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Title Page

AKT IS ESSENTIAL FOR INSULIN MODULATION OF AMPHETAMINE-INDUCED HUMAN DOPAMINE TRANSPORTER CELL SURFACE REDISTRIBUTION

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Running Title Page

Running title: Akt regulates hDAT cell surface expression

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Abbreviations: hDAT, human dopamine transporter; DA, dopamine; AMPH, amphetamine; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SERT, serotonin transporter; NET, norepinephrine transporter; NE, norepinephrine; YFP, yellow fluorescent protein.

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Abstract

Uptake by the dopamine transporter (DAT) is the primary pathway for the clearance of extracellular dopamine (DA) and consequently for regulating the magnitude and duration of dopaminergic signaling. Amphetamine (AMPH) has been shown to decrease simultaneously DAT cell surface expression and [^{3}H]DA uptake. We have shown that insulin and its subsequent signaling through the phosphatidylinositol (PI)3-kinase (PI3K) dependent pathway oppose this effect of AMPH by promoting increased cell surface expression. Here we have used HEK-293 cells stably expressing the human DAT (hDAT cells) to investigate the downstream cellular components important for this effect of insulin. Akt is a protein kinase effector immediately downstream of PI3K. Both overexpression of a dominant negative mutant of Akt (K179R) and the addition of ML9, a pharmacological inhibitor of Akt, decreased cell surface expression of DAT, suggesting a role of basal Akt signaling in the homoeostasis of DAT. Moreover, expression of a constitutively active Akt mutant reduced the ability of AMPH to decrease hDAT cell surface expression as well as [^{3}H]DA uptake. In contrast, overexpression of K179R blocked the ability of insulin to oppose AMPH-induced reduction of hDAT cell surface expression and [^{3}H]DA uptake, as did ML9. Our data demonstrate that hDAT cell surface expression is regulated by the insulin signaling pathway and that Akt plays a key role in the hormonal modulation of AMPH-induced hDAT trafficking as well as in the regulation of basal hDAT cell surface expression.

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Introduction

Dopaminergic neurotransmission is determined by extracellular DA levels, which in turn are regulated principally by DAT-mediated DA reuptake. Since DA uptake capacity depends on the turnover rate of an individual transporter as well as on the number of functional transporters expressed at the plasma membrane, regulation of DAT cell surface expression is an important mechanism for fine-tuning DA neurotransmission (Beckman and Quick, 1998; Kahlig and Galli, 2003; Robinson, 2001).

Several studies have identified signal transduction pathways that modulate DAT trafficking and activity. Activation of protein kinase C (PKC), either by phorbol esters (phorbol 12-myristate 13-acetate (PMA)) or by $G\alpha_q$-coupled substance P receptor, decreases both DAT cell surface expression and transport capacity (Daniels and Amara, 1999; Granas et al., 2003; Loder and Melikian, 2003; Melikian and Buckley, 1999; Zhang et al., 1997; Zhu et al., 1997). PKC-induced trafficking has been observed as well for other Na^+/Cl^- dependent neurotransmitter transporters (NTs), including the serotonin transporter (SERT) and the GABA transporter GAT1 (Blakely and Bauman, 2000; Robinson, 2001). In the case of DAT, PKC-down regulation has also been observed in rat striatal synaptosomes (Vaughan et al., 1997). Curiously, PKC-induced trafficking does not appear to require phosphorylation of DAT itself (Granas et al., 2003) but rather of another substrate.

Tyrosine kinases, which are activated by insulin and insulin-like growth factor (IGF) 1, have also been shown to regulate NTs function (Gonzalez and Robinson, 2004; Law et al., 2000). Prasad *et al.* have shown that activation of tyrosine kinases up-regulates the activity, cell surface expression and gene expression of the human serotonin transporter (hSERT) (Prasad et

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al., 1997). In hippocampal neurons, acute inhibition of tyrosine kinase down-regulates GAT1 function with a concomitant decrease in transporter cell surface expression (Law et al., 2000). Similarly, DAT activity and cell surface expression were also reduced by tyrosine kinase inhibitors (Doolen and Zahniser, 2001).

Insulin signaling, which enhances tyrosine kinase activity (Elmendorf and Pessin, 1999; Taha and Klip, 1999), increases NET function (Apparsundaram et al., 2001) and plays a critical role in the regulation of NE uptake by angiotensin II (Yang and Raizada, 1999). The downstream effects of insulin include the activation of PI3K. This lipid kinase phosphorylates the D-3 position of phosphoinositides to generate mainly $PI(3,4,5)P_3$ (PIP_3) (Taha and Klip, 1999), which acts at the plasma membrane as a second messenger. Self-administration of AMPH is regulated by insulin signaling (Galici et al., 2000), and PI3K has been implicated in the regulation of the behavioral actions of psychostimulants such as AMPH and cocaine (Izzo et al., 2002).

AMPH is thought to produce its behavioral effects by increasing extracellular DA levels (Koob and Bloom, 1988). AMPH achieves this increase by competing with DA for uptake by DAT, by inducing DA efflux mediated by DAT in a Na^+ and Ca^{2+} dependent manner (Gnegy et al., 2004; Khoshbouei et al., 2003), and by possibly stimulating a redistribution of DAT away from the plasma membrane (Saunders et al., 2000) (Chi and Reith, 2003; Gulley et al., 2002; Kahlig and Galli, 2003). Insulin stimulation reduced AMPH-induced intracellular accumulation of DAT (Carvelli et al., 2002). This effect of insulin appears to be mediated by PI3K because transient expression of constitutively active PI3K also reduced the AMPH-induced intracellular accumulation of DAT (Carvelli et al., 2002).

Although some progress has been made in understanding how PKC affects NTs localization and function (Gonzalez and Robinson, 2004), the signaling pathways involved in

hormonal (e.g. insulin) regulation of NTs activity and cell surface expression are not well understood. Akt is a central player in insulin and growth factor signaling, and it is thought to regulate several cellular functions including cell growth and apoptosis (Hanada et al., 2004). Three isoforms of Akt have been identified (Hanada et al., 2004), and each of the three Akt isoforms contain a pleckstrin homology (PH) domain that interacts with membrane lipid products of PI3K, and this interaction is required for Akt activation (Hanada et al., 2004). In mouse tissue, both Akt1 and Akt2 isoforms are ubiquitously expressed, whereas Akt3 is relatively highly expressed in brain and testis (Hanada et al., 2004). In hDAT cells, we have now explored the role of endogenous Akt and recombinant Akt1 in the regulation of hDAT surface expression and in insulin regulation of hDAT cell surface expression. We provide evidence that basal activity of Akt controls hDAT plasma membrane expression and that insulin requires the activation of Akt to regulate AMPH-induced hDAT cell surface redistribution.

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Material and Methods

Cell Culture

A fluorescently-tagged hDAT was constructed by fusing the C-terminus encoding region of the enhanced yellow fluorescent protein (YFP) cDNA from pEYFP-N1 (Clontech) to the N-terminus encoding region of the human synthetic DAT cDNA (Saunders et al., 2000), thereby creating the fusion construct YFP-hDAT. This construct was subcloned into a bicistronic expression vector (Rees et al., 1996) modified to express the synthetic hDAT from a cytomegalovirus promoter and a hygromycin resistance gene from an internal ribosomal entry site (pciHyg), as described previously (Saunders et al., 2000). EM4 cells, an HEK 293 cell line stably expressing macrophage scavenger receptor to increase adherence (R. Horlick, Pharmacopeia, Cranberry, NJ), were transfected with the YFP-DAT using Lipofectamine (GIBCO/BRL), and a stably transfected pool (hDAT cells) was selected in 250 μg/ml hygromycin as described (Ferrer and Javitch, 1998; Saunders et al., 2000). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C and 5% CO_2.

Uptake of [^{3}H]DA

[^{3}H]DA uptake was performed as previously described (Carvelli et al., 2002). hDAT cells were seeded into 24-well plates approximately 24 hr prior to the experiment (150,000 cells per well). After 2 hr of serum starvation in KHRT buffer containing (in mM); 120 NaCl, 4.7 KCl, 10 HEPES, 5 TRIZMA base, 2.2 $CaCl_2$, and 10 dextrose with 100 μM ascorbic acid, the cells were treated in quadruplicate wells with AMPH in KHRT buffer, at pH 7.4 and 37 °C. The plates were removed from the incubator and the cells were washed (3 washes of 5 minutes each) with 4^{o}C KHRT buffer to remove the AMPH from each well and inhibit protein trafficking (Saunders

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et al., 2000). The plates were then placed into an 18 °C incubator in KHRT buffer containing 100 μM pargyline, a monoamine oxidase inhibitor. Fifty nM [^{3}H]DA (Amersham, Piscataway, NJ, USA) together with 15 μM DA were added to reach a final volume of 250 μL. Cells were incubated for 2 min and then the solution was aspirated to terminate uptake. After three quick washes with ice-cold uptake buffer, the cells were lysed with 1ml microscint-20 fluid (Perkin Elmer Inc. Wellesley, MA). Radioactivity was measured in a Top Count scintillation counter (Perkin Elmer Inc. Wellesley, MA). Specific uptake was defined as total uptake minus non-specific uptake in the presence of 10 μM mazindol. Data were analyzed with Prism 3.02 software.

In the experiments in which insulin was applied, we add insulin (1:1000) to the incubating medium from a stock solution of 0.9% NaCl, 12 mM HCl (vehicle) containing 1 mM insulin. The vehicle itself had no significant effect on the parameters under study (data not shown).

Cell surface biotinylation

Cell-surface biotinylation experiments were performed as previously described (Kahlig et al., 2004; Saunders et al., 2000) with slight modification. hDAT cells were seeded into 6-well plates (10^6 cells/well) approximately 24 hr prior to the experiment. After 1 hr of starvation in KRHT buffer, the cells treated with AMPH in KHRT at 37 ^{o}C for the indicated time points. The cells were washed twice with ice-cold PBS containing 0.1mM $CaCl_2$ and 1mM $MgCl_2$ (PBS-Ca-Mg) and treated with Ez-link Sulfo-NHS-Biotin (1.5 mg/ml in PBS-Ca-Mg, Pierce Biotech Co., Rockford, IL) on ice for 1 hr. The reaction was quenched by washing twice with 4 ºC PBS-Ca-Mg containing 100 mM glycine (PBS-Ca-Mg-glycine) followed by an incubation with PBS-Ca-Mg-glycine for 30 min on ice. Cells were then washed twice with 4 ºC PBS-Ca-Mg before lysis

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with 1 ml of Radio Immuno Precipitation Assay (RIPA) buffer (20 mM Tris, 20 mM EGTA, 1 mM DTT , 1% Triton X-100) containing protease inhibitors (100 μM PMSF, 5μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM benzamidine) for 30 min on ice with constant shaking. Lysates were centrifuged at 14,000g for 30 min at 4ºC. The supernatants were isolated and biotinylated proteins were separated by incubation with ImmunoPure Immobilized Streptavidin beads (Pierce Biotech Co, Rockford, IL) for 1 hr at room temperature with constant mixing. Beads were washed three times with RIPA buffer containing protease inhibitors. Biotinylated proteins were then eluted with Laemmli loading buffer for 30 min at room temperature. Total cell lysates and biotinylated proteins (cell-surface) were separated by SDS-polyacrylamide electrophoresis (7.5%) and transferred into PVDF membranes (BioRad). PVDF membranes were incubated for 1.5 hr in blocking buffer (5% dry milk, 0.1 % Tween-20 in TBS) and immunoblotted with a rat monoclonal antibody directed against the N terminus of the human dopamine transporter (1:2000 in blocking buffer, Chemicon Inc., Temecula, CA). Immunoreactive bands were visualized using HRP-conjugated goat anti-rat antibody (1:5000 in blocking buffer, Santa Cruz Biotechnology, Santa Cruz, CA) with ECL-Plus on Hypersensitive ECL film (Amersham, Arlington Heights, IL). Band densities were calculated using Scion-Image software (Scion Corporation, Frederik, MD) and normalized to the appropriate total extract to control for protein loading. Data were analyzed with Prism 3.02 software and reported as mean ± SEM.

Transient Expression of Dominant Negative Akt (K179R) and Constitutively Active (myristylated) Akt (myrAkt)

hDAT cells were transiently transfected with a "kinase dead" (KD) dominant negative mutant, (K179R) of Akt1 (Akt-KD) using the PolyFect transfection method (QIAGEN). The Akt-KD mutant was made by using PCR to mutate Lys179 of Akt-HA (obtained from Dr. R.

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Roth, Stanford University) to an Arg, and the mutation was confirmed by DNA sequencing. Cells were incubated overnight in the presence of 2 μg of DNA per 35 mm dish (biotinylation assays) or 0.5 μg per well of a 24-well plate (uptake assays). A constitutively active Akt1 (myrAkt) (Ballou et al., 2001) subcloned into pcDNA3.1 was transfected into hDAT cells using PolyFect as described above. Immunoblot analysis demonstrated that transient expression of K179R or myrAkt did not alter protein levels of DAT with respect to cells transfected with the vector alone (data not shown). The cells were treated 48 h after transfection as indicated for the uptake and biotinylation experiments.

Immunoblots for phsopho Akt and total Akt were obtained using a pS^{473}-Akt Ab (1:500) and a carboxy-terminal Akt mouse Ab (1:1000)(Cell Signaling Technology).

Results

Insulin modulates AMPH-induced hDAT cell surface redistribution and dopamine uptake

AMPH causes hDAT to redistribute away from the plasma membrane (Saunders et al., 2000). This phenomenon is dynamin-dependent, as it is reduced by transient overexpression of K44A, a dominant negative mutant of dynamin (Damke et al., 1994). Recently, by means of confocal imaging, Carvelli *et al.* demonstrated that the AMPH-induced decrease in hDAT plasma membrane expression was partially restored by subsequent stimulation of the PI3K-signaling pathway with insulin (Carvelli et al., 2002). To further characterize the hormonal regulation of hDAT expression, we tested whether the timing of insulin stimulation (i.e. prior/subsequent to AMPH) was important for its modulation of the AMPH-induced hDAT cell surface redistribution. Figure 1A shows immunoblots obtained from hDAT cells treated either with vehicle (CTR), with 10 μM AMPH for 30 min (AMPH 30'), or with 1 μM insulin for 10 min followed by 10 μM AMPH for 30 min in the continued presence of insulin (Ins + AMPH).

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Although incubation of hDAT cells with AMPH significantly decreased the level of hDAT protein recovered in the surface biotinylated fractions (Fig 1A, top lane), preincubation with insulin followed by AMPH resulted in cell surface expression similar to CTR. Similarly, exposure of hDAT cells to AMPH for 40 min (AMPH 40') decreased the level of hDAT recovered in the biotinylated fraction, whereas 10 μM AMPH for 30 min followed by 1 μM insulin for 10 min in the continued presence of AMPH (AMPH + Ins) resulted in cell surface expression similar to CTR (Fig. 1B, top lane). Quantification of the biotinylated bands obtained from three different experiments as in Fig. 1A and 1B is shown in Fig. 1C and 1D, respectively. Incubation of hDAT cells with insulin (1 μM) for 40 min significantly increased hDAT cell surface expression to 145 ± 9.6 % of CTR levels (inset). Thus, whether insulin was added before or after addition of AMPH, AMPH still reduced cell surface expression with respect to insulin treatment alone, with the resulting level of surface expression similar to CTR.

Incubation of hDAT cells with 10 μM AMPH for 40 min (AMPH) decreased [^{3}H]DA uptake to 70 ± 4 % of control condition, (Fig. 2, dotted bar), consistent with the change in cell surface expression shown above. Application of 1 μM insulin during the last 10 min of AMPH exposure restored [^{3}H]DA uptake to 104 ± 6 % of control conditions (Fig. 2, closed bar; n = 3). This level of uptake was significantly lower than that seen after 10 min of 1 μM insulin alone (136 ± 6 % of control conditions (Fig. 2, stripped bar; n = 3)), which is also consistent with the surface expression data.. Similarly, incubating hDAT cells with insulin for 10 min prior to AMPH application (30 min) also led to basal levels of [^{3}H]DA uptake (98 ± 4 % of control conditions, n=4; AMPH alone was 66 ± 10 % of control condition).

Although these offsetting effects of AMPH and insulin might be achieved through different mechanisms, we hypothesized that regulation of Akt activity is the common

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mechanism. In hDAT cells, insulin stimulation of Akt activity, as measured by phosphorylation of Akt on Ser 473, was maximal after 10 min and stable up to 40 min. Band density of the immunoblots obtained using a pS^{473}-Akt Ab increased respectively to 353 ± 61 % and 345 ± 42 % of control condition after 10 min and 40 min of insulin application. In contrast, incubating hDAT cells with 10 μM AMPH for 40 min (AMPH) reduced phosphorylation of Akt on Ser 473 to 20 ± 4 % of control conditions (CTR)(inset). Data are expressed as mean ± SEM and compared to respective controls by two-tailed *t* test ($p < 0.05$; $n = 3$). In parallel experiments, neither AMPH nor insulin caused any significant change of [^{3}H]glycine uptake mediated by endogenous glycine transporters, thereby demonstrating that the regulation of DAT does not result from nonspecific bulk membrane movement (data not shown).

Expression of constitutively active Akt prevents AMPH-induced reduction of hDAT cell surface expression and [^{3}H]DA uptake.

Akt is a PH domain domain-containing serine/threonine kinase. Earlier reports suggested that Akt is activated upon translocation to the plasma membrane as a consequence of its binding to phosphoinositides (e.g. PIP_3) that are induced by insulin stimulation (Elmendorf and Pessin, 1999; Taha and Klip, 1999). Therefore, we used a membrane-targeted constitutively-active Akt (myrAkt) (Ballou et al., 2001) to probe whether Akt is part of the regulatory network required by insulin to modulate AMPH-induced trafficking of hDAT. hDAT cells were transiently transfected either with myrAkt or vector. Figure 3A shows surface biotinylated (top lane) and total (bottom lane) protein extract of transfected hDAT cells treated either with 10 μM AMPH or vehicle for 30 min. AMPH caused a decrease in the amount of hDAT proteins recovered in the biotinylated fraction of vector transfected cell (Fig. 3A). In contrast, in the cells transfected with

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myrAkt, AMPH did not decrease hDAT cell surface expression (Fig. 3A). Panel B shows quantification of the biotinylated bands obtained from three different experiments. In vector (open bar) and myrAkt (closed bar) transfected cells, AMPH decreased hDAT cell surface expression to 74 ± 7.2 and 98 ± 1.7 % of control conditions, respectively (Fig. 3B). Overexpression of myrAkt did not, however, alter basal levels of hDAT plasma membrane expression (data not shown).

Consistent with these effects on cell surface expression, in hDAT cells transfected with the empty vector, AMPH reduced [^{3}H]DA uptake to 72 ± 7 % of control (Fig. 3C, open bars). In contrast, expression of myrAkt (Fig. 3C, close bars) blocked the ability of AMPH to reduce [^{3}H]DA uptake. Also consistent with the effects on cell surface expression, myrAkt transfection did not significantly alter [^{3}H]DA uptake with respect to vector control (Fig. 3C).

Overexpression of a dominant negative mutant of Akt impairs surface expression of hDAT and insulin modulation of AMPH-induced hDAT cell surface redistribution.

AMPH application decreased both hDAT expression at the plasma membrane and phosphorylation of Akt on Ser 473 (Fig. 1,2), suggesting that basal activity of Akt is necessary to maintain normal surface levels of DAT. Therefore, we tested the effects of an Akt inhibitor, ML-9 (Fiory et al., 2004; Smith et al., 2000) originally characterized as a competitive inhibitor of a low myosin-light chain kinase (Saitoh et al., 1987) on cell surface expression of hDAT. Incubation of hDAT cells with 100 μM ML9 for 30 min produced a significant decrease in the level of hDAT protein recovered in the biotinylated fraction (Fig. 4A). Figure 4B shows quantification of the biotinylated bands obtained from 6 different experiments. In ML9 treated cells (ML9, closed bar) the biotinylated fraction was 72 % +/- 6.6 % of vehicle control (CTR,

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open bar). Consistent with these effects on surface expression, incubation of hDAT cells with 100 μM ML-9 for different time periods (between 5 and 60 min) resulted in a time-dependent decrease in [^{3}H]DA uptake (Fig. 4C). ML-9 decreased [^{3}H]DA uptake to 74.6 ± 6.1 of control conditions after 5 min of drug application.

To assess whether insulin modulation of the AMPH-induced hDAT cell surface redistribution requires Akt activation, 48 hours after transfection with Akt-KD or vector, hDAT cells were treated either with 10 μM AMPH for 40 min or with 1 μM insulin for 10 min followed by 10 μM AMPH for 30 min in the continued presence of insulin (Fig. 5). Figure 5A shows that upon insulin stimulation, the amount of hDAT recovered in the biotinylated fraction is reduced in hDAT cells overexpressing Akt-KD with respect to vector transfection. Figure 5B shows quantitation of the biotinylated bands obtained from four different experiments. Data were normalized to total protein and expressed as a percentage of vector transfected hDAT cells treated with AMPH. Insulin significantly increased hDAT cell surface expression in vector transfected cells after AMPH treatment (120 ± 17 % with respect to AMPH treatment alone; open bar). Conversely, transfection of Akt-KD blocked the insulin-mediated increase in hDAT plasma membrane expression after AMPH treatment (closed bar). The amount of hDAT recovered in the biotinylated fraction was not affected by transfecting hDAT cells with vector (Fig. 5C, top lane (left), compare Vector versus CTR). In vector transfected cells, plasma membrane hDAT was 107 ± 3 % of control condition ($n = 3$; $p > 0.05$ by two-tailed *t* test). In contrast, overexpression of Akt-KD in hDAT cells decreased cell surface expression of hDAT (Fig. 5C, top lane (right), compare Akt-KD versus vector). Similarly to ML-9 and AMPH treated cells (see Figs. 1, 4), in Akt-KD transfected cells the amount of hDAT recovered in the biotinylated fraction was 61 ± 10 % of vector control condition suggesting that basal Akt activity

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regulates hDAT cell surface expression (n = 3; level of significance equal to $p < 0.05$ by two-tailed *t* test).

ML9 also reduced the ability of insulin to modulate AMPH-induced hDAT cell surface redistribution. Figure 6A shows an immunoblot of biotinylated and total hDAT protein fraction recovered from cells treated with 1 μM insulin for 10 min followed by 10 μM AMPH for 30 min in the absence or presence of ML9. The presence of ML9 decreased the level of hDAT protein recovered in the biotinylated fraction. Figure 6B shows quantitation of the biotinylated bands obtained from four different experiments. In hDAT cells treated with AMPH, insulin significantly increased hDAT cell surface expression (128 ± 24 % of control conditions, open bar), but in hDAT cells preincubated with ML9, insulin failed to promote any significant increase in hDAT cell surface expression (closed bar).

Discussion

Akt is involved in multiple cellular functions including growth, apoptosis (Hanada et al., 2004) and glucose metabolism (George et al., 2004). Our data demonstrate that Akt plays an essential role in the homeostatic regulation of hDAT activity. A pharmacological inhibitor of Akt, ML9, decreased hDAT surface expression and DA uptake in a time dependent fashion (Fig. 4). Similar results were obtained with a dominant negative mutant of Akt (Fig. 5C). These results, for the first time, suggest that basal Akt activity maintains basal cell surface DAT levels and thereby basal level of DA uptake.

It is not yet known whether Akt activity regulates hDAT cell surface expression, and consequently DA uptake, by controlling the rate of DAT exocytosis and/or its rate of endocytosis. It is known, however, that in adipocytes and muscles insulin induces the

translocation of the intracellular GLUT4 glucose transporter to the plasma membrane (Cheatham and Kahn, 1995) and that this translocation is mediated by Akt (Elmendorf and Pessin, 1999). Therefore, we explored whether the insulin modulation of the AMPH-induced decrease in hDAT plasma membrane expression (Fig. 1) requires activation of Akt. The insulin modulation of AMPH-induced cell surface redistribution was blocked by overexpression of a dominant mutant of Akt (Fig. 5) and by preincubation with the Akt inhibitor ML9 (Fig. 6). Because AMPH inhibits Akt activity (Fig. 2), these data suggest that insulin requires activation of Akt in order to restore normal hDAT plasma membrane expression upon AMPH treatment. Moreover, increasing Akt activity by overexpressing the constitutively active myrAkt blocked AMPH-induced hDAT cell surface redistribution (Fig. 3B) as well as the ability of AMPH to decrease DA uptake (Fig. 3C), suggesting a direct correlation between insulin modulation of AMPH-induced hDAT cell surface redistribution and Akt activity.

A well-characterized signaling event elicited by insulin is the regulation of glucose homeostasis. In non-brain tissue such as skeletal muscle and adipose tissue, insulin signaling stimulates an increase of glucose reuptake (Taha and Klip, 1999). Although insulin crosses the blood-brain barrier (Banks and Kastin, 1998), neurons utilize insulin-independent mechanisms to obtain glucose. Therefore, it is conceivable that insulin in the CNS could modulate cellular processes distinct from those involved in the cellular supply and metabolism of glucose. Indeed, Patterson and coworkers have shown that insulin regulates dopaminergic neurotransmission, although the underlying mechanism remains unclear (Patterson et al., 1998). DA uptake in striatal preparations from fasted hypoinsulinemic rats was significantly decreased as compared to control rats (Patterson et al., 1998). The reduction in DA uptake resulted from a decrease in transporter V_{max} and not from a change in the K_m for DA. Such a reduction in V_{max} is consistent

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with an increased intracellular accumulation of DAT in these diabetic, insulin-deficient animals. Consistent with this hypothesis and with our data, the V_{max} of DA uptake was restored to control levels after incubation of striatal suspensions from fasted animals with 1 nM insulin for 30 min (Patterson et al., 1998). These data strongly suggest that changes in insulin levels in the limbic area of the brain could affect DA uptake, and consequently, extracellular DA levels. Notably, no changes in DA uptake were observed in the nucleus accumbens of food-deprived rats, suggesting that insulin selectively affects brain regions.

AMPH is thought to increase extracellular DA levels both by competing with DA for DAT and through facilitated exchange (Fischer and Cho, 1979). In addition, AMPH has been shown to decrease DAT cell surface expression and DA uptake, which also likely contributes to an increase in extracellular DA levels (Chi and Reith, 2003; Gulley et al., 2002; Little et al., 2002; Saunders et al., 2000). Interestingly, in pharmacologically-induced diabetic rats (i.e. alloxan-treated), the ability of acute administration of AMPH to induce anorexia, stererotyped behavior and increased locomotor activity was markedly attenuated, whereas subsequent administration of insulin reversed this attenuation (Marshall, 1978). Similarly, Galici and coauthors demonstrated that in diabetic rats AMPH self-administration and DA uptake in striatum were both reduced (Galici et al., 2003). Collectively, these studies suggest that insulin pathways in the brain may play an important role in regulating DAT activity, extracellular DA levels and the actions of AMPH.

Regulation of hDAT cell surface expression may represent an important mechanism through which insulin signaling modulates the dopaminergic system and the actions of AMPH. Here, we demonstrated that insulin offsets the ability of AMPH to decrease hDAT plasma membrane expression and DA uptake. This insulin effect did not depend on the time at which

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insulin was applied (i.e. before or after AMPH incubation), indicating that the AMPH-induced decrease in DA uptake is a consequence of hDAT cell surface redistribution rather than hDAT inactivation (Figs. 1 and 2).

Several studies have implicated PI 3-kinase (PI3K) in the regulation of trafficking and activity of the glucose and glutamate transporters (Clarke et al., 1994; Davis et al., 1998; James and Piper, 1994). Similarly, PI3K has been shown to be essential for insulin modulation of hDAT function in striatal synaptosomes and hDAT cells (Carvelli et al., 2002). PI3K inhibitors, such as LY294002, blocked the insulin-induced increase in DA uptake (Carvelli et al., 2002). Moreover, in hDAT cells, overexpression of a constitutively active mutant of PI3K increased DA uptake (Carvelli et al., 2002). These data suggested that insulin signaling in general and PI3K in particular regulate DA uptake, and we have now shown that Akt activation appears to be essential for the effect of insulin on hDAT. Interestingly, although both insulin and exogenous PI3K increased basal levels of DA uptake in hDAT cells (Carvelli et al., 2002), overexpression of constitutively active Akt did not (Fig. 3C). Thus, in addition to its effects on Akt, insulin (and likely PI3K) might also regulate DAT activity through another mechanism, although the myrAKT may not be sufficiently constitutively active to mimic the effects of insulin on Akt activation, and, therefore, Akt may be the only mechanism by which insulin effects DAT cell surface expression.

In summary, our results demonstrate that the basal activity of Akt is essential for maintaining cell surface expression of hDAT and that insulin modulation of AMPH-induced trafficking of hDAT requires Akt activation. Our results could provide a mechanism for hormonal regulation of drug abuse, and suggest that the insulin signaling pathway, including Akt, may represent a new cellular target for substance abuse therapies.

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Footnotes

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Legends for Figures

Figure 1. Insulin modulates AMPH-induced hDAT cell surface redistribution. (A); (B) Representative immunoblot for hDAT proteins recovered from biotinylated and total extract obtained from hDAT cells treated as indicated. (C); (D) Quantification of the immunoblots using the Scion Image system. The density of the biotinylated samples was normalized to the density of the parallel total extract in order to correct for difference in cell seeding and hDAT expression in different wells and expressed as percentage of control. The normalized data are expressed as mean ± SD and compared to respective controls by one way ANOVA followed by the Tukey test; * ; $^{\#}$ = level of significance $p < 0.05$. Inset Representative immunoblot for hDAT proteins recovered from biotinylated and total extract obtained from hDAT cells treated either with vehicle (CTR) or insulin (1 μM) for 40 min. The density of the biotinylated samples was normalized to the density of the parallel total extract and expressed as a percentage of control. Insulin treatment increased the density of the biotinylated samples by 45 ± 9.6 % respect to CTR. Data are expressed as mean ± SD and compared to respective controls by two-tailed *t* test ($p < 0.05$; $n = 3$).

Figure 2. Insulin modulates AMPH-induced decrease in [^{3}H]DA uptake. hDAT cells were treated either with vehicle (open bar, CTR), 1 μM insulin for 10 min (stripped bar, Ins 10'), 10 μM AMPH for 40 min (dotted bar, AMPH) or 10 μM AMPH for 40 min adding 1μM insulin during the last 10 min of the AMPH treatment (closed bar , AMPH + Ins). Data are expressed as percentage of control conditions. The normalized data are expressed as a mean ± SEM and compared against respective controls by one way ANOVA followed by the Tukey test (# = level

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of significance $p < 0.05$; n = 3). Inset Representative immunoblot obtained using a pS^{473}-Akt Ab in control conditions (CTR) or after bath application of 10 μM AMPH for 40 min (AMPH). The band density of the pS^{473}-Akt samples (top lane) was normalized to the density of the parallel total Akt extract (bottom lane) and expressed as a percentage of control.

Figure 3. Transient transfection of hDAT cells with myrAkt blocks AMPH-induced decrease in hDAT cell surface expression and prevents AMPH-induced decrease in [^{3}H]DA uptake. (A) Representative immunoblot for hDAT proteins recovered from biotinylated and total extract obtained from hDAT cells transiently transfected either with vector or myrAkt and treated as indicated. (B) Quantification of the immunoblot density using Scion Image system. The density of the biotinylated samples was normalized to the parallel total extracts and expressed as a percentage of vehicle treated vector. The normalized data are expressed as mean ± SD and compared against respective controls by two-tailed *t* test (* = level of significance $p < 0.05$; n = 3). (C) hDAT cells transfected with vector (open bars) or myrAkt (closed bars) were treated either with 10 μM AMPH or vehicle for 30 min. [^{3}H]DA uptake is expressed as a percentage of vector control as mean ± SEM and analyzed using one way ANOVA followed by the Tukey test (n =3, experiments conducted in quadruplicate; * = level of significance $p < 0.05$).

Figure 4. The Akt inhibitor ML-9 decreases both hDAT cell surface expression and [^{3}H]DA uptake in hDAT cells. (A) Representative immunoblot for hDAT proteins recovered from biotinylated and total extract obtained from hDAT cells treated either with vehicle (CTR) or 100 μM of ML-9 for 30 min. (B) Quantification of immunoblot samples using Scion Image system. The density of the biotinylated samples was normalized to the parallel total extract and expressed

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as a percentage of vehicle control (CTR) as mean ± SD. (n = 6, * = level of significance $p < 0.01$ by two-tailed *t* test). (C) hDAT cells were treated either with vehicle (CTR) or 100 μM ML-9 for the indicated time periods and then [^{3}H]DA uptake was measured. Data are expressed as percentage of control conditions and as a mean ± SEM and analyzed by one way ANOVA followed by the Tukey test (n = 4; * = level of significance $p < 0.01$).

Figure 5. Transient transfection of hDAT cells with Akt-KD blocks insulin modulation of AMPH-induced hDAT cell surface redistribution. (A) Representative immunoblot for hDAT proteins recovered from biotinylated and total extract obtained from hDAT cells transiently transfected either with vector or Akt-KD and treated as indicated. (B) Quantification of band densities using Scion Image system. The density of the biotinylated samples was normalized to the parallel total extract and expressed as a percentage of vector transfected treated with 10 μM AMPH for 30 min. Data points represent the mean ± SD and were analyzed by a two-tailed *t* test; * = level of significance at least $p < 0.01$). (C) Representative immunoblot for hDAT proteins recovered from biotinylated and total extract obtained from either hDAT cells (CTR) or hDAT cells transiently transfected either with vector or Akt-KD.

Figure 6. ML-9 blocks insulin modulation of AMPH-induced hDAT cell surface redistribution.

(A) Representative immunoblot for hDAT proteins recovered from biotinylated and total extract obtained from hDAT cells treated either with vehicle (CTR) or 1 μM insulin for 10 min followed by 10 μM AMPH for 30 min in the absence (Ins+AMPH) or presence of 100 μM ML-9 (ML-9, Ins+AMPH). (B) Quantification of immunoblot samples using Scion Image system. The density

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of the biotinylated samples was normalized by the parallel total extract to correct the difference for cell seeding and hDAT expression in different wells and expressed as a percentage of the correspondent controls as mean ± SD, and analyzed by one way ANOVA followed by the Tukey test ($n = 4$; * = level of significance $p < 0.05$).

Fig. 1

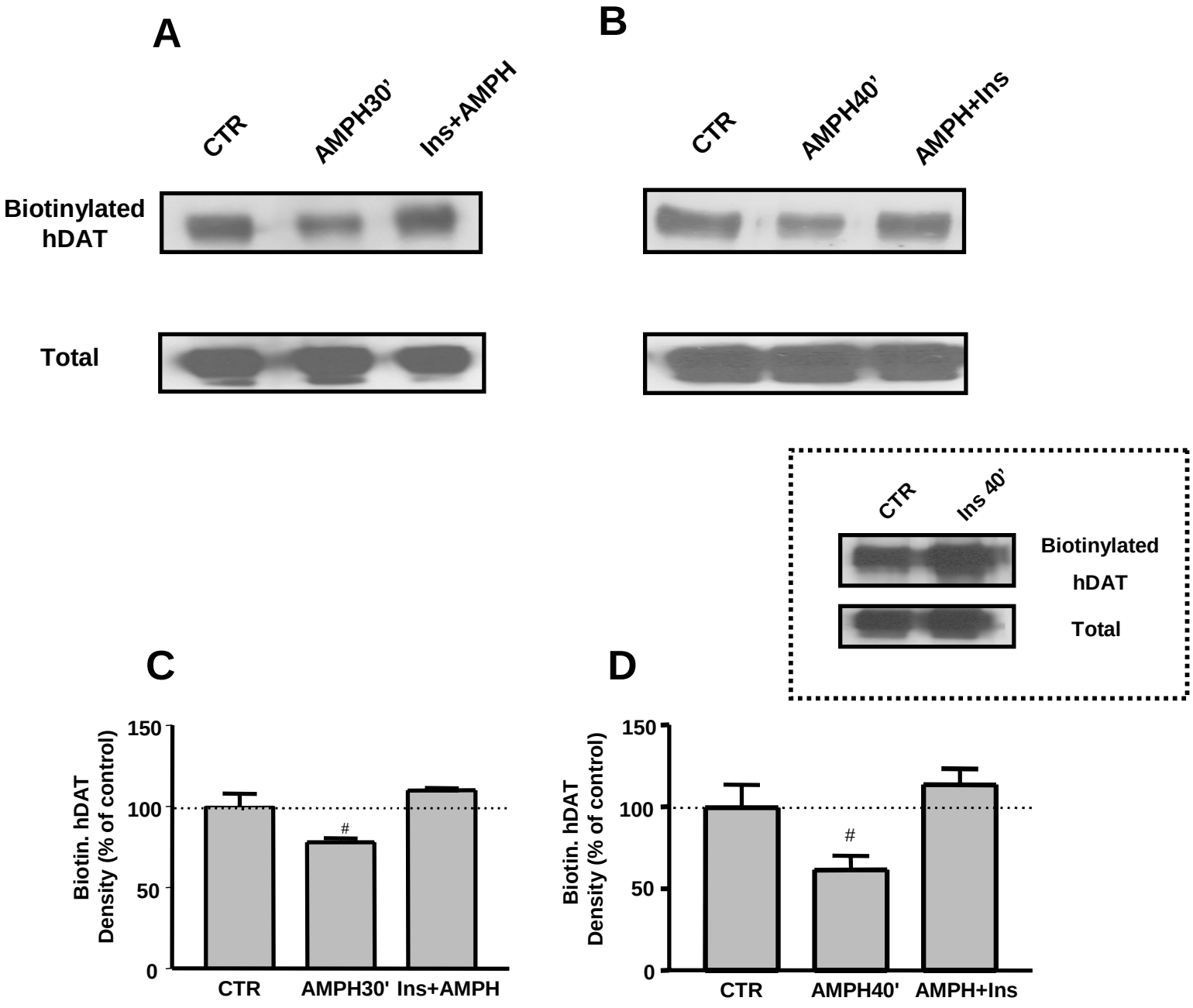

Fig. 2

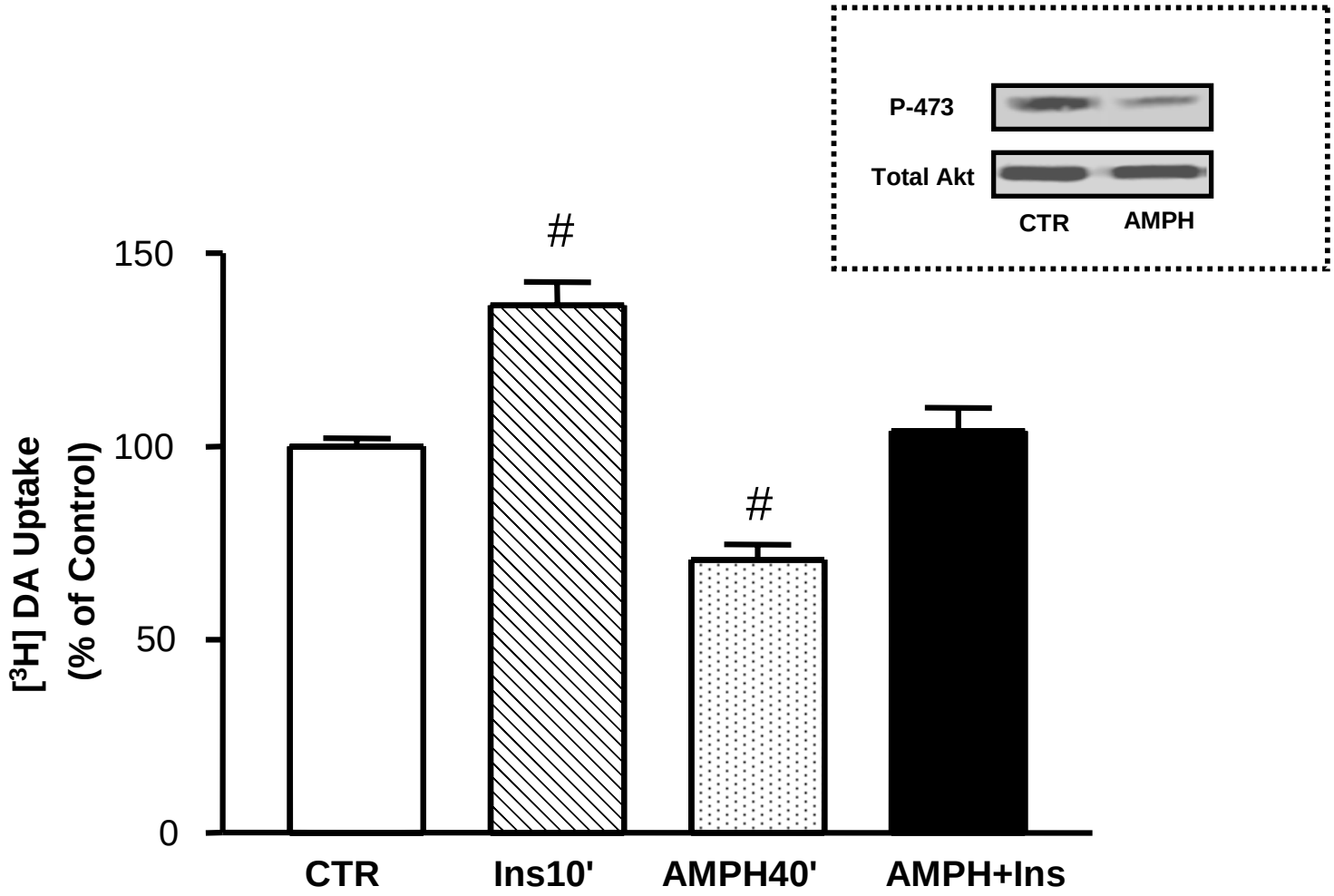

Fig. 3

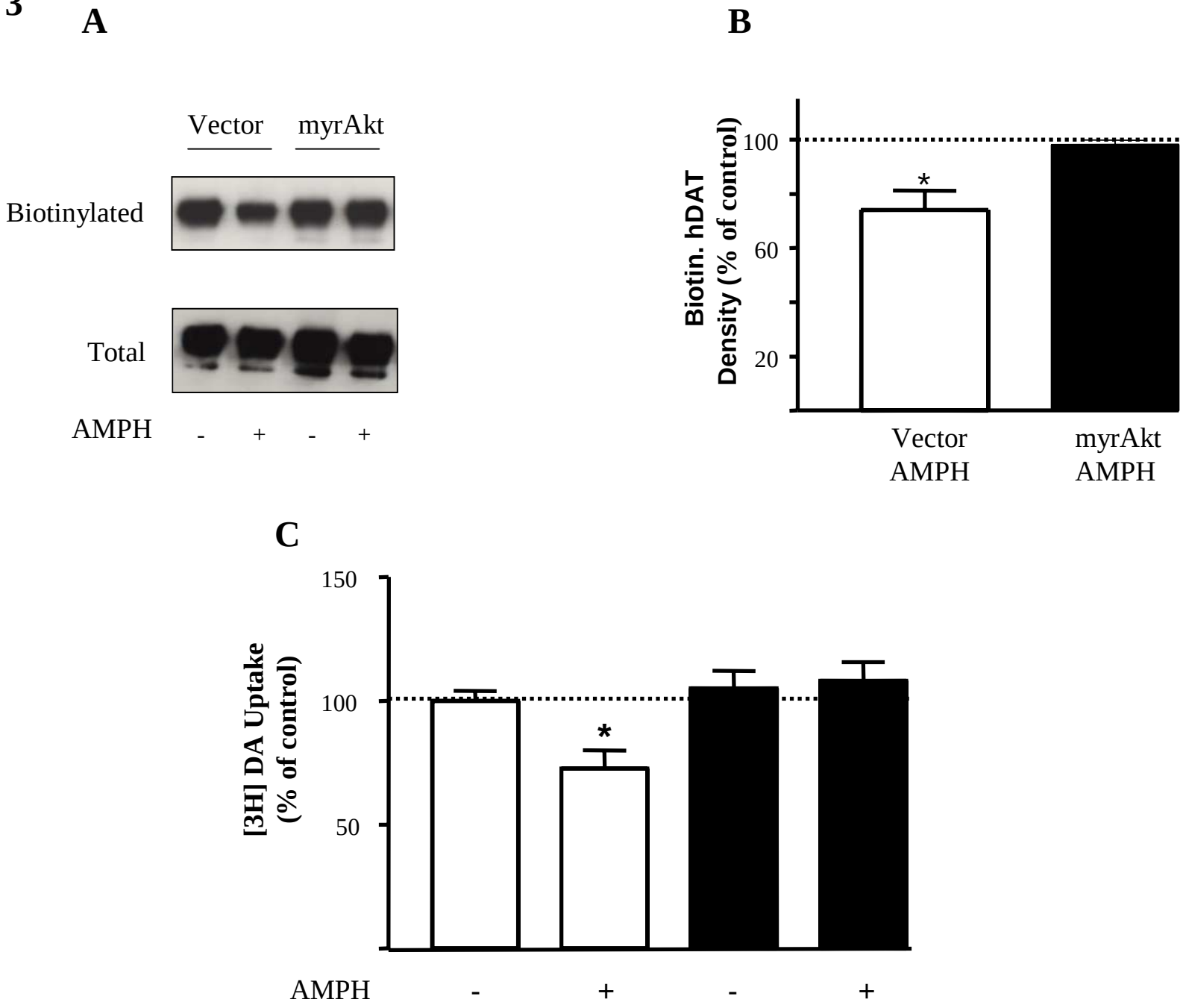

Fig. 4

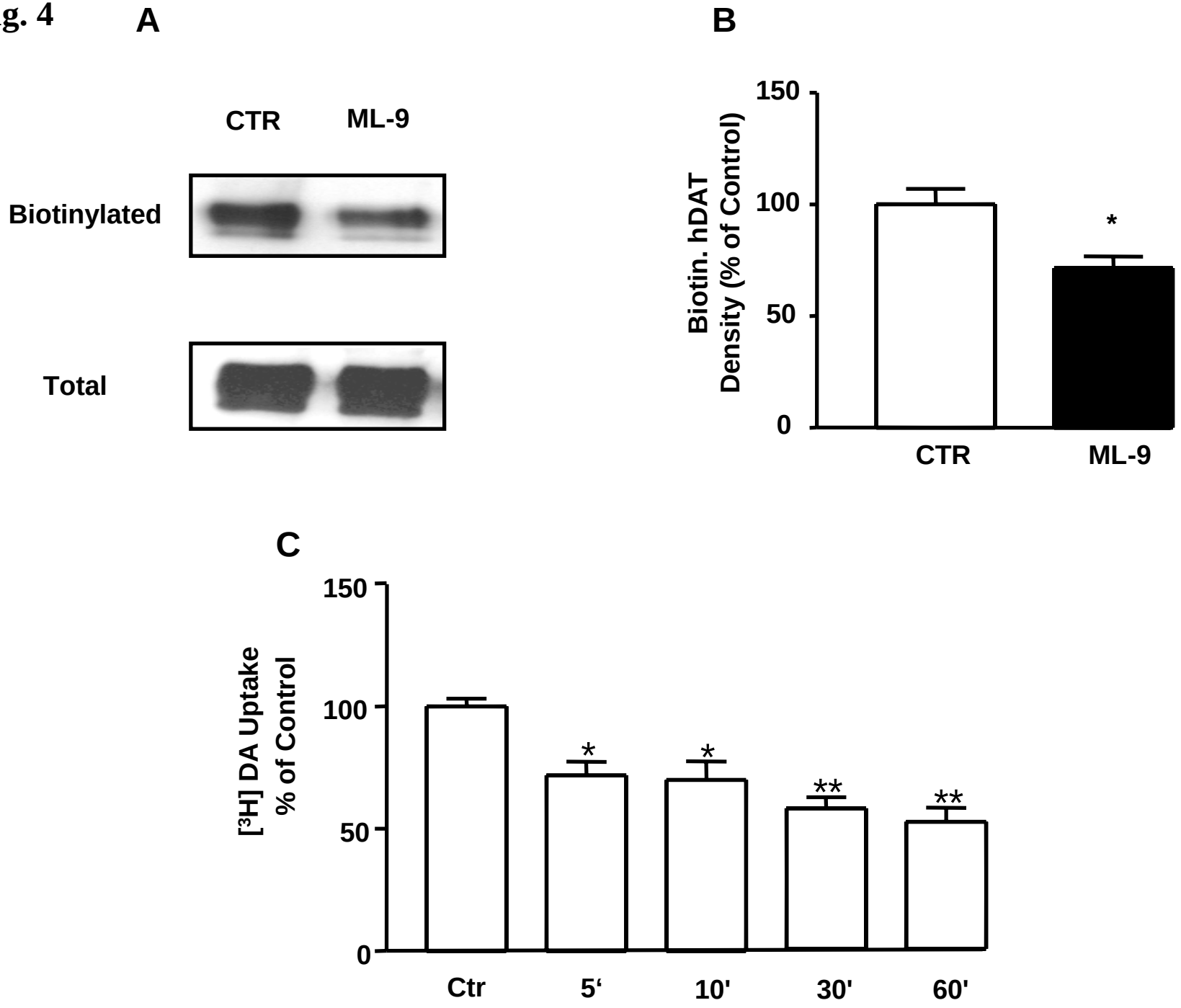

Fig. 5

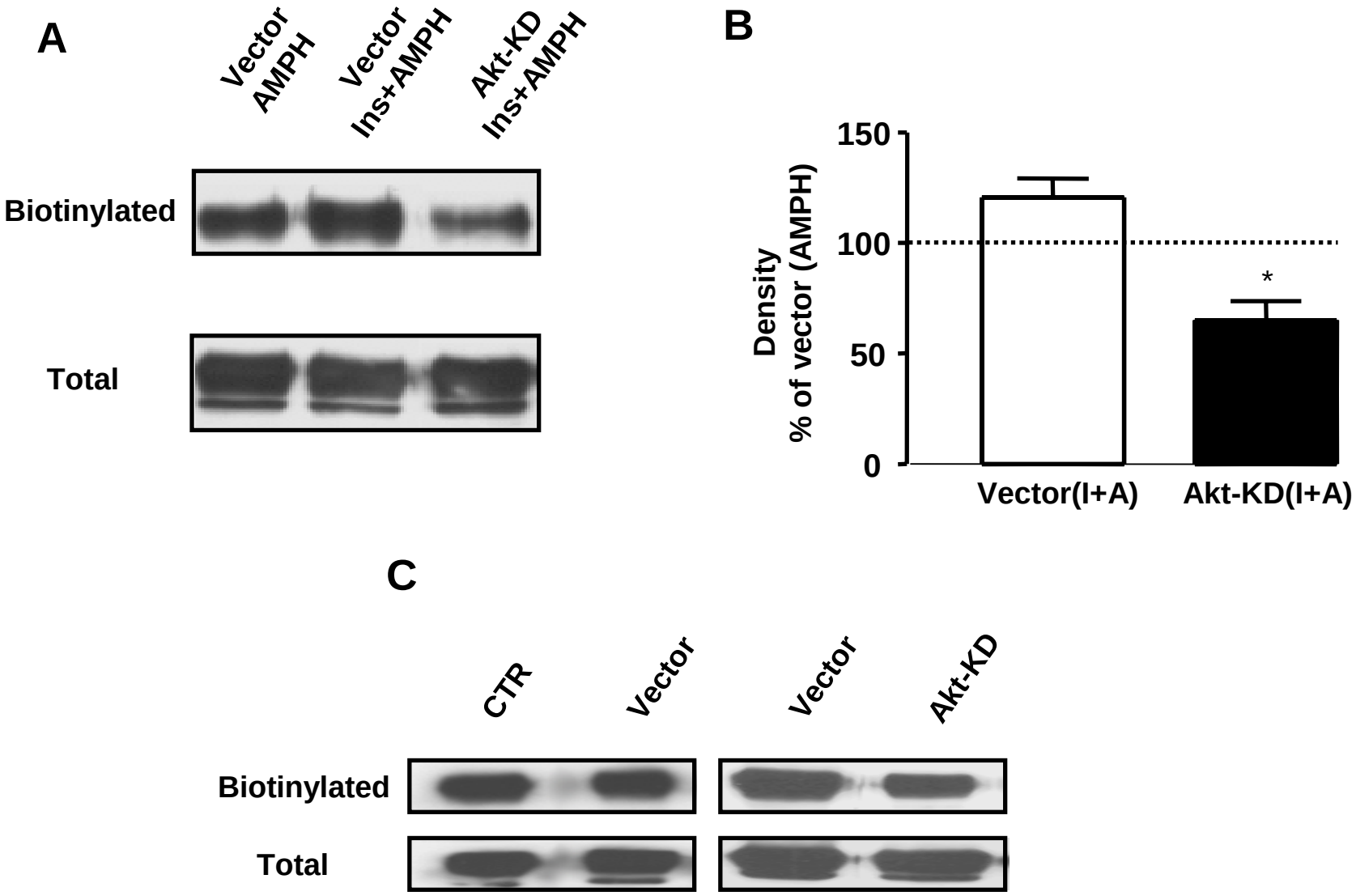

Fig. 6