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# Reserpine-induced Reduction in Norepinephrine Transporter Function Requires Catecholamine Storage Vesicles

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## Abstract

Treatment of rats with reserpine, an inhibitor of the vesicular monoamine transporter (VMAT), depletes norepinephrine (NE) and regulates NE transporter (NET) expression. The present study examined the molecular mechanisms involved in regulation of the NET by reserpine using cultured cells. Exposure of rat PC12 cells to reserpine for a period as short as 5 min decreased [$^3$H]NE uptake capacity, an effect characterized by a robust decrease in the $V_{max}$ of the transport of [$^3$H]NE. As expected, reserpine did not displace the binding of [$^3$H]nisoxetine from the NET in membrane homogenates. The potency of reserpine for reducing [$^3$H]NE uptake was dramatically lower in SK-N-SH cells that have reduced storage capacity for catecholamines. Reserpine had no effect on [$^3$H]NE uptake in HEK-293 cells transfected with the rat NET (293-hNET), cells that lack catecholamine storage vesicles. NET regulation by reserpine was independent of trafficking of the NET from the cell surface. Pre-exposure of cells to inhibitors of several intracellular signaling cascades known to regulate the NET, including $Ca^{2+}$/$Ca^{2+}$-calmodulin dependent kinase and protein kinases A, C and G, did not affect the ability of reserpine to reduce [$^3$H]NE uptake. Treatment of PC12 cells with the catecholamine depleting agent, α-methyl-*p*-tyrosine, increased [$^3$H]NE uptake and eliminated the inhibitory effects of reserpine on [$^3$H]NE uptake. Reserpine non-competitively inhibits NET activity through a $Ca^{2+}$-independent process that requires catecholamine storage vesicles, revealing a novel pharmacological method to modify NET function. Further characterization of the molecular nature of reserpine's action could lead to the development of alternative therapeutic strategies for treating disorders known to be benefitted by treatment with traditional competitive NET inhibitors.

### Keywords

norepinephrine; noradrenergic; norepinephrine transporter; antidepressant; reserpine; secretory vesicle; synaptic vesicle; storage vesicle



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The norepinephrine transporter (NET) is a $Na^+/Cl^-$-dependent transporter responsible for limiting the synaptic action of norepinephrine (NE). The function of the NET is inhibited by several psychotherapeutic drugs that directly bind to the NET, such as desipramine, cocaine, and atomoxetine. Besides acute drug effects on the NET, NET function is up- and down-regulated by exposures to several drugs, by activation of specific signaling pathways, and by physiological conditions (Mandela and Ordway, 2006b). Drugs that regulate the NET include compounds that bind directly to the NET such as desipramine, and also drugs and substances such as acetylcholine, that activate receptors and/or intracellular signaling cascades triggering changes in NET function. Abnormal function of the NET has been implicated in cardiovascular and psychiatric disorders (Benmansour et al., 2004; Hahn and Blakely, 2002; Klimek et al., 1997; Stober et al., 1999; Ungerer et al., 1996; Xu et al., 2000).

One drug that affects NET function but does not appear to bind to catecholamine transporters is reserpine (Lee et al., 1983). Reserpine binds to the vesicular monoamine transporter with high affinity ($K_i$ at subnanomolar concentration) (Henry et al., 1998), blocking neurotransmitter uptake into the vesicle and ultimately depleting catecholamines from storage vesicles (Henry et al., 1987; Kirshner, 1962a, b). The actions of reserpine on the VMAT result in an acute catecholamine release, followed by chronic inhibition of catecholamine secretion, as a result of diminished releasable stores of vesicular catecholamine (Carmichael et al., 1980; Slotkin and Edwards, 1973). Reserpine-induced depletion of catecholamines is also observed in cell systems, e.g. PC12 and adrenal chromaffin cells (Drukarch et al., 1996; Wolkersdorfer et al., 1996). Repeated treatment of rats with reserpine decreases [$^3$H]NE uptake and decreases [$^3$H]desipramine binding to the NET in the cerebral cortex, an effect partially reversed by the monoamine oxidase inhibitor, pargyline (Lee et al., 1983). It has been presumed that this effect of reserpine on the NET is secondary to its NE depleting effect. However, the cellular mechanisms involved in reserpine-induced regulation of NET function remain unknown. Furthermore, if reserpine directly affects NE uptake, rather than or in addition to inducing a regulation of NET function secondary to VMAT inhibition, such actions could contribute to the physiological effects of reserpine. For example, a reduction of NE uptake at the synaptic plasma membrane would be expected to facilitate NE depleting effects of reserpine. In addition, the interpretation of some data collected using reserpine as a tool in pharmacological studies may require reconsideration if reserpine directly affects NET function in addition to inhibition of the VMAT.

Recent studies demonstrate that reserpine inhibits dopamine transporter activity through an unknown VMAT-dependent and substrate-independent mechanism (Metzger et al., 2002; Yamamoto et al., 2007), and that VMAT-containing synaptic vesicles dock with the dopamine transporter to increase its activity (Egana et al., 2009). If these effects extend to the NET, then reserpine's effects on NE uptake as observed originally by Lee et al. (1983) may be secondary to actions at the VMAT that are unrelated to substrate-mediated compensations. The present study investigated the effect of reserpine on NET function using three cell lines that express the NET, i.e. rat PC12 cells, human SK-N-SH cells, and a kidney cell line (human embryonic kidney cells, HEK-293) transfected with the rat NET(293-rNET). PC12 cells express the native NET, but not the DAT (Lorang et al., 1994), and also synthesize and store catecholamine in an abundance of catecholamine storage vesicles (Wagner, 1985). SK-N-SH cells express the native NET (Richards and Sadee, 1986) and have high dopamine beta hydroxylase activity (Biedler et al., 1978), but have limited ability to store catecholamine and have few catecholamine storage vesicles (Smets et al., 1990; Smets et al., 1989). The 293-rNET cell is a transfected kidney cell line that does not synthesize or store catecholamines. The potential molecular mechanisms of reserpine's action on the NET were also investigated, focusing on signaling pathways known to regulate NET function (Apparsundaram et al., 1998a; Bonisch et al., 1998; Jayanthi et al., 2004;

Mandela and Ordway, 2006b; Uchida et al., 1998). Our findings demonstrate that reserpine non-competitively regulates NET activity through a $Ca^{2+}$-independent action that does not appear to involve changes in cytosolic or extracellular NE concentrations, but may involve interference with the interaction of the catecholamine storage vesicle with the NET.

## EXPERIMENTAL PROCEDURES

### Cell culture

PC12 cells and SK-N-SH cells were obtained from ATCC (ATCC, Rockville, MD, USA). HEK-293 cells transfected with the rat NET (293-rNET) were generously supplied by Dr. Heinz Bönisch (Institute of Pharmacology & Toxicology, University of Bonn). PC12 cells were grown as previously described (Mandela and Ordway, 2006a). SKN-SH cells were grown in minimum essential medium (Eagle; ATCC, Rockville, MD, U.S.A.) with 2 mM L-glutamine and Earle's BSS salts (ATCC) and fetal bovine serum (10%). Culture medium and supplements were obtained from ATCC. 293-rNET cells were grown in Dulbecco's Modified Eagle's Medium/Ham's F12 Medium (ATCC) supplemented with 4 mM *L*-glutamine, 10% fetal bovine serum, penicillin (100 u/ml) and streptomycin (100 μg/ml). Cells were grown in 75 $cm^2$ flasks and later transferred to 6 well plates, 3 days prior to initiating experiments.

### Uptake assay

Uptake assays were carried out as described by Mandela and Ordway (2006a). Both PC12 and SK-N-SH cells were then pre-incubated for 5 min at 37°C in KRH buffer (130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 10 mM HEPES, 10 mM D-glucose) for equilibration. KRH buffer was aspirated and fresh KRH buffer containing inhibitors of catecholamine metabolism (100 μM pargyline, 10 μM U-0521 and 100 μM ascorbic acid) with or without modulating agents were added to the cells. In treatment groups, drugs were present throughout the reserpine treatment phase and also during the uptake assay. Uptake assays were initiated by adding 85 nM *l*-[$^3$H]NE (Perkin-Elmer Life Science, Boston, MA, USA ) for 5 min. Uptake was terminated by two rapid washes with ice-cold KRH buffer. In some experiments, assays were carried out using a $Ca^{2+}$-free buffer by eliminating $Ca^{2+}$ salts in the buffer preparations. $Ca^{2+}$ free buffer was used in all experiments specifically designed to examine the role of $Ca^{2+}$ in reserpine-induced effects on NET function. Treatments with α-methyl-p-tyrosine (AMPT) were carried out 24 hours prior to uptake assays. Cells were lysed with 0.1% v/v Triton X-100 lysis buffer (5 mM Tris HCl, pH 7.4) and 1 ml of this fraction was used to quantify the radioactivity accumulated using a liquid scintillation counter (Beckman LS3801, Irvine, CA). An example of the DPMs collected on a single experimental day in which the effect of 50 nM reserpine on [$^3$H]NE uptake in PC12 cells was measured is: control total uptake 29,051 DPMs, control non-specific uptake 819 DPMs; reserpine total uptake 3522 DPMs, reserpine non-specific uptake 723 DPMs. Protein concentration extracted from each cell culture well was determined using the bicinchoninic acid method (Pierce Biotechnology, Inc, Rockford, IL, USA). [$^3$H]NE uptake or binding not associated with the NET (5-10% of total uptake) was determined in the presence of 10 μM desipramine, and specific uptake (as presented) was computed by subtracting nonspecific uptake from total uptake. Where a single concentration of [$^3$H]NE was used, results were expressed as a percentage of control for the paired control and treatment condition. For uptake saturation kinetics, uptake was measured with various concentrations of [$^3$H]NE ranging from 0.25 μM to 4 μM, with the time period for uptake at each concentration remaining constant (5 min).

### Reserpine-stimulated release of [$^3$H]NE

PC12 and SK-N-SH cells were incubated for 3 h with 50 nM [$^3$H]NE in 2 ml of the appropriate growth medium (see above). Cells were then washed 3 times with warm KRH buffer (37°C). After 3 washes, fresh KRH buffer was added and cells were allowed an equilibration period of 5 min. Cells were treated with KRH buffer containing reserpine (50 nM) for 30 min. After reserpine treatment, 1 ml of buffer was collected to determine radioactivity present in the extracellular medium. Cells were washed with cold KRH buffer and lysed with Triton X-100 buffer (see above) to determine intracellular stores of [$^3$H]NE. Radioactivity was assayed by liquid scintillation counting, and results were expressed as percentage of basal (without reserpine) release. Net [$^3$H]NE release is defined as reserpine-induced release minus basal release. Protein concentrations of cell lysates were measured using the bicinchoninic acid method and were used to normalize data from uptake assays.

### Whole cell binding assays

NET surface density on intact PC12 cells was measured using radioligand binding assays with a high affinity NET-selective ligand, [$^3$H]nisoxetine, in intact cells as previously described by Mandela and Ordway (2006a). Following reserpine treatment at 37°C, cells were rapidly washed 3 times with ice-cold binding buffer (NaCl 150 mM, KCl 5 mM, $MgSO_4$, 1 mM, Tris 10 mM and 0.1% BSA at pH 7.4, 4°C). The assay from this point forward was performed at 4°C. After the 3 washes, cells were incubated in the binding buffer for 5 min prior to the addition of the radioligand. Various concentrations of [$^3$H]nisoxetine (0.1 - 10 nM) were added to initiate the binding reactions. The reaction mixture was incubated at 4°C (total volume 1 ml) for 1 h. Binding assays were terminated by washing cells 3 times with ice-cold binding buffer. Cell extracts were prepared with Triton X-100 lysis buffer and bound radioactivity was quantified using scintillation counter. A portion of the cell extracts were analyzed for protein content using the bicinchoninic acid method. Nonspecific binding was determined using 10 μM desipramine.

### Homogenate binding assay

The binding of [$^3$H]nisoxetine to crude 293-rNET membranes was assayed as described by Zhu and Ordway (1997). After homogenization and washing of cell membranes, the membrane preparation (~100 μg of protein) was added to a reaction mixture at 4°C (total volume of 250 μl) containing 4.5 nM [$^3$H]nisoxetine (American Radiolabeled Chemicals, St. Louis, MO, U.S.A.), and reserpine at the concentrations specified. The presence or absence of 10 μM desipramine was used to define non-specific binding. The reaction mixtures (in triplicate) were incubated at 4°C for 4 h and processed as describe previously. Radioactivity was measured by liquid scintillation counting.

### Statistics

All experimental data are presented in the text and graphs as the mean ± SEM, with individual measurements measured in triplicate in experiments performed on 2-3 separate days. Data were analyzed by analysis of variance (ANOVA) when multiple treatment groups were compared. When the ANOVA revealed a significant treatment effect, post hoc analyses were performed using the Newman-Keuls multiple comparison test to examine individual group differences, except for time course data that was analyzed post hoc using Dunnett's multiple comparison test. $B_{max}$, $V_{max}$, $K_D$ and $K_m$ values were computed using non-linear regression analysis. The unpaired Student's t-test was used to analyze data when there were only two groups in a comparison. The p level for statistical significance was set at 0.05. All statistical and non-linear regression analyses were performed using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA, U.S.A.).

# RESULTS

## Time course of the effect of reserpine on [$^3$H]NE uptake

In initial experiments, the time course of the effect of reserpine (50 nM) on [$^3$H]NE uptake was determined. This concentration of reserpine was chosen because preliminary experiments demonstrated that it produced a robust reduction in uptake after a 30 min exposure. Exposure of PC12 cells to reserpine decreased [$^3$H]NE uptake in a time-dependent manner, reaching near maximum inhibition at 30 min (Figure 1 ). Exposure of PC12 cells to reserpine for 24 h decreased [$^3$H]NE uptake to 22 ± 4% of control, i.e. to nearly the same extent that was observed at the 30 min period of exposure. The uptake of [$^3$H]NE was saturable in both control and reserpine-treated cells (Figure 2). Nonlinear regression analyses demonstrated that the concentration for half-maximum saturation of uptake ($K_m$) was modestly reduced by reserpine (50 nM) exposure (control 0.97±0.12 μM; reserpine 0.46 ± 0.10 μM ($p < 0.05$). In contrast, reserpine treatment markedly decreased the maximum uptake velocity ($V_{max}$ ) by 58% (control 815 ±42 fmol/mg protein/min; reserpine 344±22 fmol/mg protein/min; $p<0.001$).

## Potential role of cations and protein kinase pathways in mediating the effects of reserpine on [$^3$H]NE uptake

If reserpine affects uptake by altering the ionic environment at the transporter, then uptake by other $Na^+$-dependent transporters that are similarly affected by ions (Friedrich and Bonisch, 1986; Mundorf et al., 2000) would be expected to be altered. To evaluate this possibility, [$^3$H]alanine and [$^3$H]NE uptake were measured in parallel following treatment or PC12 cells with reserpine. In contrast to [$^3$H]NE uptake, reserpine treatment did not affect [$^3$H]alanine uptake ([$^3$H]NE uptake after reserpine treatment as a percent of control, 23 ± 4; [$^3$H]alanine uptake after reserpine treatment as percent of control, 95 ± 6).

Given that reserpine displaces $Ca^{2+}$ from vesicles at concentrations near its $K_D$ for the VMAT (Martinez et al., 1998; Mundorf et al., 2000), we considered the possibility that reserpine-induced changes in NET function were mediated by $Ca^{2+}$. PC12 cells were treated with either the intracellular $Ca^{2+}$ chelator BAPTA/AM (50 nM) or the extracellular $Ca^{2+}$ chelator BAPTA (50 nM) in a $Ca^{2+}$ free environment for 30 min prior to reserpine. $Ca^{2+}$ chelators did not diminish reserpine-induced inhibition of NE uptake (Figure 3). To evaluate a potential role of CaMK, cells were treated with either KN93 (10 μM), a CaMK inhibitor, or with the inactive analogue KN92 (10 μM) for 30 min prior to reserpine treatment (Figure 4). KN93 diminished basal [$^3$H]NE uptake , and KN93 treatment prior to reserpine exposure reduced [$^3$H]NE uptake to a greater extent than either treatment alone. KN92, under the same conditions and at the same concentration, had no effect on uptake by itself and had no effect on the reserpine-induced decrease in [$^3$H]NE uptake.

One of the major molecular mechanisms regulating NET function is phosphorylation. Intracellular signaling systems like PKC, PKA, cGMP and protein phosphatases all have been shown to phosphorylate or dephosphorylate NET and thus alter its function and plasma membrane expression (Mandela and Ordway, 2006b). We considered that reserpine may recruit one or more of these systems to alter NET function. Treatment of PC12 cells with the cAMP analogue 8-bromo-adenosine 3′,5′-cyclic monophosphate (8-bromo-cAMP, 2 mM) alone for 30 min significantly diminished [$^3$H]NE uptake (Figure 5). Pretreatment with the staurosporine (100 nM) prevented the 8-bromo-cAMP-induced decrease in [$^3$H]NE uptake. PC12 cells treated with 8-bromo-cAMP for 30 min prior to reserpine treatment decreased [$^3$H]NE uptake to a greater extent than 8-bromo-cAMP or reserpine alone. Pretreatment of cells with 50 nM PMA (phorbol-12-myristate-13-acetate), a PKC agonist, alone for 30 min significantly diminished [$^3$H]NE uptake (Figure 6). Pretreatment of PC12 cells with 150 nM

staurosporine abolished the PMA-induced inhibition of [$^3$H]NE uptake. Pretreatment with PMA for 30 min prior to reserpine treatment resulted in a decrease in [$^3$H]NE uptake larger than the reductions in uptake induced by PMA or reserpine treatment alone. Pretreatment of cells with staurosporine did not alter basal [$^3$H]NE uptake and did not diminish the ability of reserpine to decrease [$^3$H]NE uptake. A 30 min pretreatment of PC12 cells with staurosporine, respectively, did not alter basal [$^3$H]NE uptake or the reserpine-induced decrease in [$^3$H]NE uptake. Treatment with 8-bromo-guanosine 3′,5′-cyclic monophosphate (8-bromo-cGMP ; 2 mM), a cGMP analogue, alone had no significant effect on [$^3$H]NE uptake (Figure 7). Similarly, PC12 cells treated with 8-bromo-cGMP for 30 min prior to reserpine treatment, did not augment or reduce the reserpine-induced decrease in [$^3$H]NE uptake. Pretreatment with the PKG inhibitor KT5823 (1 μM) did not alter basal [$^3$H]NE uptake or the reserpine-induced decrease in [$^3$H]NE uptake.

### Plasma membrane expression of NET following reserpine treatment

To determine whether reserpine might affect trafficking of the NET from the cell surface, experiments were performed to quantify saturation binding of [$^3$H]nisoxetine binding to intact PC12 cells following reserpine treatment under conditions wherein [$^3$H]nisoxetine binds to surface, but not intracellular, NET (Apparsundaram et al., 1998a; Jayanthi et al., 2004). Experiments first verified that this assay detects a 25% reduction in the binding of [$^3$H]nisoxetine (5 nM) to the NET in PC12 cells treated with 1 μM PMA, similar to that observed by others (Apparsundaram et al., 1998a; Jayanthi et al., 2004). Non-linear regression analyses of [$^3$H]nisoxetine binding to intact cells revealed that a 30 min exposure with reserpine (50 nM) did not significantly change $K_D$ or $B_{max}$ values of [$^3$H]nisoxetine at the NET. (Figure 8; Table 1). Similarly, a 24 h exposure to reserpine had no effect on the $K_D$ or $B_{max}$ values of [$^3$H]nisoxetine (Table 1).

### The role of the catecholamine storage vesicle in the effect of reserpine on [$^3$H]NE uptake

Experiments were performed to determine whether reserpine produced its effects directly at the NET, or whether the VMAT and/or catecholamine storage vesicles were required. First, membrane homogenates of 293-rNET cells were incubated with [$^3$H]nisoxetine in the absence and presence of reserpine. At concentrations ranging from 100 nM to 10 μM, reserpine did not reduce the binding of [$^3$H]nisoxetine to the NET, whereas desipramine (10 μM) reduced binding by 80% (data not shown). As opposed to PC12 cells (high capacity for catecholamine storage with many storage vesicles), concentrations of reserpine required to inhibit the uptake of [$^3$H]NE by 50% in SK-N-SH cells (low catecholamine storage capacity with few storage vesicles) were nearly 500-fold higher (Figure 9). Furthermore, reserpine had no effect on uptake in 293-rNET cells (no catecholamine storage vesicles).

Since reserpine induces release of neurotransmitter from the storage vesicles (Kanner et al., 1979; Slotkin and Edwards, 1973), we considered that reserpine effects on release may be linked mechanistically to its effects on uptake. Both PC12 and SK-N-SH cells were loaded with 50 nM [$^3$H]NE for 3 h prior to exposure to reserpine. In PC12 cells, treatment with 50 nM reserpine induced a robust time-dependent increase in [$^3$H]NE release compared to controls (Figure 10a). In contrast, exposure of SK-N-SH cells to reserpine at a concentration (5 μM) that inhibited [$^3$H]NE uptake (see Figure 9) had no effect on [$^3$H]NE release (Figure 10b). Since reserpine induced release of NE from PC12 cells, it seemed possible that unlabeled NE released into the extracellular millieu during reserpine exposure may compete with the uptake of [$^3$H]NE uptake in these cells. However, repeated cell washings after the reserpine exposure did not diminish the effect of reserpine on [$^3$H]NE uptake (data not shown). To evaluate potential contributions of intracellular NE to reserpine's effect on uptake, PC12 cells were treated with AMPT for 24 h prior to exposure to reserpine. Exposures of AMPT were those previously shown to reduce dopamine concentrations in

PC12 cells by 50% (0.1 mM), 70% (0.3 mM), and 85% (1 mM) (Drukarch et al., 1996). AMPT at 0.3 and 1 mM abolished the inhibitory effect of reserpine on [$^3$H]NE uptake (Figure 11), while simultaneously increasing [$^3$H]NE uptake. To determine whether a rise in cytosolic NE might mediate an effect of reserpine on the NET, cells were treated with AMPT (0.3 mM; to reduce vesicular NE) for 24 h, followed by reserpine (to prevent uptake of cytosolic NE into vesicles) for 30 min, then preloaded with 0.1 μM NE for 3, 10 and 30 min. Immediately following preloading, [$^3$H]NE uptake was measured under standard conditions used above to measure the effect of reserpine on uptake. Preloading the cytosol of PC12 cells with NE had no effect on [$^3$H]NE uptake (expressed as a percent of no preloading control: 3 min, 105±10; 10 min 110±10; 30 min, 92±10; n=6).

## DISCUSSION

The current study demonstrates a unique mechanism by which reserpine can modulate noradrenergic transmission. Reserpine produces catecholamine depletion from sympathetic nerve endings and from catecholamine-synthesizing cell lines, leading to a diminished catecholamine release during a secretory event (Kanner et al., 1979; Slotkin and Edwards, 1973). This effect of reserpine is secondary to blockade of catecholamine storage through its high affinity binding to the VMAT (Henry et al., 1987; Kirshner, 1962a, b). Reserpine produces an acute catecholamine release, followed by chronic inhibition of catecholamine secretions as a result of a diminished releasable pool of transmitter (Carmichael et al., 1980; Slotkin and Edwards, 1973). Lee et al. (1983) demonstrated that treatment of rats with reserpine decreases radioligand binding to the NET and decreases NE uptake, despite its a very low affinity for the NET. These authors hypothesized that NE uptake regulation by reserpine was a homeostatic mechanism by which synaptic concentrations of transmitter were adjusted following depletion of catecholamine stores. The present study demonstrated that reserpine produces a rapid reduction in the function of the NET in cultured PC12 cells, and that this effect does not require changes in levels of NET protein or the surface expression of the NET. The ability of reserpine to inhibit the NET was dependent on the presence of catecholamine storage vesicles, suggesting that the effect of reserpine on the NET is secondary to binding of reserpine to the VMAT. This effect of reserpine implicates a role of the catecholamine storage vesicle in regulating NET activity, as has been demonstrated previously for the DAT (Egana et al., 2009; Metzger et al., 2002; Yamamoto et al., 2007).

One could speculate that a reserpine-induced release of NE (Carmichael et al., 1980; Slotkin and Edwards, 1973) into the extracellular milieu of the cultured cells contributed to the reserpine-induced decrease in [$^3$H]NE uptake. However, several findings argue against this mechanism. First, reserpine robustly decreased the Vmax, while producing a slight decrease in the $K_m$ of [$^3$H]NE uptake. If the effect were secondary to increased extracellular levels of unlabelled NE, one would have expected the $K_m$ of [$^3$H]NE uptake to be primarily affected, and to be increased. Second, reserpine exposure induced [$^3$H]NE release from PC12 cells, but not from SK-N-SH cells, whereas reserpine was capable of reducing [$^3$H]NE uptake in both cell lines, albeit at a much higher concentration in SK-N-SH cells. Finally, repeated washings of cells following reserpine exposure did not diminish the effect of reserpine on uptake. These findings suggest that extracellular NE does not contribute to reserpine-induced regulation of NET function in PC12 cells.

Given that trafficking to and from the plasma membrane is an important regulatory pathway for the NET, it seemed likely that reserpine might reduce uptake by stimulating redistribution of the NET away from the cell surface. In support of this mechanism, reserpine treatment decreased $V_{max}$ [$^3$H]NE uptake. However, whole cell binding assays did not demonstrate changes in the binding of [$^3$H]nisoxetine to surface-expressed NET

following reserpine treatment. This whole cell binding assay provides accurate estimates of plasma membrane NET, as has been verified with parallel biotinylation experiments (Apparsundaram et al., 1998a; Distelmaier et al., 2004; Jayanthi et al., 2004), and its utility in detecting PMA-induced redistribution of the NET from the surface (Apparsundaram et al., 1998b; Jayanthi et al., 2004) was also verified in our laboratory. Hence, the present findings suggest that reserpine treatment decreases NET function (decreases $V_{max}$) via a mechanism independent of NET trafficking. Trafficking-independent changes of NE uptake are not uncommon as similar findings have been reported for NET and serotonin transporter regulation (Apparsundaram et al., 2001; Jayanthi et al., 1994; Mandela and Ordway, 2006b).

A number of intracellular signals that could contribute to the effect of reserpine on NET activity were ruled out. Reserpine can induce the release of $Ca^{2+}$ ions from the vesicular stores (Martinez et al., 1998; Mundorf et al., 2000), an effect that is presumably secondary to disruption of VMAT function. However, pretreatment of PC12 cells with intracellular or extracellular $Ca^{2+}$ chelators, i.e. BAPTA/AM and BAPTA, respectively, did not diminish reductions of [$^3$H]NE uptake produced by reserpine exposure. In addition, exposure of PC12 cells to reserpine did not affect of the uptake of [$^3$H]alanine via a related $Na^+$-dependent neutral amino acid transporter that shares similar ionic requirements and pH sensitivity as the NET. Several kinase pathways have been shown to regulate NET function (Apparsundaram et al., 1998b; Bonisch et al., 1998; Bryan-Lluka et al., 2001; Jayanthi et al., 2004; Mandela and Ordway, 2006b; Uchida et al., 1998). However, stimulation and/or inhibition of these pathways, including PKC, cAMP/PKA, cGMP/PKG, and CaMK pathways, did not interfere with reserpine's effect on [$^3$H]NE uptake. Inhibition of CaMK alone decreased [$^3$H]NE uptake, an effect also observed for [$^3$H] dopamine uptake by the DAT (Padmanabhan et al., 2008). The combination of reserpine and CaMK inhibition had an additive effect on inhibition of uptake, suggesting that the two treatments work through different mechanisms. Hence, CaMK function appears to be vital for basal NET activity but does not play a role in the regulation of NET function following reserpine treatment.

The high affinity of reserpine for the VMAT and the necessity of the presence of catecholamine storage vesicles for reserpine's inhibitory effect on NET activity are consistent with the notion that changes in NET function are secondary to binding of reserpine to the VMAT on the vesicle, similar to the effect of reserpine on DAT function (Metzger et al., 2002; Yamamoto et al., 2007). In fact, the potency of reserpine to inhibit [$^3$H]NE uptake was approximately 500-fold higher in PC12 cells than in SK-N-SH cells, cells that have few catecholamine storage vesicles (Smets et al., 1990; Smets et al., 1989). Reserpine had no effect on [$^3$H]NE uptake in 293-rNET cells, cells lacking VMAT and catecholamine storage vesicles. Also consistent with the role of the storage vesicle, reserpine did not displace [$^3$H]nisoxetine from the NET in membrane homogenates of 293-rNET cells.

Another possible explanation for allosteric modulation of the NET by reserpine is that reserpine binds to the VMAT and thereby hinders a normal facilitative interaction of the secretory vesicle with the NET. As mentioned above, regulatory effects of reserpine and synaptic vesicles on the function of the DAT have been reported previously. Metzger et al. (2002) demonstrated that dopamine uptake via the DAT is diminished following reserpine treatment of rats and following reserpine exposure of cultured cells expressing the DAT. Dopamine uptake is unaffected by reserpine in tissues from transgenic mice lacking VMAT2, suggesting a connection between the effect of reserpine on VMAT2 and the ultimate reduction in DAT activity. Similarly, Yamamoto et al. (2007) demonstrated a loss of reserpine-induced reduction of dopamine uptake in cultured mesencephalic neurons from homozygous, but no heterozygous, VMAT2 knockout mice. Using PC12 cells transfected with both the DAT and synaptogyrin-3, Egaña et al. (2009) provided evidence that synaptogyrin-3, a protein located in the synaptic vesicle, docks with the N-terminal end of

the DAT to increase its activity. Furthermore, this increase in activity was blocked by reserpine. Based on available data, Egana et al. speculated that synaptogyrin-3 might allow the vesicle to dock to the DAT to permit a more efficient loading of vesicles. Their data demonstrate, however, that synaptogyrin-3 does not bind to the NET. Although not suggested by Egaña et al., it seems possible that reserpine's binding to the VMAT may directly interfere with the ability of synaptogyrin-3 to dock to the DAT. The present study demonstrates that reserpine is also capable of inhibiting the NET. As is the case for the DAT, reserpine-inhibition of the NET requires the presence of the catecholamine storage vesicle. This property suggests that as for the DAT, a potential role of the catecholamine storage vesicle in regulating NET function also exists, albeit apparently through a protein different from synaptogyrin-3.

Yamamoto et al. (2007) performed preloading experiments with [$^3$H]DOPA, demonstrating that the effect of reserpine on DAT function were not substrate-dependent. Likewise, the present study demonstrated that preloading cells with unlabelled NE, in the presence of inhibitors of COMT and MAO, did not affect [$^3$H]NE uptake under conditions used to demonstrate reserpine-induced reductions in [$^3$H]NE uptake, suggesting that the effects of reserpine on NET activity are also not substrate-dependent. Nevertheless, exposure of cells to AMPT, under conditions known to reduce PC12 catecholamine concentrations by 70-85% (1 μM; Drukarch et al., 1996), abolished the inhibitory effect of reserpine on [$^3$H]NE uptake. Interestingly, [$^3$H]NE uptake was significantly elevated by exposure of cells to AMPT at concentrations that blocked reserpine's action on the NET. It is tempting to speculate that depletion of catecholamine stores increases the interaction of the storage vesicle with the NET, thereby increasing the activity of the NET. While docked, reserpine would presumably have access to VMAT sites on the vesicle that are not sterically hindered by the docking of the vesicle to the NET, but prior docking of the vesicle to the NET would prevent reserpine from binding to VMAT sites that would normally hinder the vesicle-NET interaction. Studies designed to further investigate potential mechanisms of reserpine-induced inhibition of NET function may help to unravel the role of the catecholamine storage vesicle in regulating NET activity.

It should be noted that PC12 cells use large dense core vesicles to store and release catecholamines, whereas vesicular storage in norepinephrine neurons of the brain is handled by synaptic vesicles. Nonetheless, both types of storage vesicles express the VMAT2, and inhibition of NET function by reserpine has been demonstrated in rat brain (Lee et al., 1983) and in cultured cells (present study). A caveat to studies using the PC12 cell line is that there may be subtle differences between large dense core vesicles and synaptic vesicles with regard to trafficking to the plasma membrane and the mechanism action of reserpine.

In summary, reserpine induces a rapid non-competitive inhibition of NE uptake in PC12 cells. The effect of reserpine on the NET requires the presence of VMAT and catecholamine storage vesicles, and does not appear to be secondary to changes in cytosolic or extracellular NE concentrations or in surface expression of the NET. Inhibition of catecholamine biosynthesis blocks the inhibitory effect of reserpine on NET activity. These findings contribute to growing evidence of involvement of synaptic vesicles in the regulation of NET function and provide insight into a novel action of reserpine to modulate noradrenergic transmission. Uncovering the intracellular mechanism for the VMAT/synaptic vesicle-dependent regulation of NET function could lead to the identification of a unique drug target for manipulating NET activity. The discovery of an allosteric inhibitor of NET function that lacked NE depleting properties could open the door to a novel therapeutic strategy for treating disorders, e.g. attention-deficit hyperactivity disorder and major depression, known to be benefitted by treatment with traditional competitive NET inhibitors.

## Acknowledgments

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## Abbreviations

| | |
|---|---|
| NE | norepinephrine |
| NET | norepinephrine transporter |
| VMAT | vesicular monoamine transporter |
| PMA | phorbol-12-myristate-13-acetate |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PKG | protein kinase G |
| CaMK | $Ca^{2+}$-calmodulin dependent kinase |

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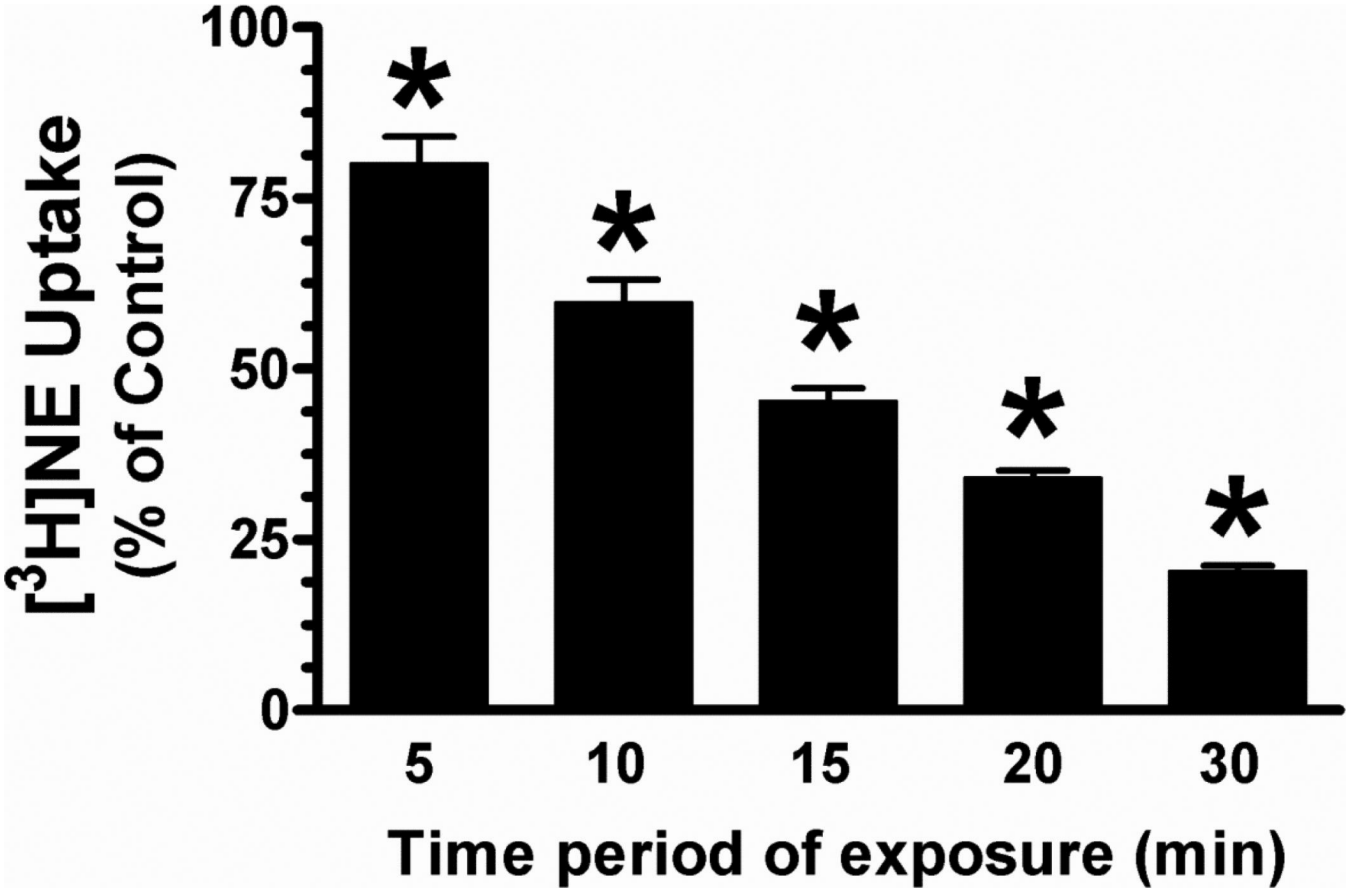


**Figure 1.**

Time course of the effect of reserpine exposure on [$^3$H]NE uptake in PC12 cells. Cells were exposed to reserpine (50 nM) and uptake was initiated after washing at different time points following exposures, as indicated under the bars. Asterisks indicates statistical significance (n=3 experiments performed on separate days; $p < 0.01$), comparing data to control values generated without reserpine exposure.

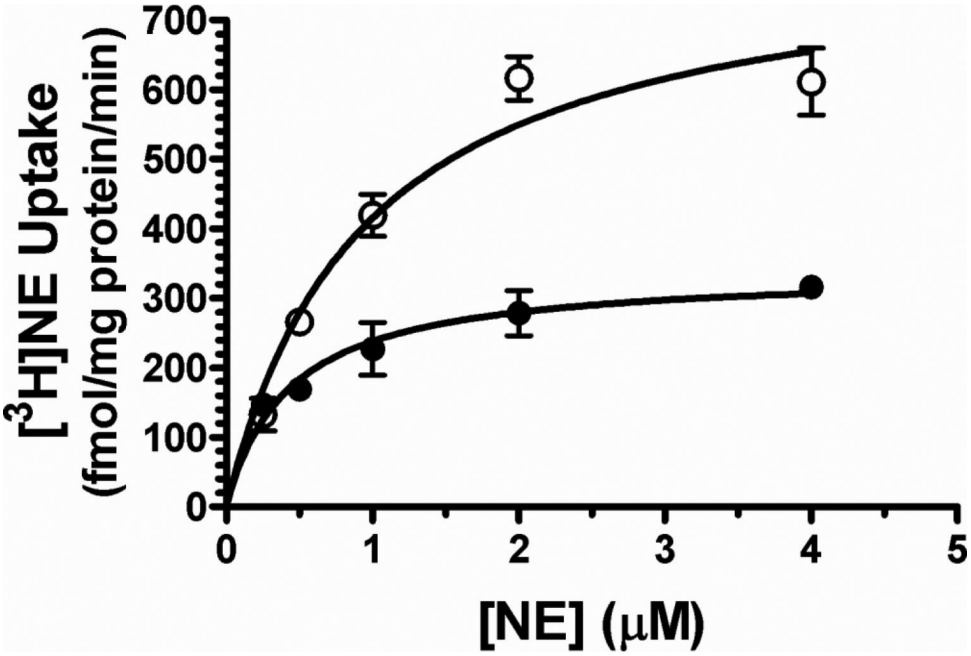


**Figure 2.**

Effect of reserpine exposure on [$^3$H]NE transport kinetics in PC12 cells. Data from control (empty symbols, no treatment) and reserpine exposure (filled symbols) conditions were analyzed by non-linear regression analyses (n=3 experiments performed on separate days). Cells were treated with 50 nM reserpine for 30 min prior to measurement of [$^3$H]NE uptake. Reserpine exposure produced a robust decrease in transport capacity ($V_{max}$) with a modest decrease in $K_m$ (see Results).

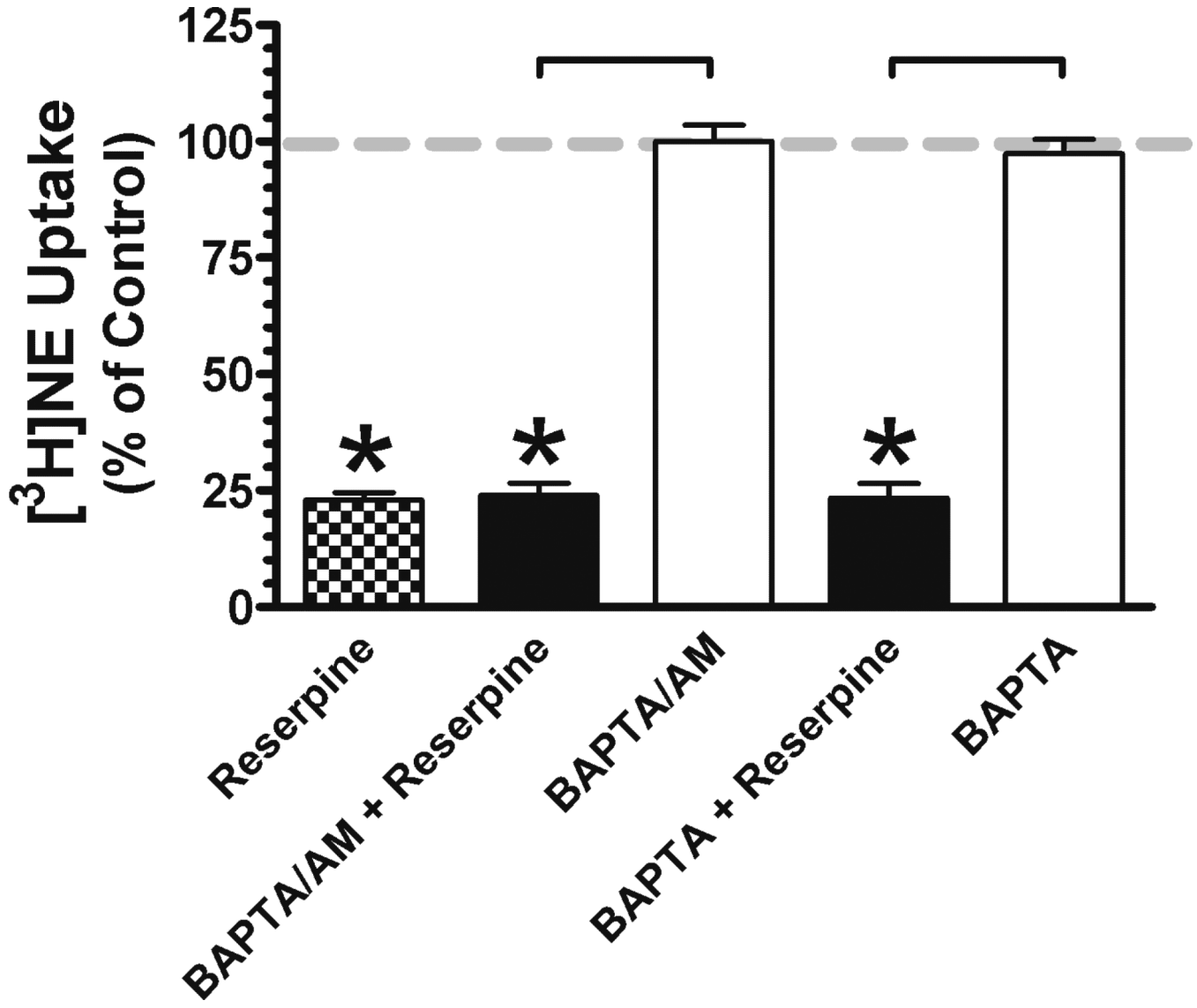


**Figure 3.**

Effect of $Ca^{2+}$ on the reserpine-induced decrease in [$^3$H]NE uptake in PC12 cells. $Ca^{2+}$-chelating agents (BAPTA/AM and BAPTA) were also present during reserpine (Res) treatment. Cells were treated with 50 nM reserpine for 30 min prior to measurement of [$^3$H]NE uptake. Asterisks above bars indicate significant differences as compared to control values (measured in the absence of drugs), and specific comparisons between groups <that were significant> are noted above bars by lined demarcations ( n=2 experiments performed on separate days, with differences between the two experiments varying <5%).

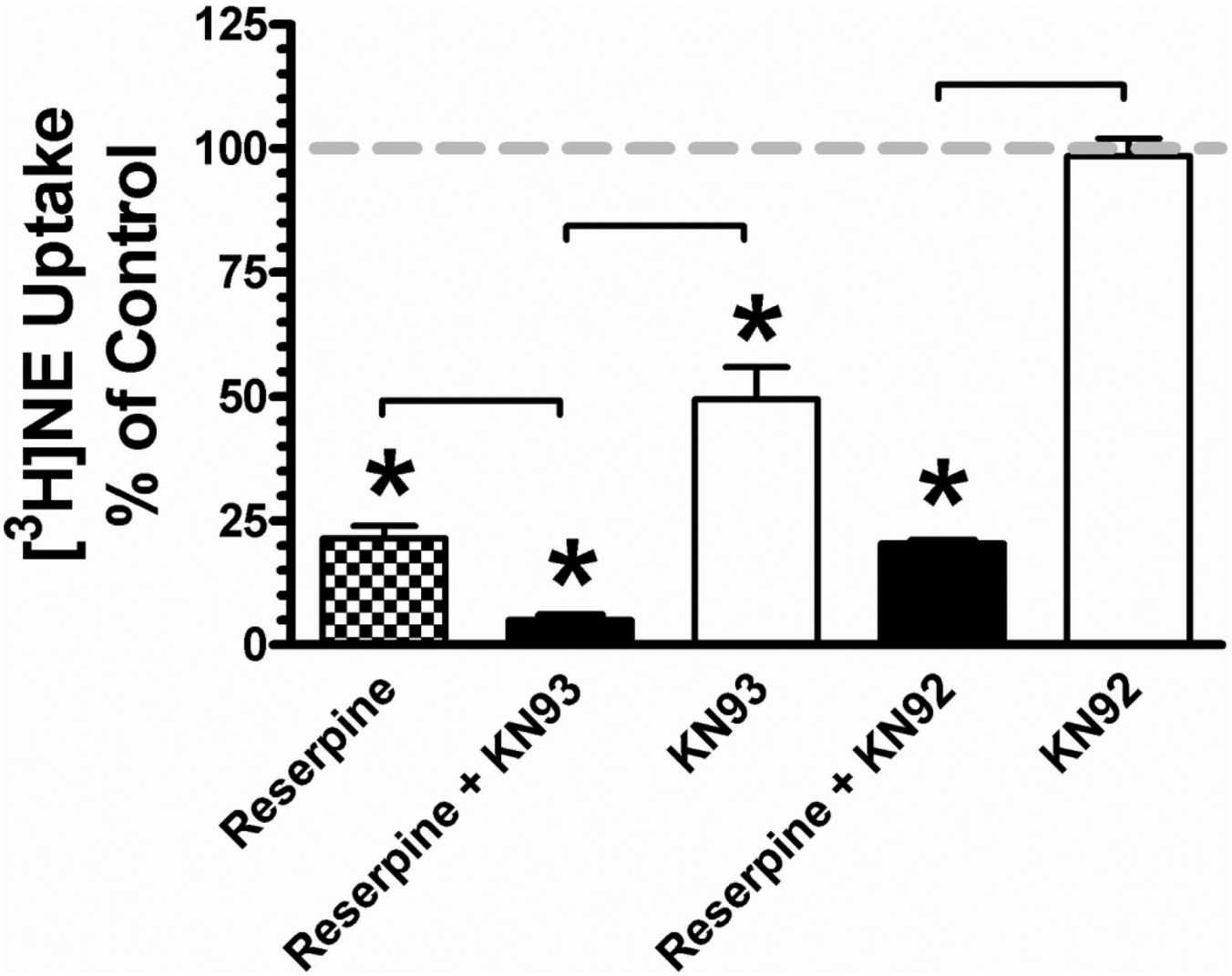


**Figure 4.**

Effect of CaMK activity on reserpine (Res) exposure-induced decrease in [$^3$H]NE uptake in PC12 cells. Cells were pretreated with 10 μM KN93 or 10 μM KN92 (inactive analog) for 30 min prior to reserpine treatment and these compounds were also present during reserpine treatment. Cells were treated with 50 nM reserpine for 30 min prior to measurement of [$^3$H]NE uptake. Asterisks above bars indicate significant differences as compared to control values (measured in the absence of drugs), and specific comparisons between groups <that were significant> are noted above bars by lined demarcations ( n=2 experiments performed on separate days, with differences between the two experiments varying <10%).

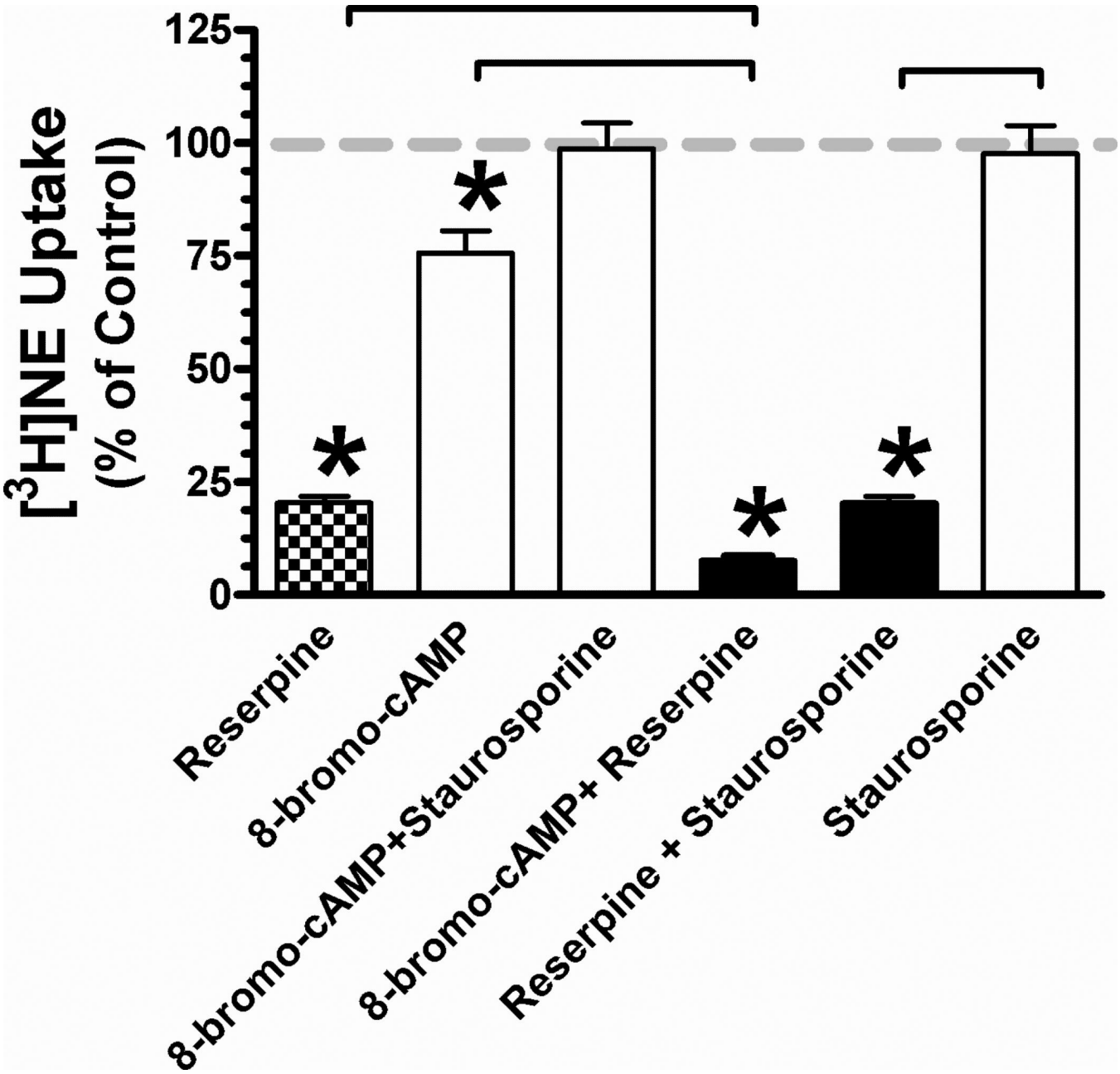


**Figure 5.**

Effect of cAMP/PKA activity on reserpine (Res) exposure-induced decrease in [$^3$H]NE uptake in PC12 cells. PC12 cells were treated with 2 mM 8-bromo-cAMP, or 100 nM staurosporine (stauro = staurosporine) prior to reserpine treatment. cAMP/PKA-regulating agents were also present during reserpine treatment. Cells were treated with 50 nM for 30 min prior to measurement of [$^3$H]NE uptake. Asterisks above bars indicate significant differences as compared to control values (measured in the absence of drugs), and specific comparisons between groups <that were significant> are noted above bars by lined demarcations (n=3 experiments performed on separate days).

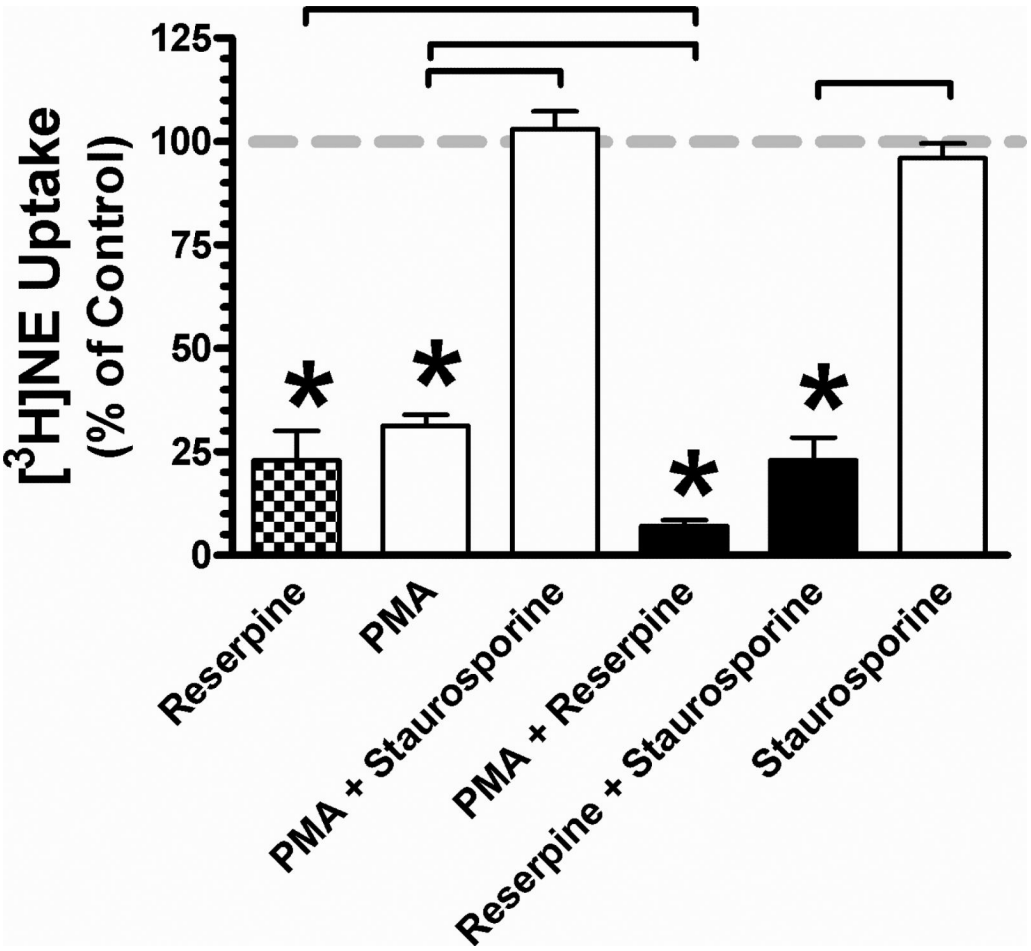


**Figure 6.**

Effect of PKC activity on the reserpine (Res) exposure-induced decrease in [$^3$H]NE uptake in PC12 cells. PC12 cells were treated with 50 nM PMA or 150 nM staurosporine (stauro = staurosporine) prior to reserpine exposure. PKC-regulating agents were also present during reserpine exposure. Cells were treated with 50 nM for 30 min prior to measurement of [$^3$H]NE uptake. Asterisks above bars indicate significant differences as compared to control values (measured in the absence of drugs), and specific comparisons between groups <that were significant> are noted above bars by lined demarcations (n=3 experiments performed on separate days).

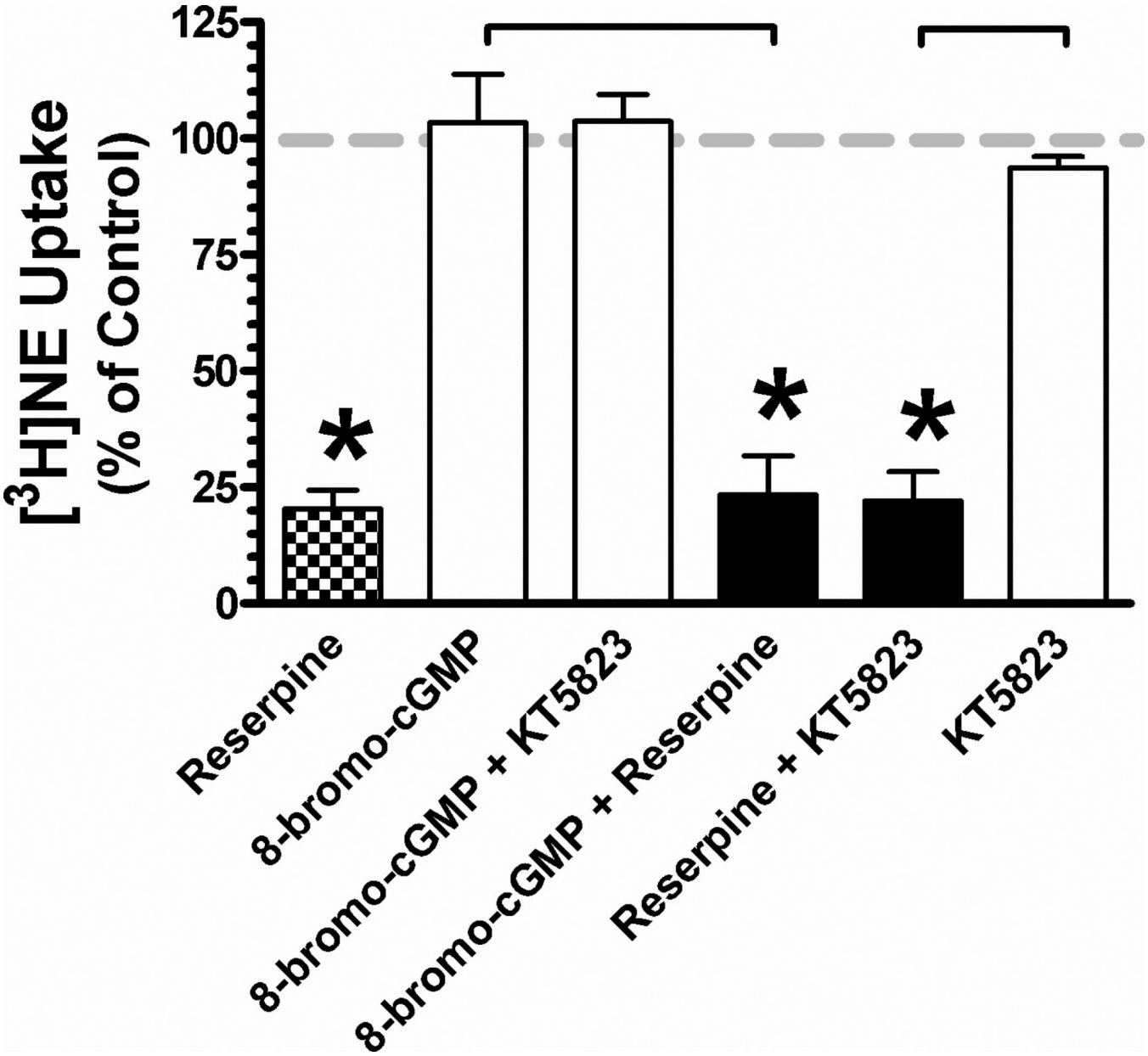


**Figure 7.**

Effect of cGMP/PKG activity on reserpine (Res) exposure-induced decrease in [$^3$H]NE uptake in PC12 cells. Cells were pretreated with 2 mM 8-Br-cGMP (8-bromo-cGMP), or 1 μM KT5823 (KT = KT5823) for 30 min prior to reserpine treatment. PKG-regulating agents were also present during reserpine treatment. Cells were treated with 50 nM reserpine for 30 min prior to measurement of [$^3$H]NE uptake. Asterisks above bars indicate significant differences as compared to control values (measured in the absence of drugs), and specific comparisons between groups <that were significant> are noted above bars by lined demarcations (n=3 experiments performed on separate days).

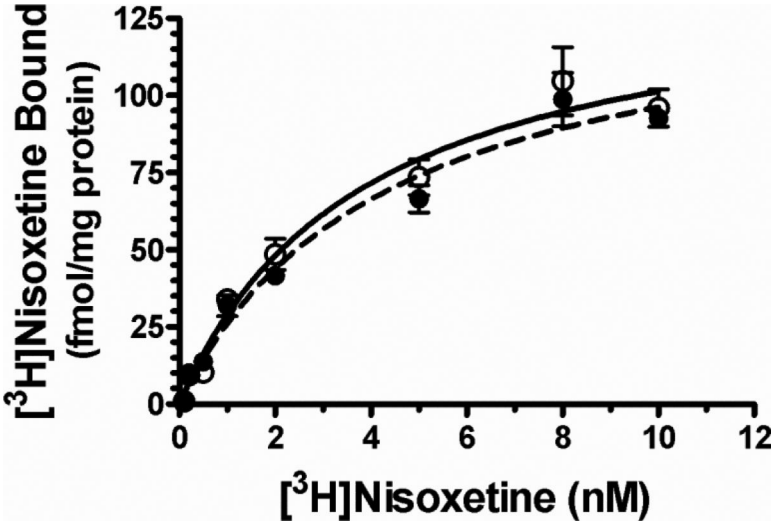


**Figure 8.**

Non-linear regression analysis of [$^3$H]nisoxetine binding to whole PC12 cells following 30 min exposure to reserpine. Filled circles with a dashed non-linear regression line represent reserpine exposure and empty circles with a solid non-linear regression line represent control conditions (no reserpine). Cells were exposed to 50 nM reserpine prior to initiating the binding assay. $B_{max}$ and $K_D$ values of [$^3$H]nisoxetine binding to surface NET are presented in Table 1 (n=3 experiments performed on separate days).

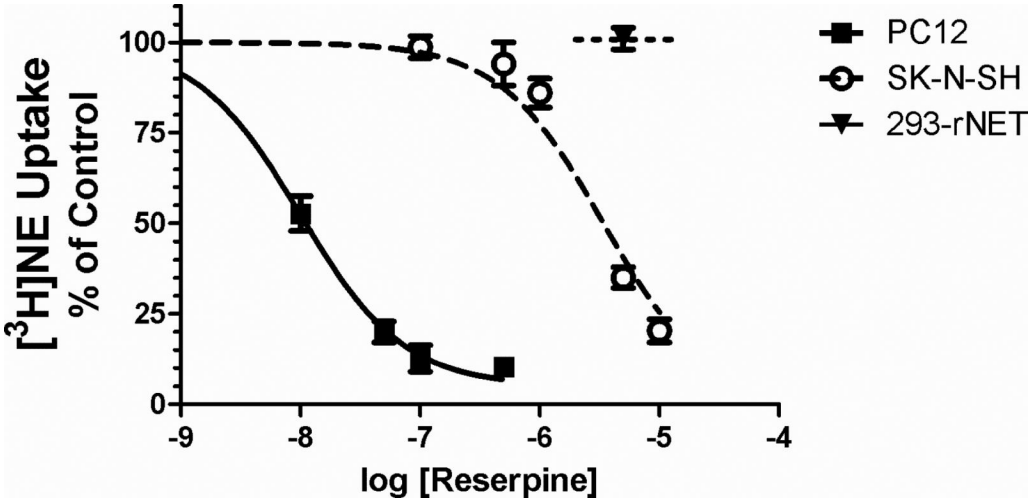


**Figure 9.**

Effect of reserpine on [$^3$H]NE uptake in three cell lines with varying amounts of catecholamine storage vesicles (PC12, high levels; SK-N-SH, low levels; 293-hNET, no storage vesicles). Cells were exposed to reserpine for 30 min exposure prior to measurement of [$^3$H]NE uptake (n=3 experiments performed on separate days).

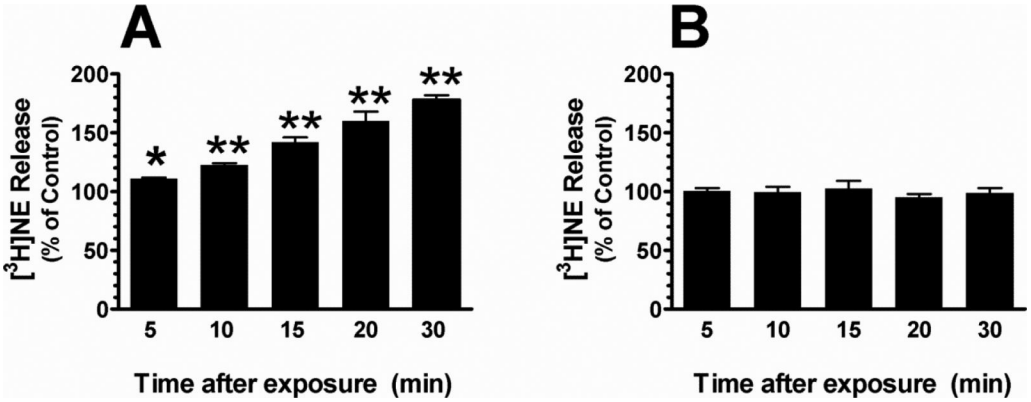


**Figure 10.**

Effect of reserpine exposure on [$^3$H]NE release from PC12 cells (A) and SK-N-SH cells (B). PC12 and SK-N-SH cells were treated with 50 nM and 5 μM reserpine, respectively, followed by the measurement of [$^3$H]NE release after different time intervals. Asterisks above bars indicate significant differences as compared to control values (measured in the absence of reserpine; * $P<0.05$, ** $P<0.01$; n=2 experiments performed on separate days, with differences between the two experiments varying <10%).

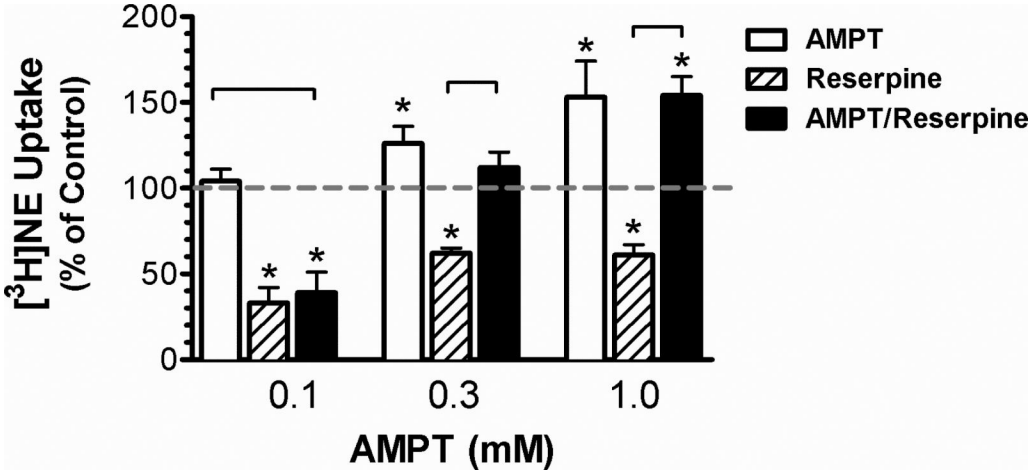


**Figure 11.**

Effect of AMPT treatment on [$^3$H]NE uptake in PC12 cells. PC12 cells were treated with AMPT or vehicle for 24 h, and then exposed to reserpine (50 nM) or vehicle 30 min prior to measuring [$^3$H]NE uptake. Data were calculated as a percentage of control values determined with vehicle exposures (n=8; experiments performed on 3 separate days). Asterisks indicate significant differences from control values and lines above bars denote significant differences between designated groups.

**Table 1**

Whole cell binding assay of PC12 cells.

| | 30 min Exposure | | | 24 h Exposure | | |
|---|---|---|---|---|---|---|
| **Experimental Condition** | **n** | **$K_D$ (nM)** | **$B_{max}$ (fmol/mg protein)** | **n** | **$K_D$ (nM)** | **$B_{max}$ (fmol/mg protein)** |
| Control | 3 | 3.9 ± 0.4 | 141 ± 6 | 2 | 4.3 ± 1.6 | 141 ± 27 |
| Reserpine | 3 | 4.8 ± 1.5 | 144 ± 21 | 2 | 4.4 ± 0.6 | 146 ± 10 |