were run in polypropylene 96-well plates (Costar; Cat. No. 3359,3930). Cell membranes (150 µl) were added to each well of a reaction plate, followed by 10 µl of compound solution. A stock solution of desipramine was prepared in dimethyl sulfoxide (DMSO; 1 mM) and delivered to triplicate wells containing membranes for a final test concentration of 1 µM. Data from wells containing desipramine (1 µM) were used to define non-specific hNET membrane binding (minimum binding in the presence of an NE reuptake inhibitor). Total radioligand bound is defined by the addition of 10 µl of binding buffer alone in the presence of [$^3$H]nisoxetine. Stock solutions of DVS were prepared in DMSO:$H_2O$ (1:1) at concentrations from 10 µM–10 mM. On the day of the assay, DVS was diluted in assay buffer according to the test range (10–30,000 nM). Compounds and membranes were pre-incubated at 4ºC for 15 min prior to initiating the binding reaction by the addition of radioligand. The binding reaction was initiated by the addition of 50 µl [$^3$H]nisoxetine diluted in binding buffer to yield a final assay concentration of 3 nM. The equilibrium binding affinity constant ($K_D$) value estimated for [$^3$H]nisoxetine was 4 nM using membranes containing hNET. The assay run under these conditions showed a 90-96% specific binding signal. The reaction was incubated on an orbital shaking platform (Bellco, Vineland, NJ) for 1 h at room temperature at a speed of 3 revolutions per min. The MultiScreen-FB opaque 96-well filtration plates containing Millipore glass fiber filters (Millipore glass fiber B, Cat. No. MAFBN0B) were used to terminate the binding reactions and to separate bound from free radioligand. The plates were presoaked with 0.5% polyethylenimine (PEI; Sigma Cat. No. P-3143) in water for a minimum of 2 h at room

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temperature to reduce non-specific binding of [$^3$H]nisoxetine during the harvest of the binding reactions. The PEI solution was aspirated using a vacuum manifold from the filter plates just prior to completion of the reaction incubation time. Aliquots of each reaction (180 µl of each 200 µl reaction well) were transferred from the reaction plates to the filter plates using a Zymark Rapid Plate-96 automated pipet station. The binding reaction was terminated by vacuum filtration through the glass fiber filters. The filter plates were aspirated at 5–10 in. Hg and the wells were washed seven times with 200 µl wash buffer (50 mM Tris-HCl, 0.9% NaCl, pH 7.4, 4ºC) using a 12-channel aspiration/wash system. Plastic bottom supports were removed from the filter plates and the plates were placed in plastic liners. A 50 µl aliquot of scintillation fluid was added to each well and the top of each plate was sealed with adhesive film. The plates were vigorously shaken on an orbital shake table (Bellco) at 5 rpm for 10–15 min to ensure adequate equilibration of aqueous-to-solvent partitioning. The collection of raw cpm data was done using a Wallac Microbeta counter (PerkinElmer).

**NE Whole-Cell Radioligand Binding Assay**

The hNET whole cell radioligand binding assay protocol was developed based on the combined methods supplied with hNET membranes (PerkinElmer, Cat. No. RBHNETM) and the hNET functional uptake assay. Twenty-four hours prior to assay, cells were plated in 96-well plates at 3000–5000 cells/well in growth medium and maintained in a cell incubator (37ºC, 5% $CO_2$). On day 2, growth medium was replaced with 75 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, 37ºC) containing 0.2 mg/ml ascorbic acid and 1 µM pargyline. Five microliter aliquots of DVS in assay buffer were

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added directly to triplicate wells to yield final test concentrations of 10–30,000 nM. Data from wells containing desipramine (1 μM) were used to define non-specific hNET binding (minimum hNET binding in the presence of an NE reuptake inhibitor). Total radioligand bound is defined by addition of 5 μl of binding buffer alone in the presence of [$^3$H]nisoxetine. The radioligand binding reaction was initiated by addition of [$^3$H]nisoxetine in 25 μl of assay buffer to each well for a final concentration of 3 nM. The $K_D$ value estimated for [$^3$H]nisoxetine was 10 nM using intact whole cells. The assay run under these conditions showed a 93-96% specific binding signal. The cells in assay buffer with test compound and radioactivity were incubated for 1.5 h at 37ºC. The supernatant was decanted to terminate the reaction. The cells were washed two times with 200 μl of assay buffer (37ºC, without pargyline and ascorbic acid) to remove unbound radioligand. The plates were inverted and left to dry for 2 min, then re-inverted and air-dried for an additional 10 min. Scintillation cocktail (50 μl) was added to each well and the plates were sealed with film tape. The plates were placed on an orbital shake table (Bellco) for 10 min to ensure adequate mixing. The plates were counted in a Wallac Microbeta counter (PerkinElmer) to collect the raw cpm data.

**hDAT Membrane Radioligand Binding Assay**

The hDAT radioligand binding assay protocol was modified from the methods supplied with hDAT membranes (PerkinElmer, Cat. No. RBHDATM), and those modifications are listed within this methods section. Frozen membrane samples from a cell line that expresses the hDAT were diluted to 7.5 ml in binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl), homogenized with a tissue-tearer (Polytron PT 1200C, Kinematica AG) and delivered at a volume of 75 μl to

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each well of a polypropylene 96-well plate. The binding reaction was carried out in polypropylene 96-well plates (Costar General Assay Plate, Cat. No. 3359; Lid, Cat. No. 3930). Homogenized membrane preparation was delivered at a volume of 75 µl to each well of a reaction plate. A stock solution of mazindol was prepared in DMSO (10 mM) and delivered to triplicate wells containing membranes for a final test concentration of 10 µM. Mazindol has been reported to be a DA transporter inhibitor (Pristupa et al., 1994), with a 50% inhibitory concentration ($IC_{50}$) value of 18.0 ± 6.0 nM in our assays. Data from wells containing mazindol (10 µM) were used to define non-specific hDAT binding (minimum hDAT binding in the presence of a DA reuptake inhibitor). Total radioligand bound is defined by the addition of 5 µl of binding buffer alone in the presence of [$^3$H]WIN-35,428. Stock solutions of DVS were prepared in DMSO:$H_2O$ (1:1) at concentrations from 10 µM–100 mM. On the day of the assay, DVS was diluted in assay buffer according to test range (100–100,000 nM), ensuring a maximal DMSO concentration of <0.5% in the assay reaction wells. Homogenized membranes were pre-incubated with DVS for 20 min at 4°C before the initiation of the binding reaction. The binding reaction was initiated by the addition of 25 µl of [$^3$H]WIN-35,428 diluted in binding buffer. The final concentration of [$^3$H]WIN-35,428 delivered was 32 nM. The $K_D$ value estimated for [$^3$H]WIN-35,428 in hDAT membranes (Lot #2227) was 29.7 nM. The assay run under these conditions showed an 82-93% specific binding signal. The plates containing the radioligand binding reactions were incubated for 2 h at 4°C on a shaking table (Bellco) at 3 revolutions per min. The MultiScreen-FB opaque 96-well filtration plates containing Millipore glass fiber filters (Millipore glass fiber B, Cat. No. MAFBN0B) were used to terminate the binding reactions and to separate bound from free radioligand. The plates were presoaked with 0.5% PEI (Sigma Cat.

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No. P-3143) in water for a minimum of 2 h at room temperature to reduce non-specific binding of [$^3$H]WIN-35,428 during the harvest procedure. Prior to harvesting the reaction plates, the PEI solution was aspirated from the filter plates using a vacuum manifold. Aliquots of each reaction (90 µl of each 100 µl reaction well) were transferred from the reaction plates to the filter plates using a Zymark Rapid Plate-96 automated pipet station. The binding reaction was terminated by vacuum filtration through the glass fiber filters. The filter plates were aspirated at 5–10 in. Hg and the wells were washed nine times with 200 µl wash buffer (50 mM Tris-HCl, 0.9% NaCl, pH 7.4, 4ºC) using a 12-channel aspiration/wash system. Plastic bottom supports were removed from the filter plates and the plates were placed in plastic liners. A 100 µl aliquot of scintillation fluid was added to each well and the top of each plate was sealed with adhesive film. The plates were vigorously shaken on an orbital shake table (Bellco) at 5 rpm for 10–15 min to ensure adequate equilibration of aqueous to solvent partitioning. The collection of raw cpm data was done using a Wallac Microbeta counter (PerkinElmer).

**5-HT Uptake Assay**

The methods for 5-HT functional reuptake using the JAR cell line were modified using a previous report (Prasad et al., 1996). On day 1, cells were plated at 15,000 cells/well in 96-well plates containing growth medium (RPMI 1640 with 10% FBS) and maintained in a cell incubator (37ºC, 5% $CO_2$). On day 2, cells were stimulated with staurosporine (40 nM) to increase the expression of the 5-HT transporter (Ramamoorthy et al., 1995). On day 3, cells were removed from the cell incubator 2 h prior to assay and maintained at room temperature to equilibrate the growth medium to ambient oxygen concentration. Subsequently, the growth medium was replaced with 200 µl of assay buffer (25 mM HEPES, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$,

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1.2 mM $MgSO_4$, 2 mg/ml glucose, pH 7.4, 37ºC) containing 0.2 mg/ml ascorbic acid and 1 µM pargyline, and the cells were incubated for 5 min at 37ºC. A stock solution of paroxetine was prepared in DMSO (10 mM) and delivered to triplicate wells containing cells for a final test concentration of 1 µM. Data from these wells were used to define non-specific 5-HT uptake (minimum 5-HT uptake in the presence of an 5-HT reuptake inhibitor). DVS was prepared in DMSO:$H_2O$ (1:1) (10 mM) and diluted in assay buffer according to the test range (1–1,000 nM). Twenty-five microliters of assay buffer (maximum 5-HT uptake) or DVS were added directly to triplicate wells containing cells in 200 µl of assay buffer. The cells were incubated with the compound for 10 min (37ºC). To initiate the reaction, [$^3$H]hydroxytryptamine creatine sulfate diluted in assay buffer was delivered in 25 µl aliquots to each well for a final test concentration of 15 nM. The cells were incubated with the reaction mixture for 9 min at 37ºC. Decanting the supernatant from the plates terminated the reaction. The cells were washed twice with 200 µl of assay buffer (37ºC) to remove free radioligand. The plates were inverted and left to dry for 2 min, then re-inverted and air-dried for an additional 10 min. Subsequently, the cells were lysed in 25 µl of 0.25 N NaOH (4ºC), then placed on a shaker table and shaken vigorously for 5 min. After cell lysis, 75 µl of scintillation cocktail was added to the wells and the plates were sealed with film tape and replaced on the shake table for a minimum of 10 min. The plates were counted in a Wallac Microbeta counter (PerkinElmer) to collect the raw cpm data.

**NE Uptake Assay**

On day 1, cells were plated at 3000 cells/well in a 96-well plate in growth medium and maintained in a cell incubator (37ºC, 5% $CO_2$). On day 2, growth medium was replaced with 200

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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, 37ºC) containing 0.2 mg/ml ascorbic acid and 10 μM pargyline. Cells were equilibrated in assay buffer for 10 min at 37ºC prior to addition of compounds. A stock solution of desipramine was prepared in DMSO (10 mM) and delivered to triplicate wells containing cells for a final test concentration of 1 μM. Data from these wells were used to define non-specific NE uptake (minimum NE uptake). DVS was prepared in DMSO:$H_2O$ (1:1) (10 mM) and diluted in assay buffer according to test range (1–10,000 nM). Twenty-five microliters of assay buffer (maximum NE uptake) or various concentrations of DVS were added directly to triplicate wells containing cells in 200 μl of assay buffer. The cells were incubated with test compound for 10 min at 37ºC. To initiate the NE uptake, [$^3$H]NE diluted in assay buffer (120 nM final assay concentration) was delivered in 25 μl aliquots to each well and the plates were incubated for 10 min at 37ºC. Decanting the supernatant from the plate terminated the reaction. The plates containing cells were washed twice with 200 μl of assay buffer (37ºC) to remove free radioligand. The plates were then inverted, left to dry for 2 min, then re-inverted and air dried for an additional 2 min. The cells were lysed in 25 μl of 0.25 N NaOH (4ºC), placed on an orbital shake table (Bellco), and vigorously shaken for 5 min. After cell lysis, 75 μl of scintillation cocktail was added to each well and the plates were sealed with film tape. The plates were returned to the shake table and vigorously shaken for a minimum of 10 min to ensure adequate partitioning of organic and aqueous solutions. The plates were counted in a Wallac Microbeta counter (PerkinElmer) to collect the raw cpm data.

**Animals**



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The brain penetration studies were done using Male Sprague-Dawley rats purchased from Taconic and housed in a room at 21°C under a reversed 12-h light/12-h dark cycle (12:00 AM on/12:00 PM off). Rats were 2 months of age on study day. Microdialysis experiments were performed using adult intact male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 280–330 g at the time of surgery. Rats were group housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International that was maintained on a 12-h light dark cycle (lights on at 0600 h) and had free access to food and water. All in vivo studies were performed in accordance with the *Guide for the Care and Use of Laboratory Animals,* as adopted and promulgated by the National Institutes of Health (Publication 85-23, 1985).

**Determination of DVS Concentrations in Plasma, Brain, and Hypothalamus**

DVS was prepared in 0.25% Tween 80 and 0.5% methylcellulose for oral administration. Three rats per time point were dosed with DVS at 30 mg/kg by oral gavage in 0.5 ml within 30 min prior to the onset of the dark cycle. Food was restricted from 4 h predosing to 20 min postdosing.

At the indicated time points, rats were anesthetized with 2–3% isoflurane/oxygen and the chest cavity was opened. Blood was drawn by cardiac puncture into the right atrium and collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes for plasma isolation. Rats were perfused with 40 ml cold phosphate-buffered saline through the left ventricle. The brain was removed and the hypothalamus was dissected. Brain and hypothalamus were placed in cold deionized water in either 5 ml or 0.25 ml, respectively, and maintained on ice until tissue



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homogenization. Brain was homogenized with a Virtis Tempest Virtishear tissue homogenizer for approximately 45 sec. The homogenization step was followed by sonication with a Heat Systems Ultrasonic sonicator for 5 sec (pulse mode was utilized set at 0.5 sec on and 0.1 sec off to reduce heating) on the probe power setting of 5.0 for brain and 4.5 for hypothalamus. Hypothalamus was homogenized by sonication only, as described above. Plasma and brain tissues were stored at –80° C until processing. For determination of the concentration of DVS in tissues, a standard curve (5–2000 ng/ml) was generated in plasma and brain tissue from untreated animals. Samples and standards (100 µl) were spiked with 20 µl of a 2500 ng/ml solution of 4-(2,4-dichloro-5-methoxy-phenylamino)-6-methoxy-7-[3-(4-methyl-piperazin-1-yl)-propoxy]-quinoline-3-carbonitrile in acetonitrile to serve as an internal standard. Protein was then precipitated by addition of 400 µl acetonitrile and centrifugation at 3400 rpm for 5 min. Supernatant was transferred and evaporated under $N_2$ atmosphere at 40°C. Material was then reconstituted in 200 µl acetonitrile:deionized (1:1) water. Twenty microliters was then subjected to high performance liquid chromatography (HPLC) using a Phenomenex Synergy MaxRP column (30 x 2.0 mm, 4 µM) and mass spectrometry (PE Sciex 4000 APCI-MRM).

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**Probe Placement in the Hypothalamus for Microdialysis Studies**

Following induction of anesthesia with 3% halothane (Fluothane; Zeneca, Cheshire, UK), animals were secured in a stereotaxic frame with ear and incisor bars (David Kopf, Tujunga, CA). Anesthesia was maintained by continuous administration of halothane (1–2%) while a microdialysis guide cannula (CMA/12, CMA Microdialysis, Stockholm, Sweden) was directed toward the preoptic area of the hypothalamus. The coordinates for these surgeries were –0.40

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mm anterior to bregma, –1.00 mm from the midline, and –6.90 mm from dura (Paxinos, 1986). Guide cannulae were secured to the skull using dental acrylic (Plastics One, Roanoke, VA) and two stainless-steel screws. Immediately following surgery, animals were individually housed in Plexiglas cages (45 cm sq.), had free access to food and water, and were allowed approximately 20–23 h of postoperative recovery.

**Experimental Procedures for Microdialysis**

Microdialysis probes (CMA Microdialysis, Stockholm, Sweden) with an active membrane surface of 2 mm were perfused with artificial cerebrospinal fluid (125 mM NaCl, 3 mM KCl, 0.75 mM $MgSO_4$, and 1.2 mM $CaCl_2$, pH 7.4) in situ for at least 18 h prior to experimentation. The microdialysis probe was then implanted via the guide cannula into the hypothalamus and perfused with artificial cerebral spinal fluid at a flow rate of 1 µl/min. A 3-h stabilization period was allowed following probe implantation, and dialysate sampling was conducted according to previously described methods (Beyer et al., 2002). In brief, five control samples (fmol/20 µl sample) were taken prior to drug injection to demonstrate a steady baseline. All subsequent values were expressed as a percentage of the mean preinjection value (percentage of baseline). After baseline sampling was complete, animals were treated with WAY-100635 (0.3 mg/kg s.c.) or vehicle (water s.c.). DVS (30 mg/kg) or vehicle (0.2% Tween and 0.5% methylcellulose dissolved in water) was orally administered 20 min later at the end of the sixth sample. For a minimum of 3 h following DVS administration, dialysis samples were collected every 20 min and analyzed to determine levels of NE, 5-HT and DA. At the end of each experiment, animals were euthanized and the probe placement was verified by histology. Data from rats with incorrect probe placement were discarded.

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**Evaluation of Neurochemical Samples**

A 20 µl sample from the hypothalamus was analyzed for NE, and 5-HT content by methods described previously **(Beyer et al., 2002),** additionally DA levels were determined. Briefly, neurochemical separation occurred by HPLC (C18, ODS3 column, 150 x 3.0 mm; Metachem, Torrance, CA, USA) and was detected using an ANTEC electrochemical detector (ANTEC, the Netherlands) set at a potential of 0.65V versus an Ag/AgCl reference electrode. Mobile phase (0.15 M $NaH_2PO_4$, 0.25 mM EDTA, 1.75 mM 1-octane sulphonic acid, 2% isopropanol, and 4% methanol, pH 4.8) was delivered by a Jasco PU1580 HPLC pump (Jasco Ltd, Essex, UK) at a flow rate of 0.5 ml/min. Neurochemical data were captured using the Atlas software package (Thermo Labsystems, Beverley, MA).

**Data Collection and Statistical Analysis**

For all in vitro experiments, except hSERT binding, a data stream of cpm values collected from the Wallac Microbeta counter was downloaded to a Microsoft Excel statistical application program. Calculations of $IC_{50}$ values were made using the transformed-both-sides logistic concentration response program written by Wyeth Biometrics Department. The statistical program uses mean cpm values from wells representing maximum binding or uptake (assay buffer) and mean cpm values from wells representing minimum binding or uptake (1 µM desipramine [hNET], 10 µM mazindol [hDAT], or 10 µM fluoxetine [hSERT]). Estimation of the $IC_{50}$ values were completed on a log scale, and the line was fit between the maximum and minimum binding or uptake values. Pooling the raw data from multiple experiments and

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analyzing the pooled data as one experiment were done to generate the $IC_{50}$ values. For hSERT binding, triplicate cpm values per concentration were collected from the PerkinElmer TopCount and analyzed by a Prism® nonlinear regression analysis program for receptor binding. Calculation of the $K_i$ values was based on the $K_D$ values of the radioligand for each bioassay. The $K_i$ value is a function of the concentration of the compound required to inhibit 50% of the radioligand ($IC_{50}$ value) divided by the free radioligand concentration [L] divided by the $K_D$ value plus one ($K_i = IC_{50}/(1+ [L]/K_D)$) (Cheng and Prusoff, 1973). Experiments evaluating DVS were completed a minimum of three times in separate experiments for all assays described within this report.

Pharmacokinetic data were subject to non-compartmental analysis using WinNonLin version 4.1 software. Tissue concentration ratios were calculated as the concentration in a given brain tissue divided by the concentration in plasma at a given time point.

In the microdialysis experiments, the fmol concentrations for the baseline samples were averaged, and this value was denoted as 100%. Subsequent sample values were expressed as a percent change from this preinjection baseline value (percent change from baseline). Neurochemical data, excluding preinjection values, were analyzed by a two-way analysis of variance with repeated measures (time). Post hoc analyses were made using the Bonferroni/Dunns adjustment for multiple comparisons. All statistical calculations were performed using the Statview software application (Abacus Concepts Inc., Berkeley, CA).

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## Results

### Determination of Affinities of DVS for Human Monoamine Transporters

**5-HT Membrane Radioligand Binding.** Competitive [$^3$H]citalopram (SSRI) scintillation proximity assays were performed using membranes prepared from HEK293 cells over-expressing hSERT to determine the affinity of DVS (Fig. 1A). Data from three separate experiments demonstrated that DVS is a potent inhibitor of [$^3$H]citalopram binding at the hSERT with a $K_i$ value of 40.2 ± 1.6 nM (Table 1).

**NE Whole-Cell and Membrane Radioligand Binding.** In MDCK-hNET6 cells, DVS competitively inhibited binding of a known NE reuptake inhibitor, [$^3$H]nisoxetine (Fig. 1B), with a $K_i$ value of 558.4 ± 121.6 nM (Table 1). In further evaluation using membranes containing the hNET, DVS competed for the binding of [$^3$H]nisoxetine with a $K_i$ value of 3385.1 ± 349.3 nM. The diminished affinity of DVS in the membrane bioassay may reflect the artificial nature of using isolated membranes in contrast to intact cells. Minor technical variations in these two radioligand binding assays may account for this discrepancy.

**DA Binding.** To determine the activity of DVS for this third human monoamine transporter, competitive binding assays using [$^3$H]WIN-35,428, a known selective DA reuptake inhibitor, were performed using purified membranes prepared from CHO cells that over-express hDAT. Data from three separate experiments demonstrated that DVS was a very weak inhibitor of [$^3$H]WIN-35,428 binding at the hDAT, with only a 61.6% ± 1.7 inhibition at the highest

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concentration tested (100 μM; Table 1). Therefore, an extrapolated logistic concentration curve was generated to determine an $K_i$ value (25 ± 5 μM) (Fig. 1C).

**Inhibitory Effects of DVS on Radioligand Uptake at hSERT and hNET.** DVS inhibited the uptake of [$^3$H]5-HT at the hSERT (Table 1). The data generated and pooled from three separate experiments show a mean $IC_{50}$ value of 47.3 ± 19.4 nM (Fig. 2A). In addition, the inhibitory action of DVS on uptake of [$^3$H]NE in MDCK-hNET6 cells was evaluated. Pooled data from nine separate experiments indicate that the $IC_{50}$ value of DVS for [$^3$H]NE uptake was 531.3 ± 113.0 nM (Fig. 2B). Desipramine, a well-characterized NE reuptake inhibitor, was tested in parallel in all NE transporter assays and showed a similar affinity in all three hNET assays with a $K_i/IC_{50}$ value of 3.4 ± 0.2 nM. DVS showed an approximate 10-fold separation between hSERT and hNET activity, with the reported activity for hSERT greater than hNET.

**Selectivity of DVS**

DVS was examined for potential affinity at a large number of other non-transporter targets to ascertain its selectivity for the 5-HT and NE monoamine transporters. DVS (10 μM) was tested for activity at 96 targets (i.e., receptors, transporters, enzymes, channels) by NovaScreen® (Table 2). No significant activity was detected for any targets with the exception of the 5-HT and NE monoamine transporters, demonstrating the selectivity of this compound for the two monoamine transporters.

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**DVS Concentrations in Plasma, Brain, and Hypothalamus After Oral Administration.** Concentrations of DVS were determined in the hypothalamus and total brain for each time point over a 24-h period (Fig. 3). Total brain represents the remainder of brain tissue after dissecting the hypothalamus. The $C_{max}$ in plasma of 940 ng/ml occurred at 30 min following DVS dosing. The terminal half-life of DVS in plasma was 2.1 h, and the compound was undetectable 24 h after the single oral dose. Extrapolated total exposure ($AUC_{0-\infty}$) in plasma was 1864 h•ng/ml. Similar to plasma, the $C_{max}$ of 963 ng/g in the hypothalamus and 771 ng/g in total brain tissue was reached 30 min after dosing. The half-life of DVS was 2.2 h in hypothalamus and 2.1 h in total brain. Extrapolated total exposure ($AUC_{0-\infty}$) in hypothalamus was 3485 h•ng/g and in total brain was 2923 h•ng/g. DVS was below the limits of detection in hypothalamus and total brain tissue in all rats at the 24 h time point. The brain-to-plasma ratio of DVS reached a maximum in hypothalamus (2.6) at 8 h and in total brain (2.2) at 2 h (data not shown). The ratio of hypothalamus AUC to plasma AUC was 1.8 and brain AUC to plasma AUC was 1.6.

**Effect of DVS on Hypothalamic Levels of Monoamines (5-HT, NE, and DA).** Acute oral administration of DVS (30 mg/kg) did not significantly [$F(2,19) = 0.74$, $P = 0.4898$] alter concentrations of 5-HT in the hypothalamus (Fig. 4A). However, pretreatment with WAY-100635 (5-$HT_{1A}$ antagonist, 0.3 mg/kg s.c.), which did not alter 5-HT levels on its own, resulted in a significant 78% increase in extracellular 5-HT levels [$F(1,9) = 36.09$, $P = 0.0001$] in the rat hypothalamus. In contrast to the observations with 5-HT levels, acute administration of DVS, when given alone, produced a significant [$F(2,16) = 6.11$, $P = 0.0107$] increase in NE concentrations (Fig. 4B). Post hoc analysis revealed significant differences in maximal NE levels



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comparing doses of 10 mg/kg (data not shown) and 30 mg/kg to vehicle (96% above baseline; *P* = 0.0221 and 118% above baseline; *P* = 0.0034, respectively), although the effects of the two doses of DVS were not significantly different from each other (*P* = 0.4470). Pretreatment with WAY-100635 (0.3 mg/kg s.c.) did not significantly increase NE in this brain region when tested alone [F (2,15) = 1.986, *P* = 0.1768]. However, when combined with DVS, pretreatment with WAY-100635 did not alter the ability of DVS to significantly elevate extracellular NE levels [F (1,9) = 8.11, *P* = 0.0192]. The neurochemical effects of DVS were found to be specific for noradrenergic and serotonergic systems since the acute administration of DVS (30 mg/kg p.o.) alone, or in combination with WAY-100635 (0.3 mg/kg s.c.), did not significantly alter concentrations of DA in the hypothalamus [F (2,23) = 0.18, *P* = 0.8343; **Fig. 4C**].

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## Discussion

DVS is a novel succinate salt monohydrate of desvenlafaxine, the major active metabolite of venlafaxine. The results of these in vitro experiments show that DVS exhibits selective inhibitory activity of neurotransmitter uptake at the human 5-HT and NE monoamine transporters and increases extracellular levels of NE and 5-HT (in the presence of a 5-$HT_{1A}$ antagonist) when compared with baseline levels of monoamines in the hypothalamus of rats. Higher affinity was noted for the hSERT when compared with the affinity for the hNET, whereas weak affinity for the hDAT was noted. DVS (10 μM) demonstrated no significant activity at numerous non-transporter targets. In addition, oral administration of DVS resulted in measurable concentrations in the brain. Taken together, these data support that this new chemical entity would be classified as a new SNRI.

The results demonstrate that DVS potently interacts with hSERT and, to a lesser degree, with hNET. The in vitro functional assay indicated that DVS is approximately 10-fold more potent at inhibiting 5-HT uptake than NE uptake. DVS competes for the binding of a known NE reuptake inhibitor, [$^{3}$H]nisoxetine, in cells containing the hNET. These data support the hypothesis that DVS interacts with the hNET to inhibit NE uptake. Additionally, DVS inhibits functional NE uptake in the same cells, supporting a functional consequence of the interaction of DVS with the hNET. Competition of DVS for the binding of [$^{3}$H]WIN-35,428, a known selective DA reuptake inhibitor, only showed inhibitory activity at high micromolar concentrations (>10 μM). Based on these assays that were performed to evaluate the binding interaction of DVS, this weak binding



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interaction would suggest that no functional consequence on dopamine levels would occur at concentrations required to inhibit hNET and hSERT.

Studies comparing the receptor and transporter binding profile of several SSRIs and SNRIs have illustrated that these drugs and their metabolites each have unique functional activity ratios (Owens et al., 1997; Bymaster et al., 2001). However, in vitro assays may not accurately depict in vivo activity because of factors such as pharmacokinetics, blood/brain permeability, protein binding, and clearance of compound. Additionally, the amino acid binding sites for specific drugs versus different radioligands can influence apparent in vitro affinities. Although DVS showed modest affinity for the hNET, its action at these sites is likely to be functionally important. Based on currently available data, it is unclear what levels of 5-HT and NE are required to restore "normal" function. Although the efficacy of other SNRIs has been shown for the treatment of specific disorders, the optimal activity ratio for the hSERT and hNET has not been determined for any specific disease indication.

It is postulated that at clinically relevant doses, acute treatment with SSRIs and SNRIs do not acutely elevate extracellular 5-HT levels in terminal brain areas. The postulated reason for this lack of serotonergic effect is that increases in terminal neurotransmitter concentrations, resulting from blockade of transporter, are paralleled by increases in the somatodendritic cell body region of the dorsal raphe. Local increases in raphe 5-HT, via activating the inhibitory 5-$HT_{1A}$ autoreceptors in this region, will suppress serotonergic neuronal cell firing and result in little or no 5-HT release in terminal brain regions (see Discussion section of Beyer et al, 2002). Reversing this inhibitory feedback loop with 5-$HT_{1A}$ receptor antagonists (e.g., WAY-100635)

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or chronic antidepressant treatment (i.e., 5-$HT_{1A}$ receptor desensitization) causes an immediate increase in extracellular 5-HT in the presence of an SSRI or SNRI, likely mimicking their long-term effects (Chaput et al., 1986; Jolas et al., 1994; Kreiss and Lucki, 1995; Le Poul et al., 1995; Le Poul et al., 2000; Beyer et al, 2002). This neurochemical hypothesis is corroborated by preclinical findings showing that acute combination treatment with a 5-$HT_{1A}$ antagonist and antidepressants produces similar increases in extracellular 5-HT to those seen following repeated antidepressant treatment alone (Kreiss and Lucki, 1995). Since it is speculated that combining 5-$HT_{1A}$ antagonism with acute DVS treatment would mimic the neurochemical effects of chronic DVS treatment, the results of the present studies suggest that chronic treatment with DVS is likely to result in elevations in 5-HT in this brain region over time. Similar to additional microdialysis studies conducted in other areas of the brain, including the frontal cortex (Wyeth, unpublished findings), DVS produced significant increases in extracellular NE concentrations in the hypothalamus. As expected, and similar to reports with other transport blockers (e.g., SSRIs), the addition of WAY-100635 did not affect the ability of DVS to increase extracellular NE levels when compared with baseline NE levels (Beyer et al., 2002).

The present study evaluated brain concentrations and brain-to-plasma concentration ratios of DVS over time following oral administration in male rats. DVS demonstrates rapid brain penetration with brain concentrations in excess of those noted in the plasma following a single oral dose of 30 mg/kg. This work supports the notion that DVS can exert direct effects on neuronal systems of the brain and that it can have utility in treating CNS-related dysfunctions. Additionally, increasing 5-HT and NE levels in the local brain environment may result in increased levels of these key neurotransmitters in the circulation. These transmitters play an

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important role in the peripheral nervous system, as well as in the periphery (Teschemacher, 2005), and thus may have utility in treating disorders both centrally and peripherally that are associated with modified levels of 5-HT and NE, such as various pain modalities (Willis and Westlund, 1997; Sommer, 2004), inflammatory processes (Kaneko et al., 2005; Maleki et al., 2005) and urinary incontinence (Fraser and Chancellor, 2003).

Compounds with dual-acting 5-HT and NE monoamine transporter inhibition have been shown to have multiply utilities (Sayar et al., 2003; Schober and Ansani, 2003; Stearns et al., 2003; Arnold et al., 2004; Iyengar et al., 2004; Ladd et al., 2005; Mariappan et al., 2005). DVS, a new molecule in this class, is brain penetrable and increases key neurotransmitters in the hypothalamus, a region of the brain involved in important physiological functions, including regulation of mood, temperature, and pain sensation. In addition the hypothalamus is important in regulation of the sleep cycle, stress response, and sexual behavior. Based on these preclinical findings, DVS appears to be a selective and potent SNRI that warrants further evaluation in a variety of therapeutic end points associated with disrupted 5-HT and NE levels. The utility and precise functional activity at both the serotonergic and noradrenergic transporters of this new chemical entity in disorders implicated to have decreased neurotransmitter levels and altered functional activity needs to be explored clinically.

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concentrations were performed by William Adams, and the study reported was issued by Christina Huselton from the Drug Safety and Metabolism Group, Collegeville, PA. The authors would like to thank Dr. Mary E. Hanson from Cardinal Health in the preparation and critical review of this manuscript.

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## Footnote

*Funding for this study was provided by Wyeth Research.

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## Figure Legends

**Fig. 1**. DVS competes for the binding of radioligands specific to the serotonin (membrane) and norepinephrine (whole cell) transporters, whereas weak competition was noted for the radioligand specific to the dopamine (membrane) transporter. For each transporter bioassay a known comparator was used (fluoxetine [5-HT reuptake inhibitor]; desipramine [NE reuptake inhibitor]; mazindol [DA reuptake inhibitor]). The $IC_{50}$ value was generated from each of these curves and used to generate the $K_i$ values. Each data point depicted represents the mean ± S.E.M. of three independent experiments performed in triplicate. The $IC_{50}$ value was generated from each of the curves illustrated and used to generate the $K_i$ values. The $K_i$ values for DVS and the comparators are shown in Table 1.

**Fig. 2**. Functional activity of desvenlafaxine demonstrates inhibition of radioligand uptake of serotonin or norepinephrine. For each uptake transporter bioassay a known comparator was used (fluoxetine [5-HT reuptake inhibitor]; desipramine [NE reuptake inhibitor]). Each data point depicted represents the mean ± S.E.M. of three independent experiments performed in triplicate. The $IC_{50}$ values for desvenlafaxine are shown in Table 1.

**Fig. 3**. Orally administered desvenlafaxine (30 mg/kg) to intact male rats is found in plasma and penetrates the brain and hypothalamus over time. Each time point depicted in the figure represents the mean concentration ± S.E.M. of desvenlafaxine from three individual rats. A standard curve (5–2000 ng/ml) was generated in plasma, brain, and hypothalamus from vehicle-treated rats.

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**Fig. 4.** DVS increases extracellular levels serotonin (A) and norepinephrine (B) levels without increasing dopamine (C) levels in the rat hypothalamus when compared with baseline. The first and second arrows represent time of subcutaneous (WAY-100635) and oral (desvenlafaxine) injections, respectively. *Significant ($P < 0.05$) treatment effects compared with vehicle. $N = 6–9$ per treatment group.

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TABLE 1
Characterization of desvenlafaxine at the human serotonin, norepinephrine, and dopamine transporters

| | Radioligand binding $K_i$ values (nM ± S.E.M.) | | | | **Neurotransmitter uptake** **$IC_{50}$ values (nM ± S.E.M.)** | |
|---|---|---|---|---|---|---|
| | hSERT membrane | hNET membrane | hNET whole cell | hDAT membrane | hSERT whole cell | hNET whole cell |
| **Desvenlafaxine** | 40.2 ± 1.6 | 3385.1 ± 349.3 | 558.4 ± 121.6 | 25,000 ± 5000 | 47.3 ± 19.4 | 531.3 ± 113.0 |
| **Comparator** | 7.0 ± 1.2 fluoxetine | 4.1 ± 1.1 desipramine | 2.1 ± 0.4 desipramine | 18.0 ± 6.0 mazindol | 10.3 ± 1.7 fluoxetine | 3.9 ± 0.5 desipramine |

$IC_{50}$, concentration inhibiting 50% of the radioligand uptake; hSERT, human serotonin transporter; hNET, human norepinephrine transporter; hDAT, human dopamine transporter.

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

Comparators: fluoxetine (selective serotonin reuptake inhibitor); desipramine (norepinephrine reuptake inhibitor); mazindol (dopamine reuptake inhibitor).

Data presented were generated in a minimum of two separate assays conducted on different test days. Each test concentration was run in triplicate.



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TABLE 2

DVS selectively inhibits serotonin and norepinephrine uptake*

| **Desvenlafaxine selectivity profile[†]: Lack of significant affinity at numerous targets examined** | | | |
|---|---|---|---|
| **Neurotransmitter Related**<br>Adenosine transporter<br>Adenosine $A_1$ and $A_2$<br>Benzodiazepam periphery (h)<br>Cannabinoid CB1 and CB2 (h)<br>Dopamine transporter<br>Dopamine 1–5 (h) | **Neurotransmitter Related**<br>Opiate (h)<br>Mu, delta 2, Kappa<br>Purinergic, P2Y (h)<br>Serotonin transporter<br>5-HT nonselective<br>Serotonin receptors (5-HT) 1A, 1D, 2A, 2C, 3, 4, 5A, 6 | **Ion Channels**<br>Ca channel L (Benz)<br>Ca channel L (Dihyd)<br>Ca channel N<br>K+ channel, ATP and CA2<br>Sodium, sites 1 and 2<br>GABA, chloride | **Growth Factors/Hormones**<br>Oxytocin<br>Corticotropin-releasing factor<br>Platelet-activating factor<br>Thyrotropin-releasing hormone<br>Neurokinin 1–3 (h)<br>Neuropeptide 1–2 (h) |
| Glutamate (AMPA, Kiante[jlm1])<br>GABA A, agonist<br>GABA A, BDZ<br>GABA B<br>Histamine 1–3 (h)<br>Imadazoline, I1, I2 | **Brain/Gut Peptides**<br>AT1 (h) and AT2<br>BK2<br>CCK A and B<br>ET A and B (h)<br>Galanin | **Prostaglandins**<br>Leukotriene B4, D4<br>Thromboxane A2 (h) | Neurotensin<br>Somatostatin<br>Vasoactive intestinal peptide (human)<br>Vasopressin |
| Melatonin<br>Muscarinic 1–5 (h)<br>Nicotinic<br>Norepinephrine transporter<br>Adrenergic receptors<br>Alpha 1A, 1B, 2A, 2B<br>Beta non-selective | **Steroids**<br>Estrogen<br>Glucocorticoid<br>Progesterone<br>Testosterone (cytosolic and nuclear) | **Second Messengers**<br>NOS<br>Adenylate cyclase<br>IP3<br>Protein kinase C | **Other**<br>Complement e5a (h)<br>Sigma 1 and 2 |

$IC_{50}$>10 µM; (h), human.

*Desvenlafaxine (10 µM) was tested for activity at numerous drug targets by NovaScreen® (Hanover, Maryland). No significant activity was detected with the exception of the 5-HT and NE transporters.

[†]NovaScreen®.



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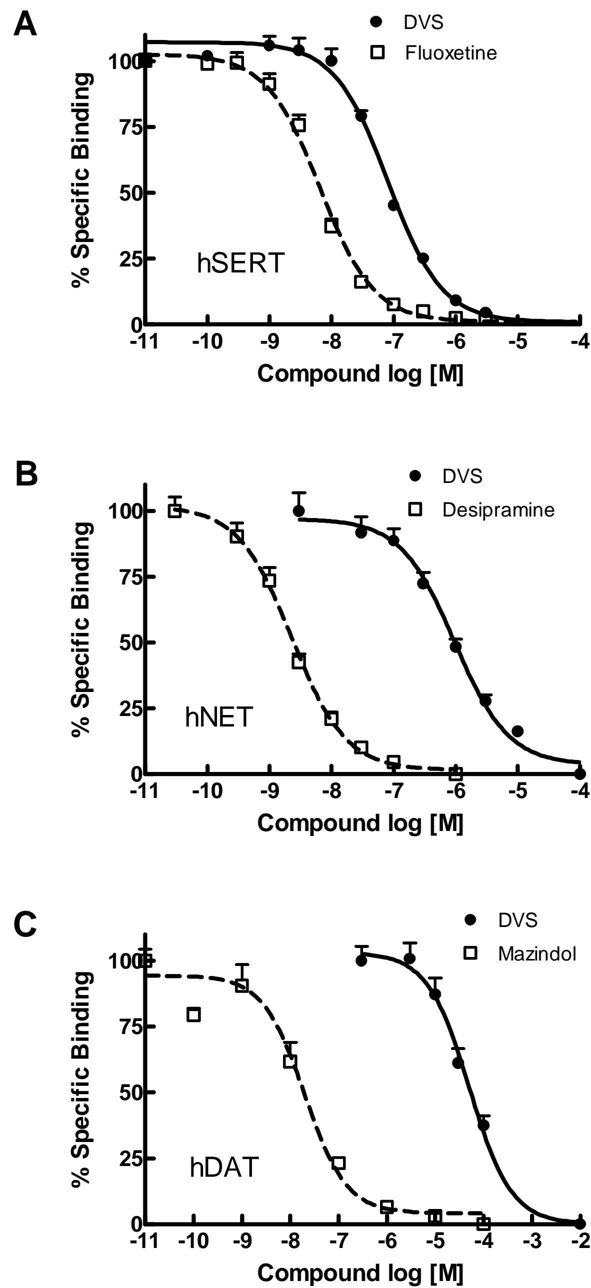
# Figure 1

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# Figure 2

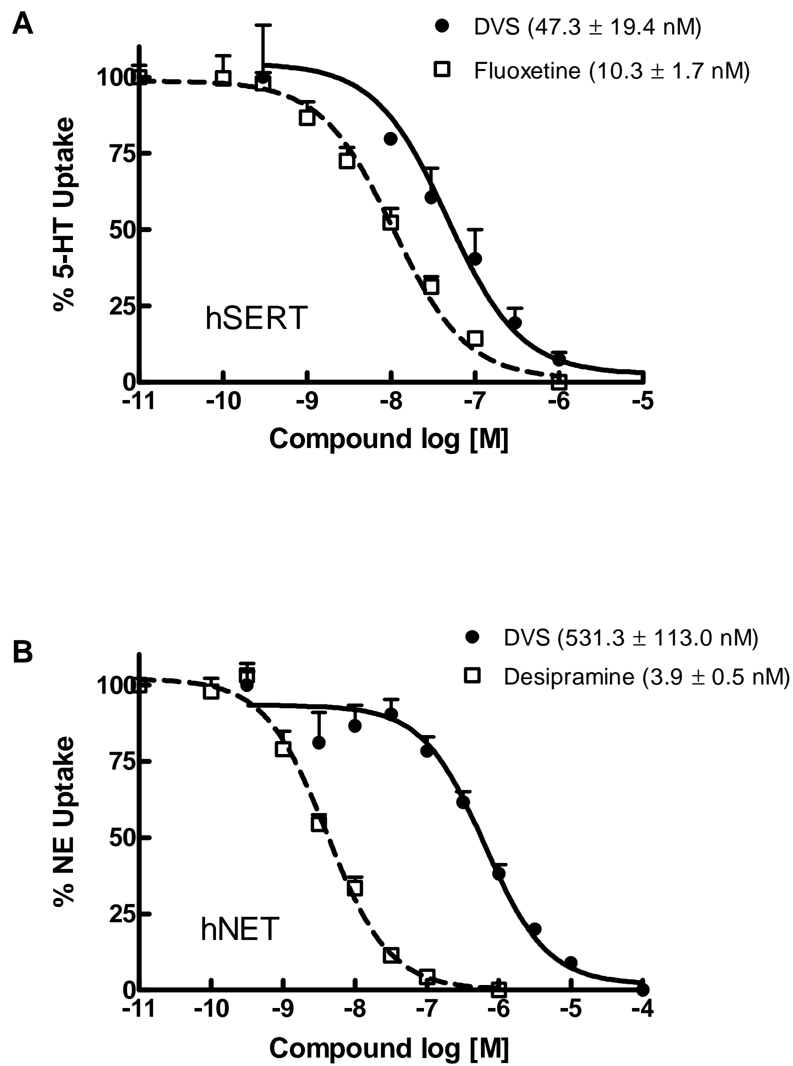


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Figure 3

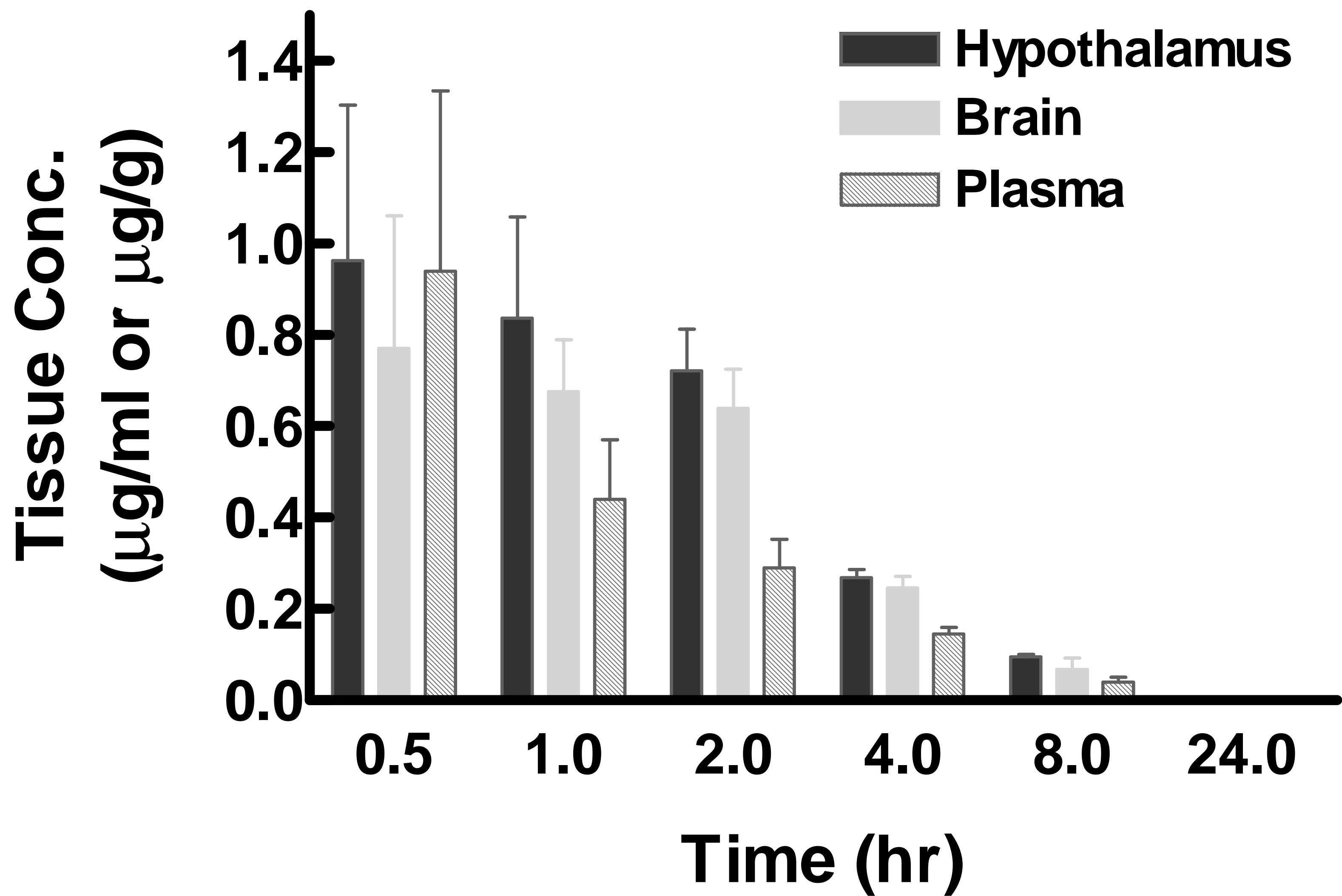


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Figure 4A

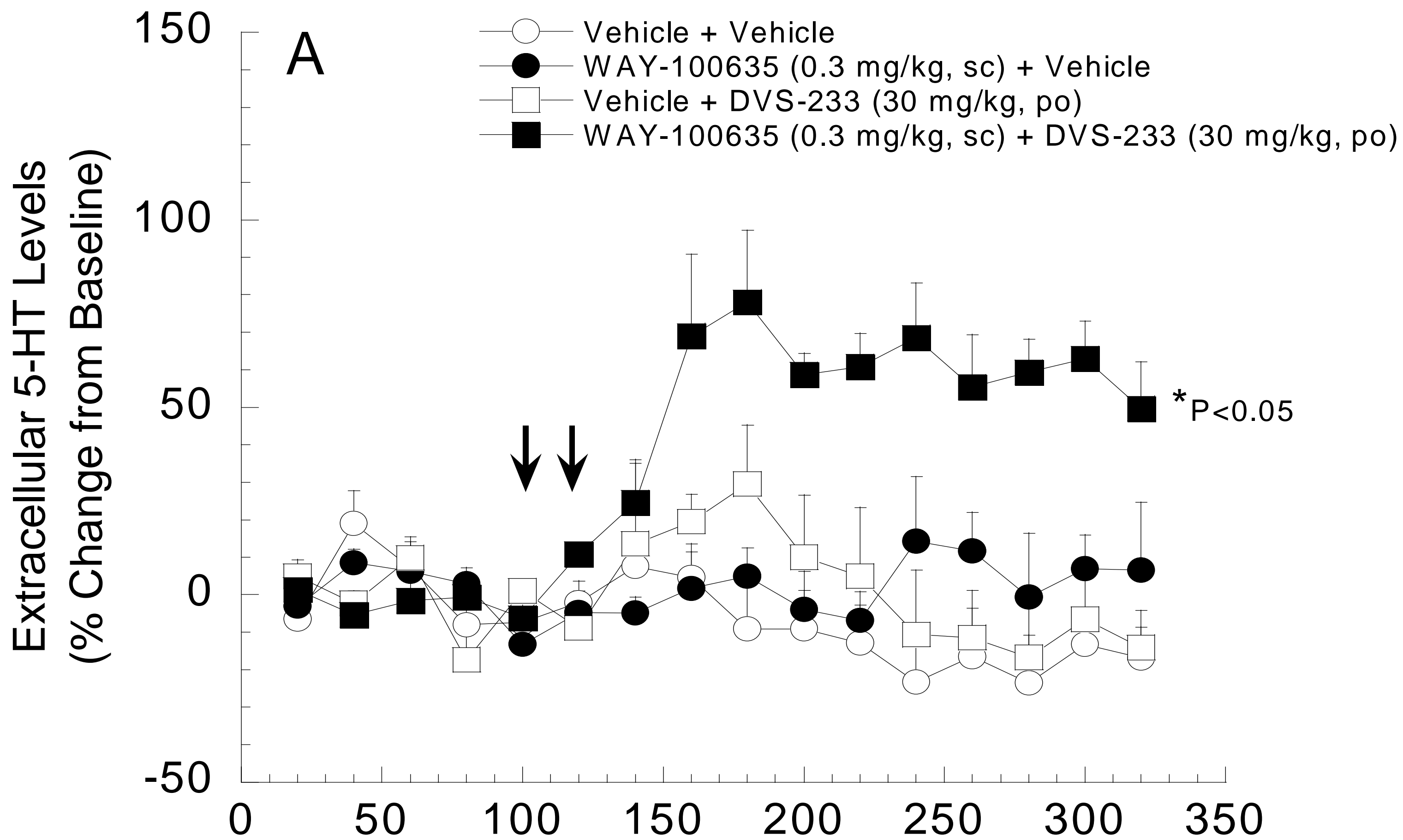


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Figure 4B

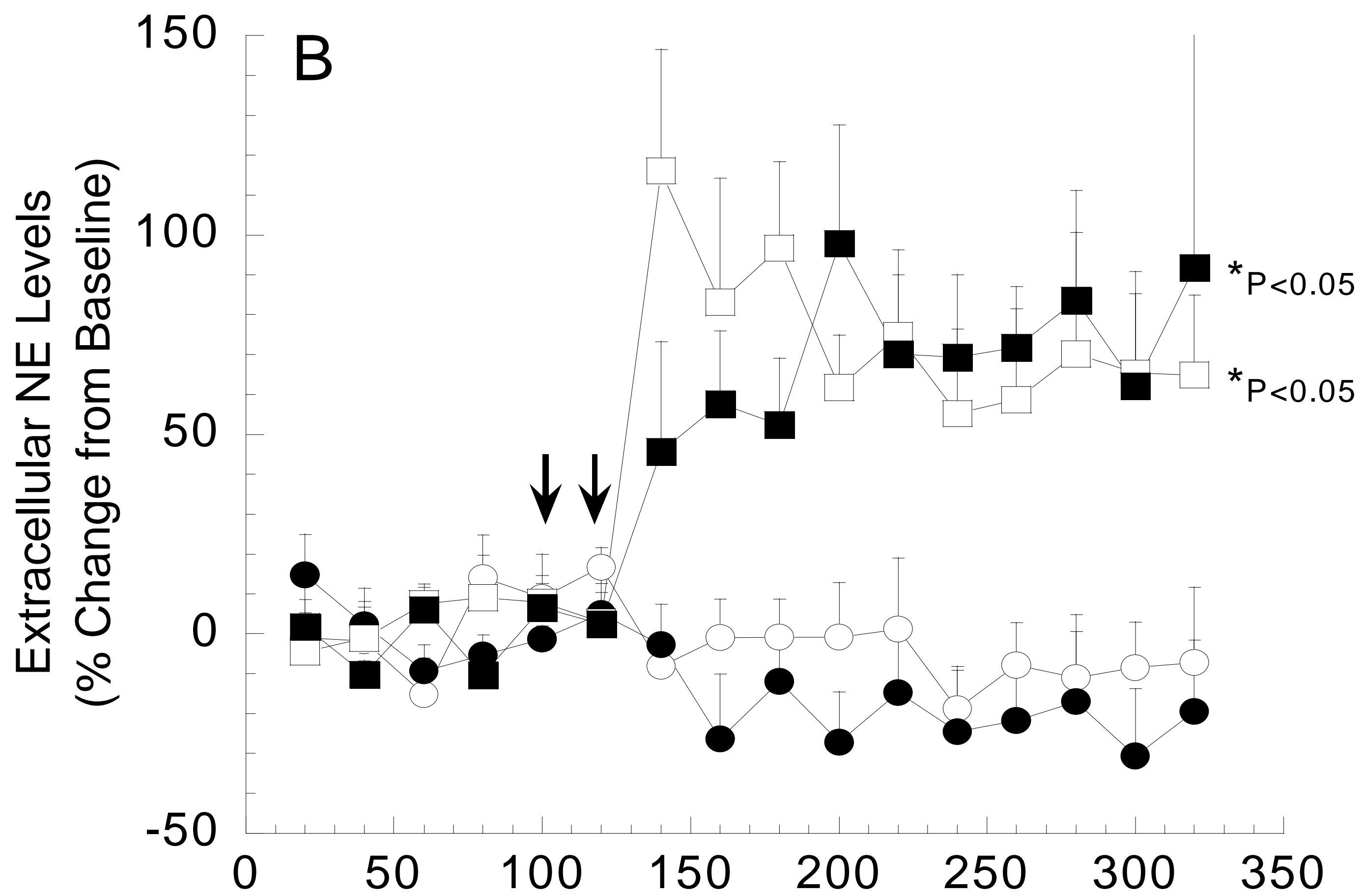


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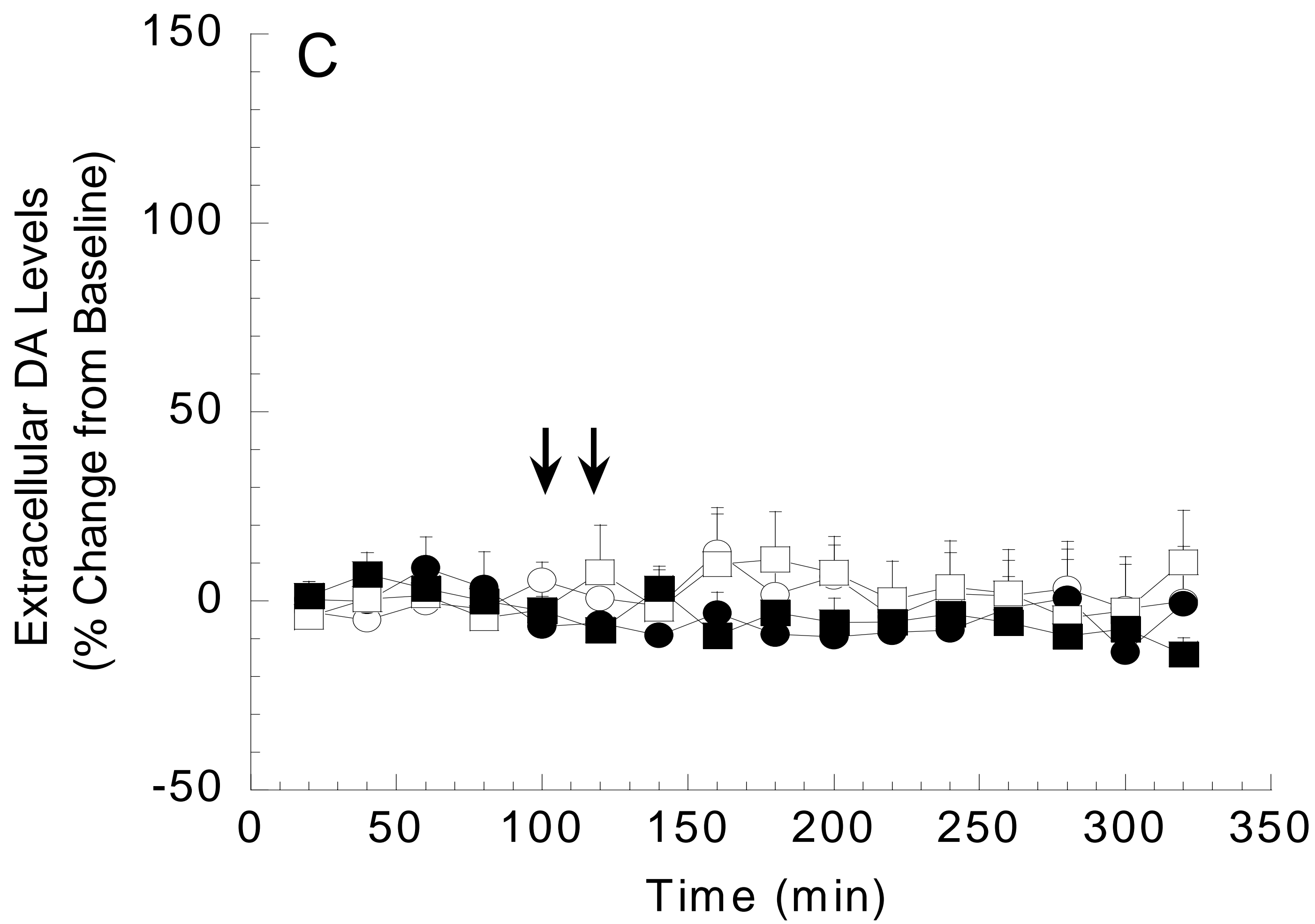
Figure 4C

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