

Transport of dopamine and levodopa and their interaction in COS-7 cells heterologously expressing monoamine neurotransmitter transporters and in monoaminergic cell lines PC12 and SK-N-SH

W. Hashimoto¹, S. Kitayama², K. Kumagai¹, N. Morioka¹, K. Morita¹ and T. Dohi¹ *

¹Department of Dental Pharmacology, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima 734-8553, Japan

²Department of Dental Pharmacology, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8525, Japan

*Correspondence should be addressed to:

Toshihiro Dohi

Department of Dental Pharmacology, Hiroshima University Graduate School of Biomedical Sciences

Kasumi 1-2-3, Minami-ku, Hiroshima 734-8553, Japan

(Tel) +81-82-257-5640

(Fax) +81-82-257-5644

(E-mail-address) todohi@hiroshima-u.ac.jp

Suggested section heading: Neuroscience

Abstract

The present study investigated the effects of levodopa, a precursor of dopamine (DA) therapeutically used for the treatment of Parkinson's disease, on DA transport in the two different systems, COS-7 cells heterologously expressing rat monoamine transporter cDNA and in monoaminergic cell lines PC12 and SK-N-SH. Levodopa enhanced uptake of [³H]DA and [³H]norepinephrine (NE) but not [³H]serotonin in the transfected COS-7 cells in a concentration-dependent manner. On the other hand, in PC12 and SK-N-SH cells where NET is functionally expressed, levodopa enhanced [³H]DA and [³H]NE uptake at low concentrations and inhibited the uptake at higher concentrations. The effects of levodopa on catecholamine transporters in the opposite direction suggest a different mechanism at the intra- and extracellular sites in a levodopa transport-dependent and independent manner.

Keywords: Dopamine, Levodopa, Monoamine Transporter, Norepinephrine, Serotonin, Cocaine, Parkinson's disease

Introduction

Levodopa (L-DOPA), a precursor of dopamine (DA), is used for the treatment of Parkinson's disease (PD) in order to compensate for the decreased dopaminergic function (Horykiewicz and Kish, 1987; Steiger and Quinn 1995). In early or middle stages of PD, remaining DA neurons require newly synthesized DA to support the altered dopaminergic activity. The DA from levodopa should be stored in synaptic vesicles for subsequent release, resulting in the activation of dopaminergic synaptic transmission. Therefore, it is vital to explore the relation between DA and levodopa transport in the remaining DA neurons.

Levodopa is transported by amino acid transport systems, such as the L-system (Wade and Katzman, 1975; Anclus and Borchardt, 1986), and DA is transported by DAT (Iversen, 1971). Levodopa is not a transportable substrate for DAT, but transport of levodopa and DA through various transporters may affect each other within the cell in a direct or indirect manner. To answer this question, the present study explored the relationship between levodopa and DA transport in various cell systems expressing the monoamine neurotransmitter transporters.

Methods and Materials

Cell culture

Cell lines used were COS-7 (monkey kidney cells), SK-N-SH (human neuroblastoma cells), and PC12 (rat adrenal pheochromocytoma cells). All cells were cultured at 37 °C under 5 % CO₂ / 95 % air. Culture medium was composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS), 100 unit/ml penicillin G, 100 µg/ml streptomycin and 0.25 µg/ml fungisone for COS-7 cells, minimal essential medium-alpha (αMEM) supplemented with 10 % fetal calf serum, 100 unit/ml penicillin G, 100 µg/ml streptomycin and 0.25 µg/ml fungisone for SK-N-SH cells, DMEM supplemented with 5 % FCS, 10 % horse serum, 100 unit/ml penicillin G, 100 µg/ml streptomycin and 0.25 µg/ml fungisone for PC12 cells.

Cell transfection

COS-7 cells at subconfluence were harvested and transfected with cDNA by electroporation (Shimada et al., 1991; Kitayama et al., 1992; Sato et al., 2000). Parallel transfection with pcDNA3 vector alone was performed every time for the negative control. After electroporation, cells were diluted in the culture medium, plated in 24- or

48-well culture plates and cultured for 2-3 days.

Uptake assay

Cells were washed three times with oxygenated Krebs Ringer HEPES-buffered solution (KRH; 125 mM NaCl, 5.2 mM KCl, 1.2 mM CaCl₂, 1.4 mM MgSO₄, 1.2 mM KH₂PO₄, 5 mM glucose, 20 mM HEPES, pH 7.3 ± 0.1) and incubated for 10 min at 37 °C with [³H]DA or other radiolabeled ligands, as described previously (Kitayama et al., 1992; Sato et al., 2000). After removal of excess radioligands by aspiration, the cells were washed three times rapidly with ice-cold KRH and any radioactivity remaining in the cells was extracted with NaOH and measured by liquid scintillation spectrometry. Nonspecific uptake was determined in the cells transfected with empty plasmid vector (mock) and also in each plate in the presence of 100 μM cocaine. For kinetic analysis, cells were incubated in KRH containing 10 nM [³H]DA and 0.1-100 μM cold DA. Data analyzed by Eadie-Hofstee plot using k-cat (BioMetallics).

Statistical analyses were performed using analysis of variance (ANOVA) with pairwise comparison by Bonferroni method (Wallenstein et al., 1980).

Materials

Drugs used were: levodopa (Wako Pure Chemical Industry Co., Ltd., Tokyo, Japan), cocaine hydrochloride (Takeda Chemical Industries, Ltd., Osaka, Japan), DA, pargyline hydrochloride (Nakalai Tesque, Kyoto, Japan), benserazide, m-hydroxybenzylhydrazine (HBH), OR486, 2-(-)-endoaminobicycloheptane-2-carboxylic acid (BCH) (Sigma Chemical Co., St. Louis, MO, USA).

[³H]DA (1176.6 GBq/mmol), [³H]norepinephrine (NE) (462.5 GBq/mmol), and [³H]serotonin (5HT) (1028.6 GBq/mmol) were purchased from NEN Life Science Products, Inc. (Boston, MA, USA), and [³H]levodopa (814 GBq/mmol) from Moravek Biochemicals, Inc. (Brea, CA, USA).

Results

Effects of levodopa on dopamine uptake in COS-7 cells heterologously expressing monoamine transporter

Initial examination of the effects of levodopa on DA transport was performed for COS-7 cells transfected with rat DAT cDNA. Simultaneous incubation with [³H]DA and levodopa increased the uptake of [³H]DA over [³H]DA alone, increasing with

incubation time (Fig. 1). The effect of levodopa was concentration-dependent (Fig. 2) and reversible (data not shown). Pretreatment with levodopa did not alter the facilitatory effect of levodopa (data not shown). Fig. 3 shows the kinetic analysis of the effect of levodopa on [³H]DA uptake. Levodopa increased V_{max} value without affecting apparent affinity for DA (K_m).

[³H]levodopa transport in COS-7 cells

Next, we examined the transport of [³H]levodopa in COS-7 cells transfected with rat DAT cDNA or empty plasmid vector (mock-transfection). Fig. 4 clearly shows similar uptake of [³H]levodopa in both transfected cells, indicating that expression of DAT did not alter levodopa transport. This conclusion was supported by the finding that DAT inhibitor cocaine failed to affect the levodopa uptake (Fig. 4). On the other hand, a specific inhibitor of L-system amino acid transporter, BCH (Wade and Katzman, 1975; Audus and Brochardt 1986), significantly inhibited [³H]levodopa uptake (Fig. 4).

Characterization of the effects of levodopa on dopamine transport in COS-7 cells

To assess whether the effect of levodopa is specific for DAT, we examined the effect of levodopa on the transport of other monoamines in COS-7 cells heterologously

expressing rat NE transporter (NET) or 5HT transporter (SERT). Levodopa significantly enhanced [³H]NE uptake in COS-7 cells expressing rat NET, while it did not alter [³H]5HT uptake in COS-7 cells expressing rat SERT (Fig. 5). These results indicate that the effect of levodopa is specific for catecholamine transporters.

To evaluate the one-way cross-talk between DA and levodopa, we examined the effect of an inhibitor of the levodopa-transportable L-system and inhibitors of levodopa metabolizing enzymes on the levodopa facilitation of [³H]DA uptake. BCH, an L-system inhibitor, did not affect [³H]DA uptake by itself, and did not affect the facilitatory action of levodopa on [³H]DA uptake (Fig. 6). Benserazide, an inhibitor of L-DOPA decarboxylase (DDC) (Lotti and Porter 1970; Goodale and Moore, 1976), slightly inhibited [³H]DA uptake by itself, and a facilitatory effect of levodopa on [³H]DA uptake was observed in the presence of benserazide (Fig. 7). m-Hydroxybenzylhydrazine (HBH), another inhibitor of DDC, did not eliminate the facilitatory effect of L-DOPA, although HBH by itself caused [³H]DA uptake inhibition (Fig. 7). OR486, an inhibitor of catechol-O-methyltransferase (COMT) (Nissinen et al., 1988), did not alter the effect of levodopa on [³H]DA uptake (data not shown).

Effects of levodopa on dopamine uptake in SK-N-SH and PC12 cells

Since the effect of levodopa was specific for catecholamine transporters, we further investigated the effect of levodopa in two cell lines which endogenously express human or rat NET. SK-N-SH cells derived from human neuroblastoma and PC12 cells derived from rat pheochromocytoma both reveal a significant uptake of [³H]DA as well as [³H]NE in a cocaine-sensitive manner (Greene and Tischler, 1976 Richards and Sadee 1986). In both cells, levodopa significantly enhanced [³H]NE uptake at low concentrations (Fig. 8A). Interestingly, however, at higher concentrations levodopa inhibited [³H]NE uptake in a concentration-dependent fashion (Fig. 8A). A similar effect of L-DOPA on [³H]DA uptake was observed in SK-N-SH cells (data not shown). The inhibitory action of levodopa was more significant with longer incubation times (Fig. 8B). Pretreatment with levodopa and concomitant presence of levodopa with [³H]DA did not alter the inhibitory action of levodopa observed in simultaneous incubation with levodopa and [³H]DA. However, washout of cells after pretreatment with levodopa did not recover the [³H]DA uptake to control levels (data not shown). These results suggest that levodopa may enter the cell to exert its inhibitory action on [³H]DA uptake in these cell lines.

[³H]levodopa transport in SK-N-SH cells

To evaluate the possibility mentioned above, we examined the uptake of [³H]levodopa in SK-N-SH cells. Fig. 9 shows the uptake of [³H]levodopa in a BCH-sensitive fashion, similar to that observed in COS-7 cells (see Fig. 4). The time-course of [³H]levodopa uptake paralleled the inhibitory effect of levodopa on [³H]DA uptake as observed in Fig. 8B.

Discussion

The present study demonstrated that levodopa enhanced uptake of [³H]DA and [³H]NE but not [³H]5HT in COS-7 cells transfected with their transporter cDNA. Pharmacological characterization of the effects of levodopa suggests that levodopa acts on catecholamine transporters to facilitate their transport activity. This conclusion was supported by the finding that levodopa enhanced [³H]NE and [³H]DA uptake at low concentrations in PC12 and SK-N-SH cells where NET is functionally expressed. The failure of benserazide and HBH, inhibitors of AADC, to affect levodopa effects on DA transport suggest that metabolites of levodopa such as DA are not involved in this mechanism. The finding that BCH, an inhibitor of L-system, which is known to

transport levodopa, failed to affect DA uptake in COS cells expressing DAT also excludes the involvement of intracellular conversion of levodopa to DA.

It is unclear of the precise mechanism underlying the facilitatory action of levodopa on DAT and NET in both COS-7 cells heterologously expressing catecholamine transporters and monoaminergic cell lines PC12 and SK-N-SH. Eshleman et al. (1997) demonstrated that COMT inhibitors potentiate the uptake of DA and NE but not 5HT in the cell lines heterologously expressing recombinant monoamine transporters, and proposed the hypothesis that prevention of the metabolism of DA to the more lipophilic 3-methoxytyramine reduced its diffusion out of the cells, thereby resulting in apparent retention (uptake) of [³H]DA. L-DOPA is a substrate for COMT, so that it may inhibit COMT activity competitively for DA to produce an apparent potentiation of DA uptake. However, we believe this is not the case in our situation for the following reasons. First, OR-468, a COMT inhibitor, failed to affect [³H]DA uptake by itself, nor did it alter the facilitatory effect of levodopa on [³H]DA uptake in COS-7 cells expressing rat DAT. Second, the hypothesis proposed by Eshleman et al. (1997) is based on the idea that intracellular COMT activity metabolizes the [³H]DA taken up. However, BCH, an inhibitor of the levodopa-transportable L-system, failed to affect

the facilitatory effect of levodopa on [³H]DA uptake, indicating that levodopa did not necessarily enhance uptake by entering the cells. Although the inhibition of COMT by levodopa in its effect on [³H]DA uptake cannot be completely excluded, the present results suggest that levodopa by itself acts on the catecholamine transporters to facilitate their transport at least.

Higher concentrations of levodopa inhibited the uptake of [³H]DA in both PC12 and SK-N-SH cells. This inhibition was greater at longer incubation times in association with a significant uptake of [³H]levodopa. Furthermore, washout of the cells pretreated with levodopa failed to recover the [³H]DA uptake to the control level. These results suggest that levodopa enters the cells, converts to DA, and thereby inhibits [³H]DA uptake. The cytosolic total DA concentration ([³H]DA plus DA synthesized from levodopa) becomes saturated at the lower extracellular [³H]DA concentrations. Intra- and extracellular metabolism of neurotransmitters, therefore, should be closely associated with transmitter transport through plasma membrane. It was demonstrated that NE metabolism by MAO or COMT changed with the activities of uptake₁ and uptake₂ for NE in neuronal and non-neuronal cells, respectively (see review of Trendelenburg, 1991). Blockade of vesicular storage has been found to alter

the kinetics of DA transport by synaptosomes (Parish and Shenk, 1996). We also found that inhibitors of vesicular monoamine transporter, such as reserpine, inhibited DA uptake, probably through the increase in cytosolic accumulation of DA (Hashimoto et al., unpublished observation). Therefore, increase in intracellular concentration is critical for the regulation of DAT transport activity.

In summary, the present study demonstrated the effects of levodopa on catecholamine transporters in opposite directions, such as enhancement in COS cells expressing catecholamine transporter and in PC12 and SK-N-SH cells at low concentrations, and inhibition in PC12 and SK-N-SH cells at high concentrations. These results suggest the probability of different mechanism at the intra- and extracellular sites in a levodopa transport-dependent and -independent manner.

Acknowledgement

This study was supported in part by the Grant-in-Aid for Scientific program from the Ministry of Education, Culture, Science, Sports and Technology (TD, SK).

References

- Audus K.L., Borchardt R.T., 1986. Characteristics of the large neutral amino acid transport system of bovine brain microvessel endothelial cell monolayers. *Journal of Neurochemistry* 47, 484-488.
- Eshleman A.J., Stewart E., Evenson A.K., Mason J.N., Blakely R.D., Janowsky A., Neve K.A., 1997. Metabolism of catecholamines by catechol-O-methyltransferase in cells expressing recombinant catecholamine transporters. *Journal of Neurochemistry* 69, 1459-1466.
- Goodale D.B., Moore K.E., 1976. A comparison of the effects of decarboxylase inhibitors on L-dopa-induced circling behavior and the conversion of dopa to dopamine in the brain. *Life Sciences* 19, 701-706.
- Greene L.A., Tischler A.S., 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proceedings of the National Academy of Sciences of the United States of the America* 73, 2424-2428.
- Hornykiewicz O., Kish S.J., 1987. Biochemical pathophysiology of Parkinson's disease. In: *Advances in Neurology*, vol.45: Parkinson's disease (Yahr M. and

- Bergmann K. eds.) pp19-34, Raven Press, New York.
- Iversen L.L., 1971. Role of transmitter uptake mechanisms in synaptic neurotransmission. *British Journal of Pharmacology* 41, 571-591.
- Kitayama S., Shimada S., Xu H., Markham L., Donovan M.D., Uhl G.R., 1992. Dopamine transporter site-directed mutations differentially alter substrate transport and cocaine binding. *Proceedings of the National Academy of Sciences of the United States of the America* 89, 7782-7785.
- Lotti V.J., Porter C.C., 1970. Potentiation and inhibition of some central actions of L(-)-dopa by decarboxylase inhibitors. *Journal of Pharmacology and Experimental Therapeutics* 172, 406-415.
- Nissinen E., Linden I.B., Schultz E., Kaakkola S., Mannisto P.T., Pohto P., 1988. Inhibition of catechol-O-methyltransferase activity by two novel disubstituted catechols in the rat. *European Journal of Pharmacology* 153, 263-269.
- Parish J.J., Schenk J.O., 1996. Kinetic analyses of striatal dopaminergic transport in vitro: linkage to metabolism and vesicular sequestration. *Society for Neuroscience Abstract* 22, 370.
- Richards M.L., Sadee W., 1986. Human neuroblastoma cell lines as models of catechol

- uptake. *Brain Research* 384, 132-137.
- Sato T., Kitayama S., Mitsuhashi C., Ikeda T., Morita K., Dohi T., 2000. Selective inhibition of monoamine neurotransmitter transporters by synthetic local anesthetics. *Naunyn-Schmiedeberg's Archives of Pharmacology* 361, 214-220.
- Shimada S., Kitayama S., Lin C.L., Patel A., Nanthakumar E., Gregor P., Kuhar M.J., Uhl G.R., 1991. Cloning and expression of a cocaine-sensitive dopamine transporter complementary DNA. *Science* 254, 576-578.
- Steiger M.J., Quinn N.P., 1995. Levodopa-based therapy. In: *Therapy of Parkinson's Disease*, 2nd ed. (Koller W.C., Paulson G. eds), pp105-143, Marcel-Dekker, New York.
- Trendelenburg U., 1991. Functional aspects of the neuronal uptake of noradrenaline, *Trends in Pharmacological Sciences* 12, 334-337.
- Wade L.A., Katzman R., 1975. Synthetic amino acids and the nature of L-DOPA transport at the blood-brain barrier. *Journal of Neurochemistry* 25, 837-842.
- Wallenstein S., Zucker C.L., Fleiss J.L., 1980. Some statistical methods useful in circulation research. *Circulation Research* 47, 1-9.

Figure legends

Fig.1 Effects of L-DOPA and cocaine on the [³H]dopamine (DA) uptake in COS cells transfected with rat DA transporter (DAT) cDNA. Cells transfected with rat DAT cDNA were incubated with [³H]DA in the presence (closed symbols) or absence (open symbols) of 100 μM L-DOPA. Total (circle) and nonspecific (triangle) uptake was determined in the absence or presence of 100 μM cocaine. Values represent mean ± SEM of 3 experiments each performed in triplicate. **P*<0.05 compared with total uptake in the absence of L-DOPA.

Fig.2 Concentration-response relationship of the facilitatory effects of L-DOPA on [³H]DA uptake in COS cells expressing rat DAT. Cells were incubated at 37 °C for 5 min with 10 nM [³H]DA in the presence or absence of various concentrations of L-DOPA. Specific uptake of [³H]DA was expressed as % of control. Control uptake in the absence of L-DOPA was 136.4 ± 33.1 fmol/well. Values represent mean ± SEM of 3 experiments each performed in triplicate. **P*<0.05, ***P*<0.01 ****P*<0.001 compared with control, respectively.

Fig.3 Changes in the kinetics of [³H]DA uptake induced by L-DOPA in COS cells expressing rat DAT. Cells were incubated at 37 °C for 5 min with 10 nM [³H]DA and various concentrations of unlabeled DA (0.01-30 μM) in the presence (closed circle) or absence (open circle) of 100 μM L-DOPA. Representative results from single experiment performed in triplicate are shown. K_m of [³H]DA uptake in the absence and presence of 100 μM L-DOPA was 2.51 ± 0.24 and 2.56 ± 0.16 (no significant difference, n=3~4), and V_{max} was 119.1 ± 6.3 and 158.4 ± 5.5 (P<0.05, n=3~4), respectively.

Fig.4 Uptake of [³H]L-DOPA in COS-7 cells transfected with rat DAT cDNA or with empty plasmid vector (Mock). Cells were incubated with 10 nM [³H]L-DOPA in the presence or absence of 1 mM L-DOPA, 1 mM BCH or 100 μM cocaine. Values represent mean ± SEM of 3 experiments each performed in triplicate. *P<0.001 compared with total uptake.

Fig.5 Effects of L-DOPA on the uptake of [³H]DA, [³H]norepinephrine (NE) and

[³H]serotonin (5HT) in COS cells expressing rat DAT, NE transporter (NET) or 5HT transporter (SERT). Cells transfected with cDNA of rat DAT NET or SERT were incubated at 37 °C for 10 or 20 min with 10 nM [³H]DA, 20 nM [³H]NE or 20 nM [³H]5HT in the presence (striped bar) or absence (open bar) of 100 μM L-DOPA. Specific uptake was calculated by subtracting uptake in the presence of 100 μM cocaine from total uptake in the absence of cocaine and presented as % of control uptake measured at 10 min incubation (in the absence of L-DOPA). The control specific uptake of [³H]DA, [³H]NE and [³H]5HT was 69.0 ± 3.1, 171.6 ± 1.9 and 90.2 ± 1.4 fmol/well for 10 min incubation, respectively. Values represent mean ± SEM of 3 experiments each performed in triplicate determinations. **P*<0.01 compared with the uptake in the absence of L-DOPA.

Fig.6 Effect of L-system inhibitor on levodopa-induced enhancement of [³H]DA uptake in COS-7 cells expressing rat DAT. Cells were incubated at 37 °C for 10 min with 10 nM [³H]DA in the presence or absence of 100 μM L-DOPA and/or 1 mM BCH, an L system inhibitor. Specific uptake was determined by subtracting the nonspecific uptake in the presence of 100 μM cocaine and expressed as control uptake which was 101.1 ±

4.4 fmol/well for 10 min incubation. Values represent mean \pm SEM of 3 experiments each performed in triplicate. * P <0.01 compared with control.

Fig.7 Effects of inhibitors of aromatic amino acid decarboxylase (AADC) on levodopa-induced enhancement of [3 H]DA uptake in COS-7 cells expressing rat DAT. Cells were incubated at 37 °C for 10 min with 10 nM [3 H]DA in the presence or absence of 100 μ M L-DOPA and/or 100 μ M benserazide or 100 μ M m-hydroxybenzylhydrazine (HBH), inhibitors of AADC. Values represent mean \pm SEM of 3 experiments each performed in triplicate. Control specific uptake of [3 H]DA was 119.8 ± 1.7 fmol/well for 10 min incubation. * P <0.05 compared with control or AADC inhibitor alone.

Fig.8 Effects of L-DOPA on [3 H]NE or [3 H]DA uptake in SK-N-SH cells and PC12 cells. A. Concentration-response relationship for the effects of L-DOPA on [3 H]NE uptake in SK-N-SH cells and PC12 cells. Cells were incubated with 20 nM [3 H]NE in the presence or absence of various concentrations of L-DOPA. Values represent mean \pm SEM of 3 experiments each performed in triplicate and expressed as % of control in the absence of L-DOPA. Control specific uptake of [3 H]NE in SK-N-SH cells and PC12

cells was 590.2 ± 74.8 and 125.4 ± 8.6 fmol/well for 10 min incubation, respectively.

* $P < 0.05$ compared with control. B. Time-course of the inhibitory effect of L-DOPA on

[^3H]DA uptake in SK-N-SH cells. Cells were incubated with 10 nM [^3H]DA in the

presence or absence of 100 μM L-DOPA. Values represent mean \pm S.E.M of 3

experiments each performed in triplicate and were expressed as % of the control

measured for 5 min incubation without L-DOPA, which was 98.4 ± 15.4 fmol/well.

* $P < 0.01$ compared with control, respectively.

Fig.9 [^3H]L-DOPA uptake in SK-N-SH cells. Cells were incubated at 37 $^{\circ}\text{C}$ for various

times with 10 nM [^3H]L-DOPA in the presence or absence of 1 mM BCH, an L system

inhibitor. Values represent mean \pm SEM of 3 experiments each performed in triplicate.

* $P < 0.001$ compared with the total uptake in the absence of BCH.

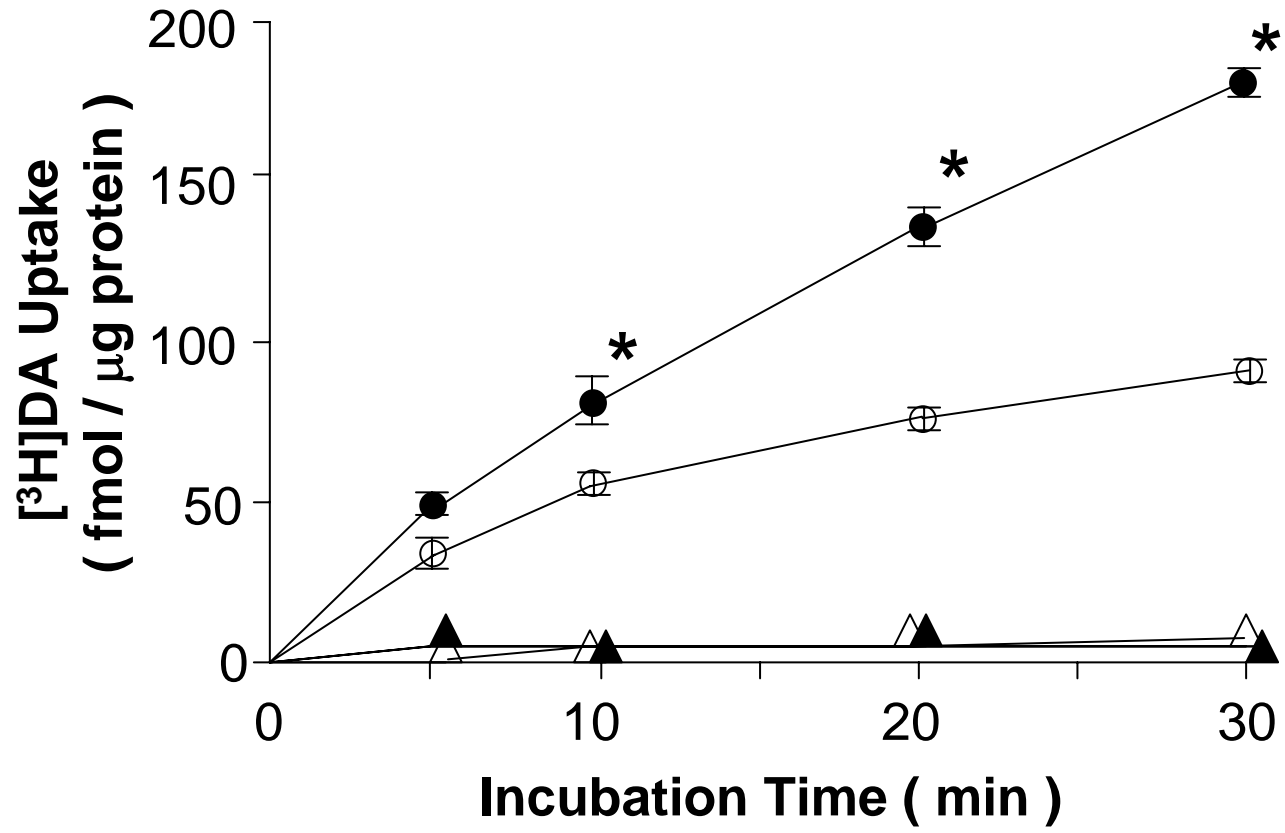


Fig. 1

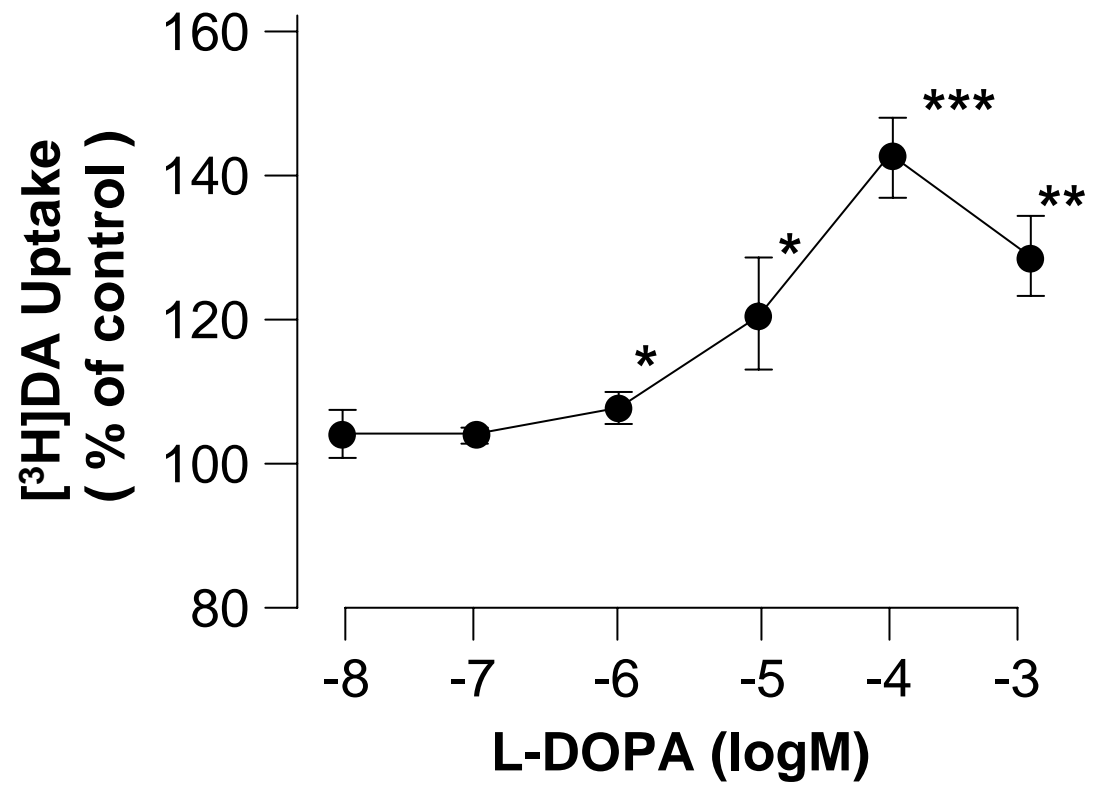


Fig. 2

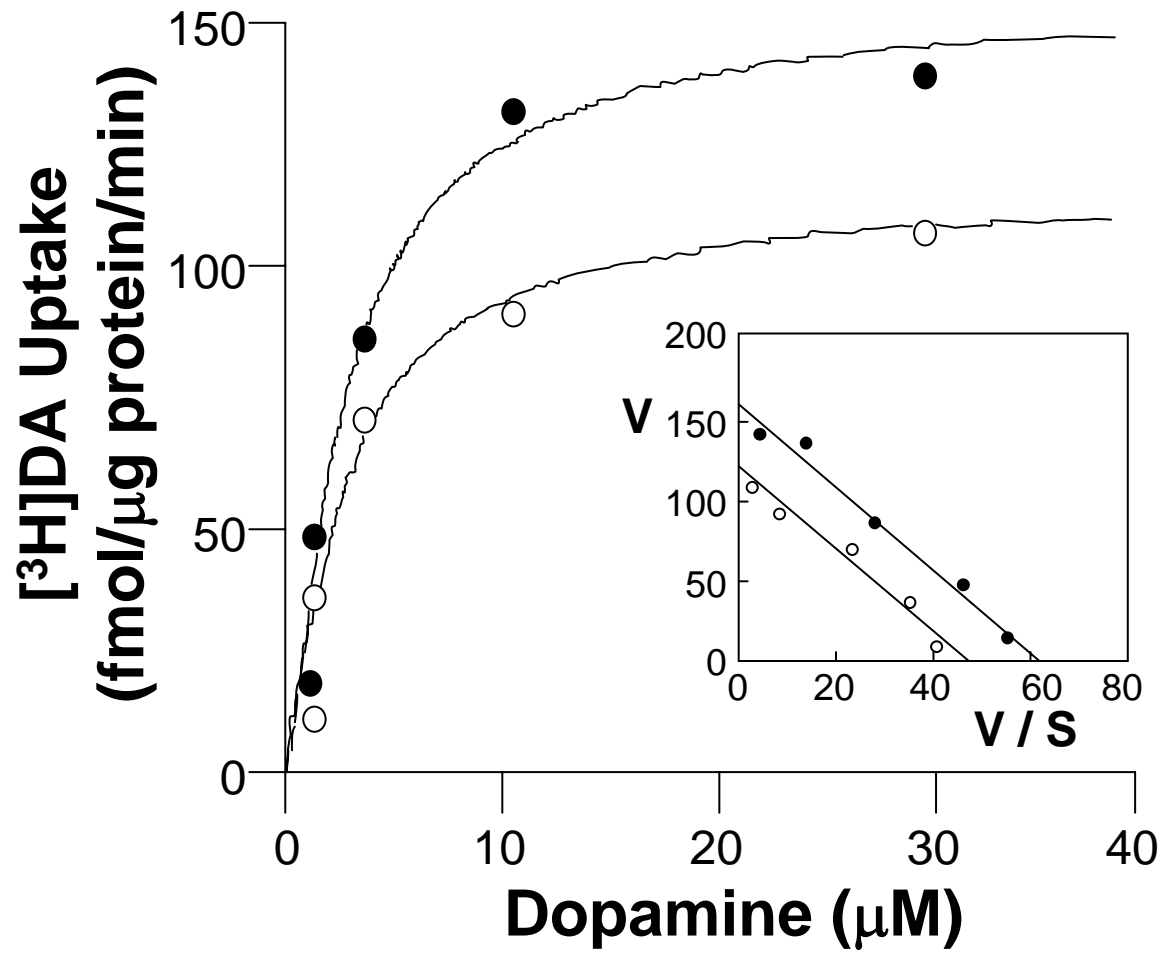


Fig. 3

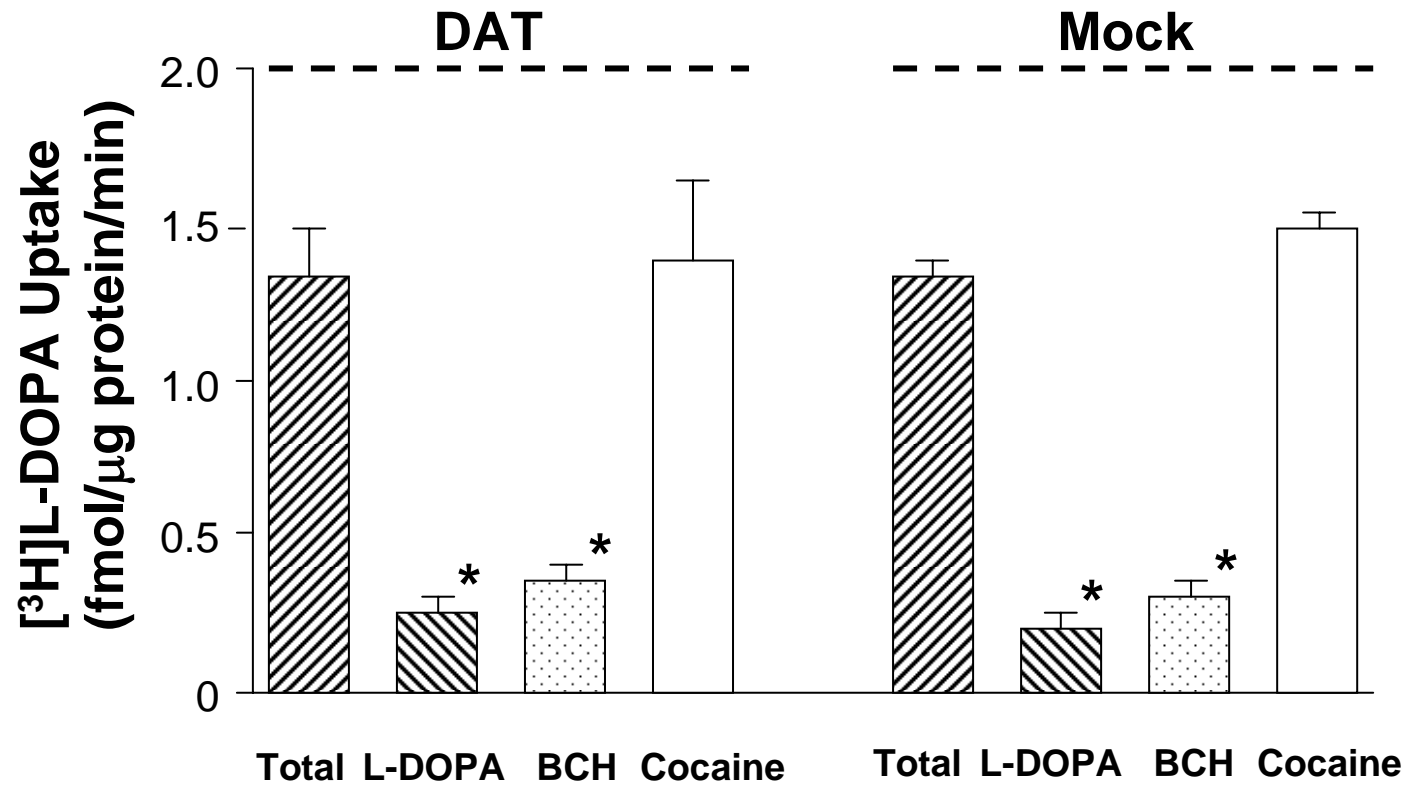


Fig. 4

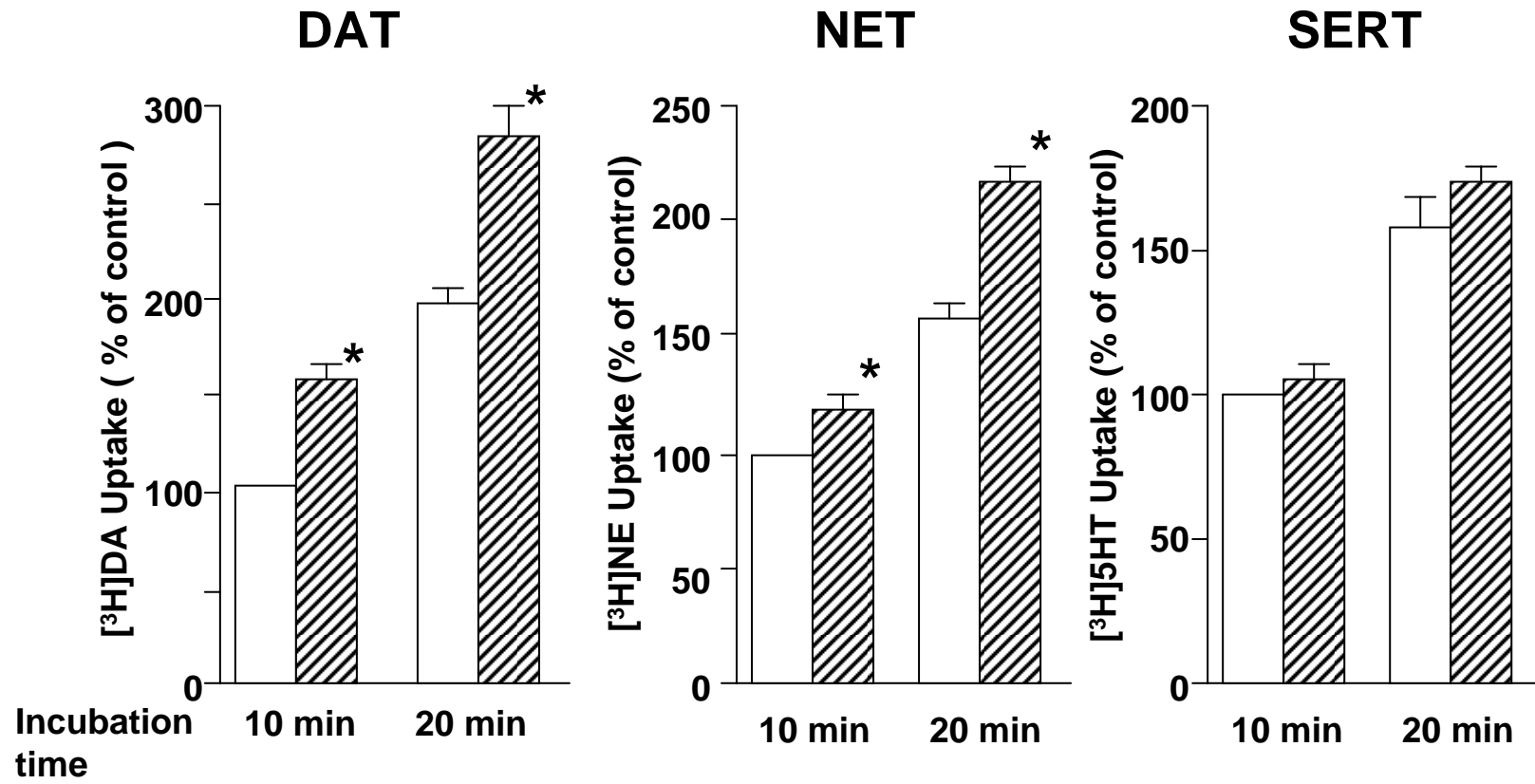


Fig. 5

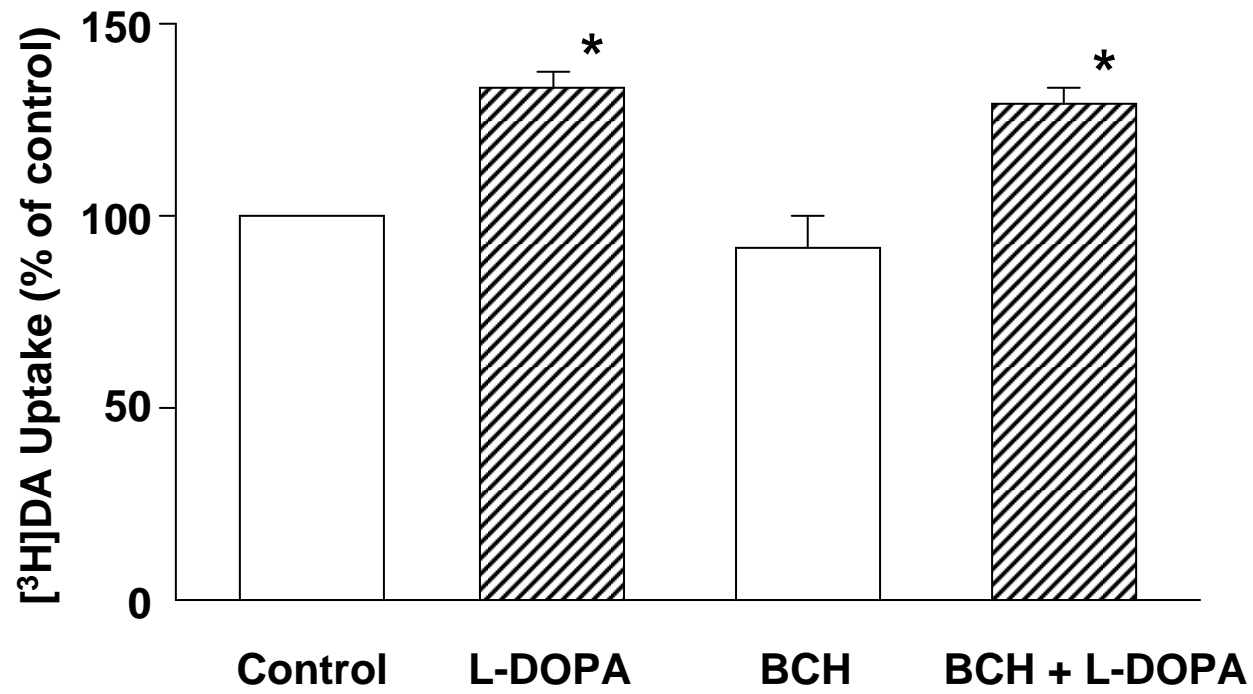


Fig. 6

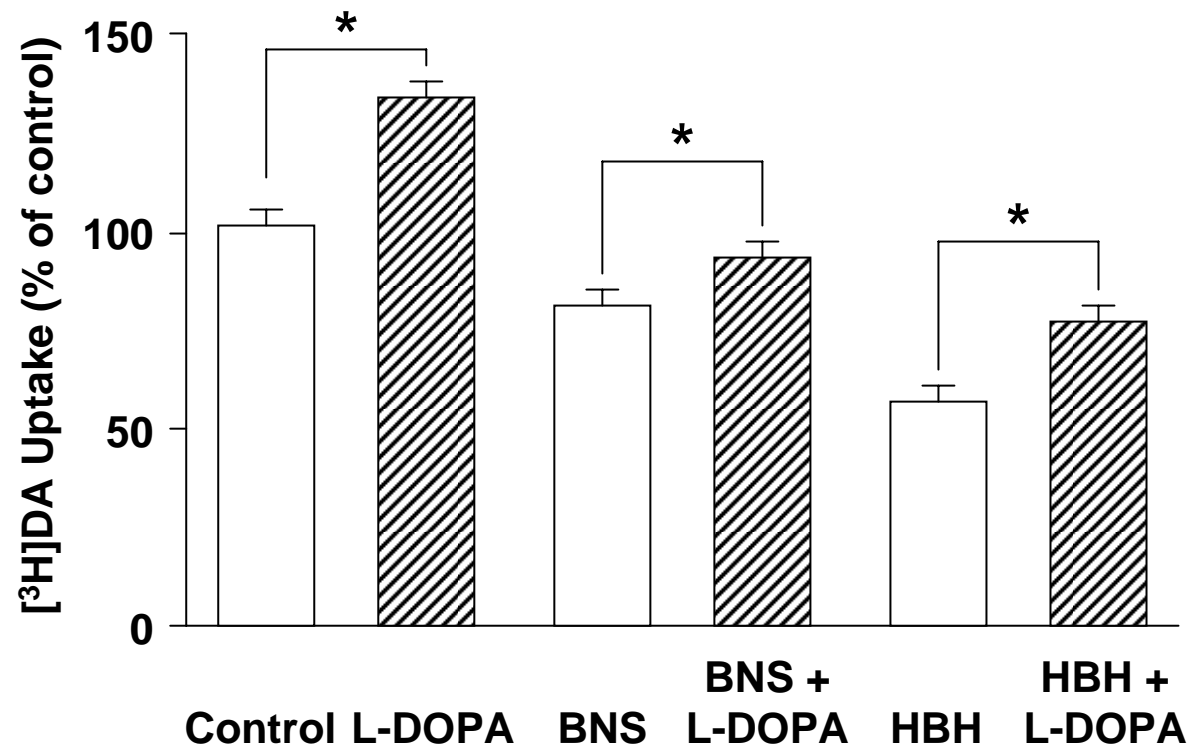


Fig. 7

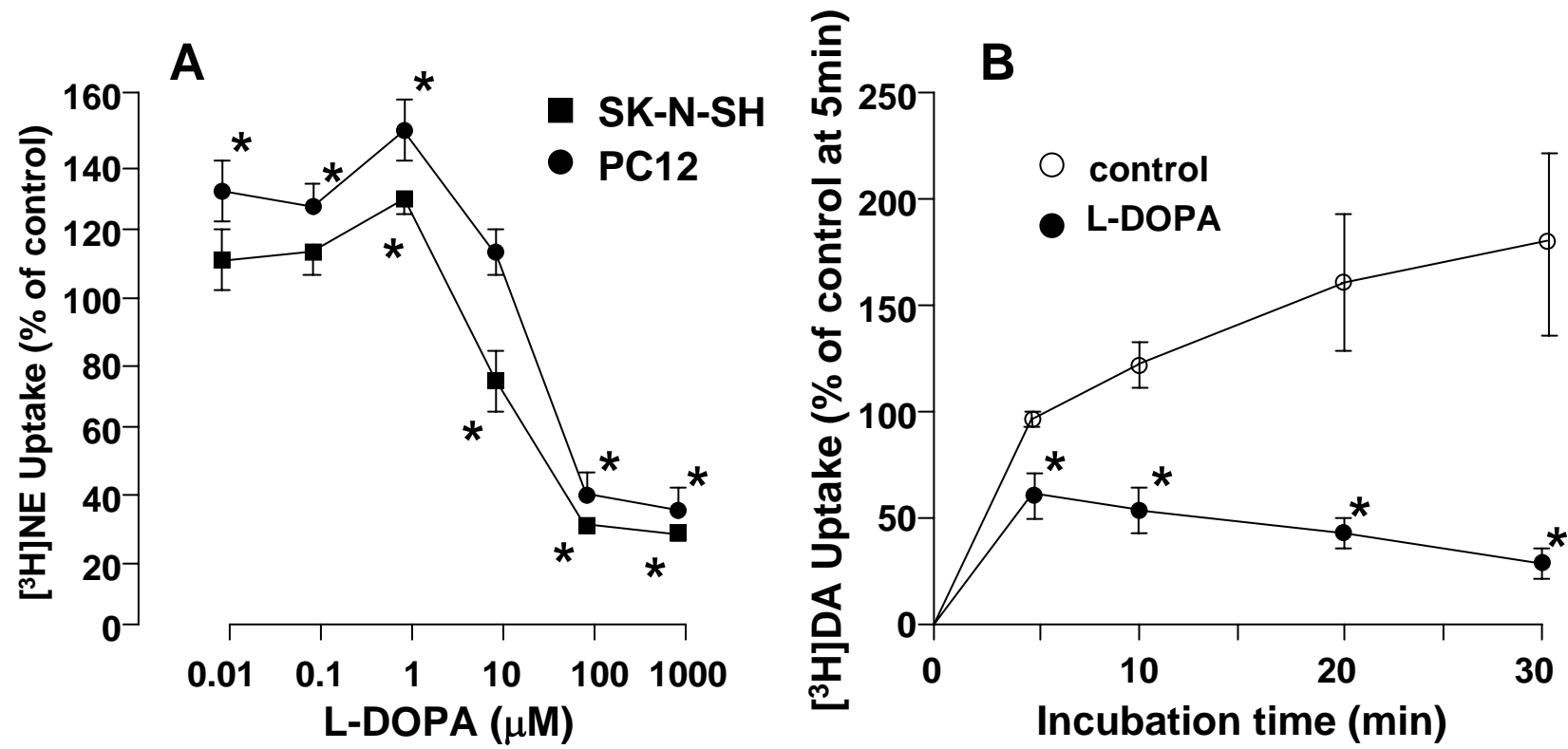


Fig. 8

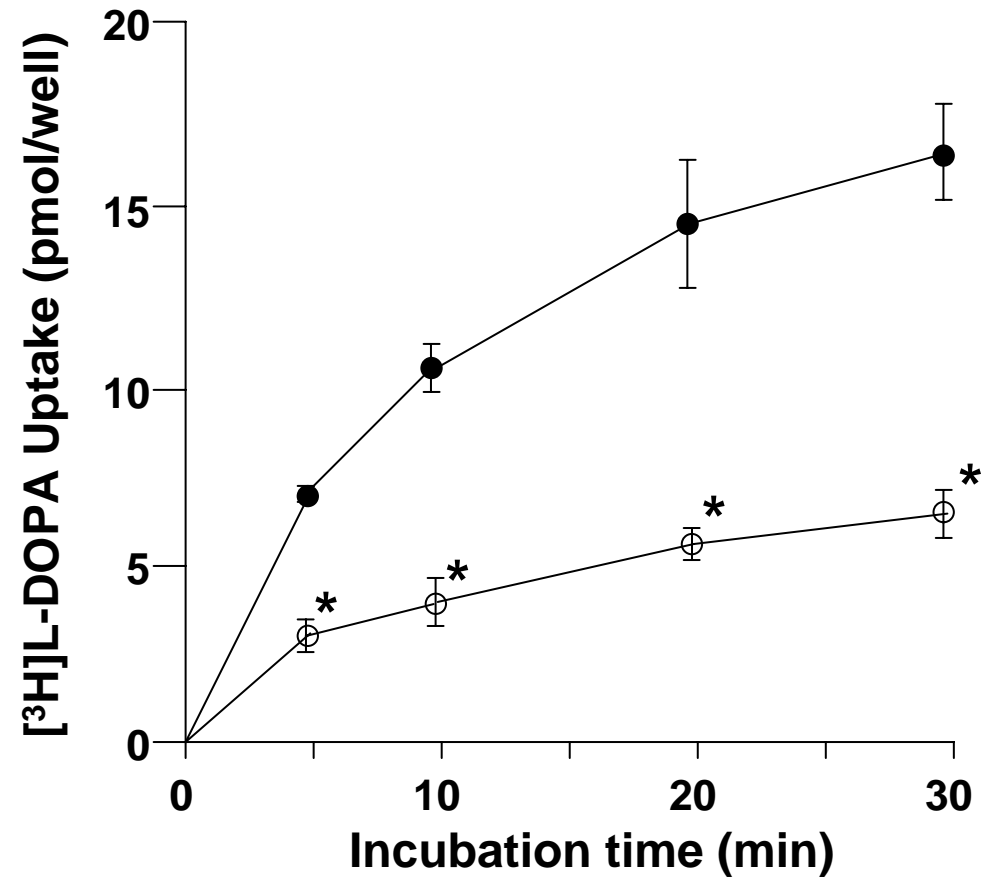


Fig. 9