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Nicotine regulates DARPP-32 phosphorylation at multiple sites in neostriatal neurons

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Running title: Regulation of DARPP-32 phosphorylation by nicotine

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Abbreviations used:

cAMP, cyclic AMP; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of M_r 32 kDa; DHβE, dihydro-β-erythroidine; nAChRs, nicotinic acetylcholine receptors; PKA, cAMP-dependent protein kinase; PP-1, protein phosphatase-1; TTX, tetrodotoxin.

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Abstract

Nicotinic acetylcholine receptors (nAChRs), by modulating the release of neurotransmitters, regulate dopaminergic signaling in the striatum. We have recently reported that nicotine stimulates the release of dopamine via α4β2* nAChRs and/or α7 nAChRs, leading to regulation of DARPP-32 at Thr34, the site involved in regulation of protein phosphatase-1 (PP-1). In this study, we investigated the regulation of DARPP-32 phosphorylation at its other sites, Thr75 (Cdk5 site), Ser97 (CK2 site), and Ser130 (CK1 site), that serve to modulate T34 phosphorylation and dephosphorylation. In neostriatal slices, nicotine (100 μM) increased phosphorylation of DARPP-32 at Ser97 and Ser130 at an early time point (30 s), and decreased phosphorylation of DARPP-32 at Thr75 at a late time point (3 min). The increase in Ser97 and Ser130 phosphorylation was mediated through the release of dopamine via activation of α4β2* nAChRs and α7 nAChRs and the subsequent activation of dopamine D1 and D2 receptors. The decrease in Thr75 phosphorylation was mediated through the release of dopamine via activation of α4β2* nAChRs and the subsequent activation of dopamine D1 receptors. These various actions of nicotine on modulatory sites of phosphorylation would be predicted to result in a synergistic increase in the state of phosphorylation of DARPP-32 at Thr34 and thus would contribute to increased dopamine D1 receptor/DARPP-32 Thr34/PP-1 signaling.

Introduction

Dopamine plays a central role in the regulation of psychomotor function in the brain. Many of the actions of dopamine are mediated through signal transduction pathways that involve DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of M_r 32 kDa) (Svenningsson et al., 2004). When DARPP-32 is phosphorylated on Thr34, it is converted into a potent inhibitor of protein phosphatase-1 (PP-1), and thereby controls the phosphorylation state and activity of many downstream physiological effectors. We have recently reported that nicotine regulates DARPP-32 phosphorylation at Thr34 by stimulating the release of dopamine in medium spiny neurons in the neostriatum (Hamada et al., 2004). Nicotine at a low concentration (1 μM) stimulates dopamine D2 receptor signaling in striatopallidal/indirect pathway neurons, likely by activating β2 subunit-containing nicotinic acetylcholine receptors (nAChRs), presumably α4β2* nAChRs, at dopaminergic terminals. Nicotine at a high concentration (100 μM) stimulates dopamine D1 receptor signaling in striatonigral/direct pathway neurons, likely by activating (i) α4β2* nAChRs at dopaminergic terminals and (ii) α7 nAChRs at glutamatergic terminals, which, by stimulating the release of glutamate, activates NMDA/AMPA receptors at dopaminergic terminals. The differential regulation of DARPP-32 Thr34 phosphorylation by low and high concentrations of nicotine may contribute to its regulation of psychomotor functions (Calabresi et al. 1989; Picciotto, 2003; Laviolette and van der Kooy, 2004).

Phosphorylation of DARPP-32 at Thr34 is critical for inhibition of PP-1. Mouse DARPP-32 is also phosphorylated at Thr75 by cyclin-dependent kinase 5 (Cdk5), Ser97 (Ser102 in rat sequence) by CK1, and Ser130 (Ser137 in rat sequence) by CK2, and the phosphorylation by each kinase modulates the functions of DARPP-32 (Svenningsson et al., 2004). DARPP-32 phosphorylated at Thr75 inhibits PKA activity and thereby reduces the efficacy of dopamine D1 receptor/PKA signaling (Bibb et al., 1999). DARPP-32 Ser97 phosphorylation increases the efficacy of Thr34 phosphorylation by PKA (Girault et al., 1989), and DARPP-32 Ser130 phosphorylation decreases the rate of dephosphorylation of Thr34 by PP-2B (calcineurin) (Desdouits et al., 1995a; Desdouits et al., 1995b). As a consequence, the phosphorylation of DARPP-32 by CK1 or CK2 increases the state of phosphorylation of DARPP-32 at Thr34. In this study, we found that nicotine at a high concentration (100 μM) increases DARPP-32 phosphorylation at Ser97 and

Ser130 and decreases DARPP-32 phosphorylation at Thr75. These changes likely contribute synergistically to an increase in DARPP-32 Thr34 phosphorylation and potentiation of dopamine D1 receptor/PKA/DARPP-32 Thr34/PP-1 signaling.

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Materials and Methods

Preparation and incubation of neostriatal slices. Male C57BL/6 mice at 6-8 weeks old were purchased from Japan SLC (Shizuoka, Japan). All mice used in this study were handled in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and the specific protocols were approved by the Institutional Animal Care and Use Committee of Kurume University School of Medicine. Mice were sacrificed by decapitation. The brains were rapidly removed and placed in ice-cold, oxygenated Krebs-HCO_3^- buffer (124 mM NaCl, 4 mM KCl, 26 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1.25 mM KH_2PO_4, 1.5 mM $MgSO_4$ and 10 mM D-glucose, pH 7.4). Coronal slices (350 μm) were prepared using a vibrating blade microtome (VT1000S, Leica Microsystems, Nussloch, Germany). Striata were dissected from the slices in ice-cold Krebs-HCO_3^- buffer. Each slice was placed in a polypropylene incubation tube with 2 ml of fresh Krebs-HCO_3^- buffer containing adenosine deaminase (10 μg/ml). The slices were preincubated at 30°C under constant oxygenation with 95% O_2/5% CO_2 for 60 min. The buffer was replaced with fresh Krebs-HCO_3^- buffer after 30 min of preincubation. Slices were pretreated with a dopamine uptake inhibitor, nomifensine (10 μM), for 10 min, and then treated with drugs as specified in each experiment. Treatment with nomifensine alone did not affect the levels of phospho-Thr34, phospho-T75, phospho-Ser97, or phospho-Ser130 DARPP-32. Drugs were obtained from the following sources: α-bungarotoxin (α-BgTx), dihydro-β-erythroidine (DHβE), N,2,3,3-tetramethylbicyclo[2.2.1]heptan-2-amine hydrochloride (mecamylamine), (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK801), (-)-1-methyl-2-(3-pyridyl)pyrrolidine (nicotine), 1,2,3,4-tetrahydro-2-methyl-4-phenyl-8-isoquinolinamine maleate salt (nomifensine), 3,5-dichloro-N-(1-ethylpyrrolidin-2-ylmethyl)-2-hydroxy-6-methoxybenzamide tartrate salt (raclopride), R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH23390), (±)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide (SKF81297) from Sigma-Research Biochemicals International (St. Louis, MO); tetrodotoxin (TTX) from Wako Pure Chemical (Osaka, Japan); 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) from

Tocris Cookson (Bristol, UK); cyclosporin A from LC Laboratories (Woburn, MA). After drug treatment, slices were transferred to Eppendorf tubes, frozen on dry ice, and stored at -80^{o}C until assayed.

Immunoblotting. Frozen tissue samples were sonicated in boiling 1% SDS containing 50 mM sodium fluoride and boiled for an additional 10 min. Small aliquots of the homogenate were retained for protein determination by the BCA protein assay method (Pierce, Rockford, IL) using bovine serum albumin as standard. Equal amounts of protein (100 µg) were separated by SDS/PAGE (12% polyacrylamide gels), and transferred to nitrocellulose membranes (0.2 µm) (Schleicher and Schuell, Keene, NH) as described (Towbin et al., 1979). The membranes were immunoblotted using phosphorylation state-specific antibodies raised against DARPP-32 phospho-peptides: phospho-Thr34, the site phosphorylated by PKA (mAb-23; 1:750 dilution) (Snyder et al., 1992); phospho-Thr75, the site phosphorylated by Cdk5 (1:5000 dilution) (Bibb et al., 1999c); phospho-Ser97, the site phosphorylated by CK2 (1:500 dilution) (see below); phospho-Ser137 (Ser130 in mouse sequence), the site phosphorylated by CK1 (1:1000 dilution) (Liu et al., 2001c). A monoclonal antibody (C24-5a; 1:7,500 dilution) generated against DARPP-32 (Hemmings and Greengard, 1986), which is not phosphorylation state-specific, was used for reblotting the membrane in order to determine the total amount of DARPP-32. None of the experimental manipulations used in the present study altered the total amount of DARPP-32.

Antibody binding was revealed by incubation with a goat anti-mouse horseradish peroxidase-linked IgG (1:2,000 dilution) (Pierce, Rockford, IL) or a goat anti-rabbit horseradish peroxidase-liked IgG (1:2000-4000 dilution) and the ECL immunoblotting detection system (Amersham, Arlington Heights, IL). Chemiluminescence was detected by autoradiography using Kodak autoradiography film, and phospho-Thr34, phospho-Thr75, phospho-Ser97, and phospho-Ser130 DARPP-32 bands were quantified by densitometry using NIH Image 1.61 software. Samples from control and drug-treated slices were analyzed on the same immunoblots. For each experiment, values obtained for treated slices were calculated relative to the value for the control slices. Normalized data from multiple experiments were averaged and statistical analysis was carried out as described in the figure legends.

Generation of phosphorylation state-specific antibody for phospho-Ser97 DARPP-32. A polyclonal antibody specific for phospho-Ser97 DARPP-32 was raised in rabbits by immunizing with the peptide, Ac-CNQA(pS)EEE-amide, coupled to carrier protein (Quality Controlled Biochemicals, Hopkinton, MA). The antiserum was confirmed to be phospho-specific using dephospho- and phospho-recombinant DARPP-32. 6X-His tagged recombinant human DARPP-32 was expressed in *E. coli* using a pET21 vector and purified by Ni-NTA affinity chromatography. The purified DARPP-32 was phosphorylated by incubation with CK2 (Biomol, Plymouth Meeting, PA) for 1 hour at 30°C. Equal amounts of either phosphorylated or unmodified DARPP-32 were loaded on a 3-10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with either preimmune serum or immune serum (Fig. 1A). This serum was further purified via affinity chromatography before use.

Striatum was dissected from wild-type (+/+) or DARPP-32 null (-/-) mice and homogenized in 1% SDS. Equal amounts of protein (25 µg) were loaded on a 10% bis-trispolyacrylamide gel (BioRad, Hercules, CA), and transferred to nitrocellulose. The membranes were immunoblotted with either affinity-purified phospho-Ser97 DARPP-32 antibody or DARPP-32 antibody (Cell Signaling, Beverly, MA). Affinity-purified phospho-Ser97 DARPP-32 antibody was found to recognize a single polypeptide of 32 kDa in striatal extracts from wild-type mice, but no crossreactive proteins were detected in striatal extracts from DARPP-32 null mice (Fig. 1B), indicating that phospho-Ser97 DARPP-32 antibody specifically reacts with DARPP-32 phosphorylated at Ser97.

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Results

Effect of nicotine on DARPP-32 phosphorylation at Thr75, Ser97 and Ser130

We have recently reported (Hamada et al., 2004) that nicotine at 100 μM transiently stimulates DARPP-32 Thr34 phosphorylation by 7-fold within 15 sec of incubation (reproduced in Fig. 2A for comparison), and that, in contrast, nicotine at 1 μM decreases DARPP-32 Thr34 phosphorylation within 3 min of incubation. In this study, we investigated the effect of nicotine on other phosphorylation sites of DARPP-32 in neostriatal slices. Treatment with nicotine at 100 μM decreased the level of phospho-Thr75 DARPP-32 at 3 min of incubation (Fig. 2B). Nicotine at 1 μM did not affect the level of phospho-Thr75 DARPP-32 (data not shown). As observed for Thr34 phosphorylation, nicotine at 100 μM rapidly and transiently stimulated DARPP-32 phosphorylation at both Ser97 (2-fold, Fig. 2C) and Ser130 (1.6-fold, Fig. 2D) within 30 sec of incubation, and the increased levels of phospho-Ser97 and phopho-Ser130 returned to basal values at 3 min. Nicotine at 1 μM did not affect either the level of phospho-Ser97 or phospho-Ser130 DARPP-32 (data not shown).

Analysis of the decrease in DARPP-32 Thr75 phosphorylation caused by nicotine

We next examined the mechanism by which nicotine (100 μM) decreased DARPP-32 Thr75 phosphorylation at 3 min of incubation. To examine the possible involvement of dopamine, the effect of nicotine (100 μM) was examined in the presence of a Na^+ channel blocker, TTX (1 μM), a dopamine D1-type receptor antagonist, SCH23390 (1 μM), or a dopamine D2-type receptor antagonist, raclopride (1 μM) (Fig. 3A). Pretreatment of slices with TTX or SCH23390 did not affect the basal level of phospho-Thr75 DARPP-32, but abolished the inhibitory effect of nicotine on DARPP-32 Thr75 phosphorylation. Pretreatment with raclopride (1 μM) did not affect either the basal level of phospho-Thr75 DARPP-32 or the nicotine-induced decrease in DARPP-32 Thr75 phosphorylation. These results suggest that the effect of nicotine on DARPP-32 Thr75 phosphorylation is mediated through the TTX-sensitive release of dopamine from dopaminergic terminals and activation of dopamine D1 receptors in neostriatal neurons.

To identify the nAChRs involved in the decrease in DARPP-32 Thr75 phosphorylation by nicotine, the effect of nicotine was examined in the presence of various nAChR antagonists: a

nonselective nAChR antagonist, mecamylamine (100 μM), a specific inhibitor of β2 subunit-containing nAChRs, DHβE (50 μM), or an α7 nAChR antagonist, α-bungarotoxin (10 nM) (Fig. 3B). Pretreatment of slices with mecamylamine, DHβE or α-bungarotoxin did not affect the basal level of phospho-Thr75 DARPP-32. The inhibitory effect of nicotine on DARPP-32 Thr75 phosphorylation was antagonized by mecamylamine and DHβE, but not by α-bungarotoxin, suggesting that the effect of nicotine is mediated through β2 subunit-containing nAChRs. We have previously reported that activation of ionotropic glutamate NMDA and AMPA receptors in neostriatal neurons stimulates the dephosphorylation of phospho-Thr75 DARPP-32 by PP-2A and decreases DARPP-32 Thr75 phosphorylation (Nishi et al., 2002). We therefore examined the effect of nicotine in the presence of an NMDA receptor antagonist, MK801 (100 μM), plus an AMPA receptor antagonist, CNQX (20 μM) (Fig. 3B). Pretreatment with MK801 plus CNQX did not affect the nicotine-induced decrease in DARPP-32 Thr75 phosphorylation. These results suggest that nicotine stimulates dopamine release via activation of β2 subunit-containing nAChRs, presumably α4β2* nAChRs, at dopaminergic terminals, and activates a dopamine D1 receptor/PKA/PP-2A cascade in neostriatal neurons (Nishi et al., 2000), leading to the decrease in DARPP-32 Thr75 phosphorylation.

Analysis of the increase in DARPP-32 Ser97 phosphorylation caused by nicotine

The mechanism by which nicotine (100 μM) increased DARPP-32 Ser97 phosphorylation at 30 s of incubation was analyzed. To examine the possible involvement of dopamine, the effect of nicotine was studied in the presence of TTX (1 μM), SCH23390 (1 μM) or raclopride (1 μM) (Fig. 4A). Pretreatment of slices with TTX did not affect the basal level of phospho-Ser97 DARPP-32, but abolished the stimulatory effect of nicotine on DARPP-32 Ser97 phosphorylation. Pretreatment of slices with SCH23390 or raclopride did not affect the basal level of phospho-Ser97 DARPP-32, but both SCH23390 and raclopride abolished the stimulatory effect of nicotine on DARPP-32 Ser97 phosphorylation.

To identify the nAChRs involved in the increase in DARPP-32 Ser97 phosphorylation by nicotine, the effect of nicotine was examined in the presence of mecamylamine (100 μM), DHβE (50 μM) or α-bungarotoxin (10 nM) (Fig. 4B). Pretreatment of slices either with mecamylamine, DHβE or α-bungarotoxin did not affect the basal level of phospho-Ser97 DARPP-32. The stimulatory

effect of nicotine on DARPP-32 Ser97 phosphorylation was antagonized by mecamylamine, DHβE, and α-bungarotoxin, suggesting that the effect of nicotine is mediated through β2 subunit-containing nAChRs and α7 nAChRs. To examine the possible involvement of glutamate, the effect of nicotine was examined in the presence of MK801 (100 μM) plus CNQX (20 μM). Pretreatment with MK801 plus CNQX abolished the nicotine-induced increase in DARPP-32 Ser97 phosphorylation. These results suggest that nicotine activates β2 subunit-containing nAChRs at dopaminergic terminals and α7 nAChRs at glutamatergic terminals, which, by stimulating the release of glutamate, activates NMDA and AMPA receptors at dopaminergic terminals, resulting in the release of dopamine. The released dopamine activates both dopamine D1 and D2 receptors in neostriatal neurons, leading to the increase in DARPP-32 Ser97 phosphorylation.

Analysis of the increase in DARPP-32 Ser130 phosphorylation caused by nicotine

The mechanism by which nicotine (100 μM) increased DARPP-32 Ser130 phosphorylation at 30 s of incubation was examined. Pretreatment of slices with TTX (1 μM) did not affect the basal level of phospho-Ser130 DARPP-32, but abolished the stimulatory effect of nicotine on DARPP-32 Ser130 phosphorylation (Fig. 5A). Pretreatment with SCH23390 (1 μM) or raclopride (1 μM) did not affect the basal level of phospho-Ser130 DARPP-32, but both SCH23390 and raclopride abolished the stimulatory effect of nicotine on DARPP-32 Ser130 phosphorylation.

Pretreatment of slices with mecamylamine (100 μM), DHβE (50 μM) or α-bungarotoxin (10 nM) did not affect the basal level of phospho-Ser130 DARPP-32 (Fig. 5B). The stimulatory effect of nicotine on DARPP-32 Ser130 phosphorylation was antagonized by mecamylamine, DHβE, and α-bungarotoxin, suggesting that the effect of nicotine is mediated through β2 subunit-containing nAChRs and α7 nAChRs. The effect of nicotine was examined in the presence of MK801 (100 μM) plus CNQX (20 μM). Pretreatment with MK801 plus CNQX abolished the nicotine-induced increase in DARPP-32 Ser130 phosphorylation. These results suggest that, similarly to the stimulation of Ser97 phosphorylation, nicotine stimulates DARPP-32 Ser130 phosphorylation via activation of β2 subunit-containing nAChRs and α7 nAChRs, dopamine release and activation of both dopamine D1 and D2 receptors in neostriatal neurons.

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Discussion

In our previous study (Hamada et al., 2004), we demonstrated that nicotine at a high concentration (100 μM) stimulates the release of dopamine by activating both α4β2* nAChRs at dopaminergic terminals and α7 nAChRs at glutamatergic terminals, leading to regulation of DARPP-32 phosphorylation at the Thr34 site. In the present study, we have found that nicotine (100 μM) modulates DARPP-32 phosphorylation at three additional phosphorylation sites (Thr75, Ser97 and Ser130) in addition to Thr34 (Table 1). Treatment of mouse neostriatal slices with nicotine (100 μM) increased Ser97 and Ser130 phosphorylation at an early time point (30 s), as it did for Thr34 phosphorylation, and decreased Thr75 phosphorylation at a later time point (3 min). Each of the changes in DARPP-32 phosphorylation at Thr75, Ser97 and Ser130, induced by nicotine, is likely to contribute to the activation of PKA/DARPP-32 Thr34/PP-1 signaling in neostriatal neurons (Table 1).

Since Ser97 phosphorylation is known to increase the efficiency of phosphorylation of Thr34 by PKA (Girault et al., 1989) and Ser130 phosphorylation to reduce the rate of dephosphorylation of Thr34 by PP-2B (Desdouits et al., 1995a), the increase in Ser97 and Ser130 phosphorylation would be expected to contribute to the increase in Thr34 phosphorylation by nicotine. Nicotine reduced DARPP-32 phosphorylation at Thr75 at a later time point (3 min). DARPP-32 at Thr75 is highly phosphorylated *in vivo* with a stoichiometry of ~26% (Bibb et al., 1999). Therefore, the reduction in Thr75 phosphorylation would remove the tonic inhibition of PKA. Since dis-inhibition of PKA is observed after the peak of Thr34 phosphorylation, the reduction of Thr75 phosphorylation is not directly involved in the early increase in Thr34 phosphorylation. However, it would be expected to increase the state of phosphorylation of DARPP-32 at Thr34 and other PKA substrates at later time points following stimulation with nicotine.

The stimulatory effect of nicotine on DARPP-32 Ser97 and Ser130 phosphorylation is mediated through release of dopamine via activation of both α4β2* and α7 nAChRs. The mechanism underlying the nicotine-induced release of dopamine required for the regulation of Ser97 and Ser130 phosphorylation appears similar to that required for the regulation of Thr34 phosphorylation: nicotine (100 μM) stimulates both α4β2* nAChRs at dopaminergic terminals and α7 nAChRs at glutamatergic terminals; activation of α7 nAChRs results in the release of glutamate; activation of α4β2* nAChRs and activation of NMDA/AMPA receptors by glutamate at

dopaminergic terminals synergistically induce robust dopamine release (Kaiser and Wonnacott, 2000; Hamada et al., 2004). In contrast, the decrease in DARPP-32 Thr75 phosphorylation is mediated through dopamine release solely via activation of α4β2* nAChRs at dopaminergic terminals. Activation of α7 nAChRs or NMDA/AMPA receptors is not required for the regulation of Thr75 phosphorylation by nicotine. These results suggest that depending on the anatomical localization of nicotinic receptors there may be distinct pools of DARPP-32 that are differentially regulated by dopamine released either via activation of both α4β2* and α7 nAChRs, or via activation of only α4β2* nAChRs. These two pools might be expressed in different types of cells, for example direct and indirect pathway medium spiny neurons, or in the same type of cells, but with different intracellular localization.

The effects of nicotine on all four sites of DARPP-32 phosphorylation are mediated through the release of dopamine and activation of dopamine receptors in neostriatal neurons. The effects of nicotine on Thr34 and Thr75 phosphorylation are mediated through activation of dopamine D1 receptors in neostriatal neurons. The results on Thr75 phosphorylation are in agreement with our previous findings showing that activation of D1 receptors leads to activation of PKA and subsequent activation of PP-2A, leading to the dephosphorylation of phospho-Thr75 DARPP-32 (Nishi et al., 2000). Dopamine D1 and D2 receptors have opposing effects on DARPP-32 phosphorylation (Nishi et al., 1997; Nishi et al., 2000), but the ability of D1 receptors to increase Thr34 phosphorylation and to decrease Thr75 phosphorylation are predominant. In contrast to DARPP-32 Thr34 and Thr75 phosphorylation, which are regulated solely by activation of dopamine D1 receptors, activation of both dopamine D1 and D2 receptors is required for the regulation of Ser97 and Ser130 phosphorylation by nicotine. The regulatory mechanisms underlying Ser97 and Ser130 phosphorylation are largely unknown. Elucidation of the mechanism of D1 and D2 receptor interaction will be important for the further understanding of dopamine signaling in the neostriatum. DARPP-32 Ser130 phosphorylation can also be regulated both by group I metabotropic receptors (Liu et al., 2001) and by 5-HT_2 receptors (Svenningsson et al., 2002) through a mechanism involving activation of a PLC/PP-2B/CK1 signaling cascade (Liu et al., 2001a; Liu et al., 2002).

We previously found that nicotine at a low concentration (1 μM) stimulates dopamine D2 receptor signaling and subsequently decreases DARPP-32 Thr34 phosphorylation in

striatopallidal/indirect pathway neurons by activating α4β2* nAChRs at dopaminergic terminals (Hamada et al., 2004). Our observation that nicotine (1 μM) did not affect the phosphorylation of Thr75, Ser97 or Ser130 at any time point (data not shown) supports the conclusion that the effect of D2 receptor activation is unique with respect to Thr34 phosphorylation and does not require the involvement of other phosphorylation sites on DARPP-32. The effect of 1 μM nicotine and selective activation of D2 receptors on Thr34 dephosphorylation presumably reflects the specific signaling pathway(s) that acts only on Thr34 under these conditions. In contrast, the results from the present study indicate that synergistic interactions of multiple pathways acting on Thr75, Ser97 and Ser130 are likely required for the pro-dopaminergic effect of high nicotine concentration that is ultimately mediated by increased phosphorylation of Thr34.

In conclusion, nicotine regulates the state of phosphorylation of DARPP-32 at multiple sites. Phosphorylation of these individual sites synergistically contributes to the enhancement of dopamine D1 receptor/PKA/DARPP-32 Thr34/PP-1 signaling. The enhancement of dopamine signaling may play a role in the ability of nicotine to modulate psychomotor functions.

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Footnotes

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Legends of figures

Fig. 1. Antibodies specific for phospho-Ser97 DARPP-32. (A) Equal amounts of either phosphorylated (lanes 1 and 3) or unmodified (lanes 2 and 4) recombinant human DARPP-32 (prepared as described in Materials and Methods) were probed with either preimmune serum (lanes 1 and 2) or immune serum (lanes 3 and 4). A robust phospho-specific signal was obtained only with phospho-DARPP-32 (lane 3). (B) This serum was further purified via affinity chromatography before use. Affinity-purified phospho-Ser97 DARPP-32 antibody recognized a single polypeptide of 32 kDa in striatal extracts from wild-type mice (+/+), but no crossreactive proteins were detected in striatal extracts from DARPP-32 null mice (-/-).

Fig. 2. Effect of nicotine on DARPP-32 phosphorylation at Thr34, Thr75, Ser97 and Ser130 in the neostriatum. Neostriatal slices were pre-incubated with nomifensine (10 μM) for 10 min, followed by the addition of nicotine at a concentration of 100 μM for the indicated times. Immunoblots for detection of phospho-Thr34 (A), phospho-Thr75 (B), phospho-Ser97 (C), and phospho-Ser130 (D) DARPP-32 are shown in upper panels. The levels of phospho-Thr34 phospho-Thr75, phospho-Ser97, and phospho-Ser130 DARPP-32 were quantified by densitometry, and the data were normalized to values obtained for nomifensine alone. Data represent means ± SEM for 11 to 13 experiments. *$p<0.05$, **$p<0.01$ compared with 0 min; analysis of variance and Newman-Keuls test.

Fig. 3. Role of dopamine receptors, nAChRs and NMDA/AMPA receptors in nicotine-induced decrease in DARPP-32 Thr75 phosphorylation. (A) Neostriatal slices were pre-incubated with TTX (1 μM), a D1-type receptor antagonist, SCH23390 (1 μM), or a D2-type receptor antagonist, raclopride (1 μM) for 10 min, followed by the addition of nicotine (100 μM) for 3 min. (B) Neostriatal slices were pre-incubated for 60 min with a nonselective nAChR antagonist, mecamylamine (10 μM), a specific inhibitor of β2 nAChRs, DHβE (50 μM), or α-bungarotoxin (10 nM), used as an α7 nAChR antagonist, or for 10 min with an NMDA receptor antagonist, MK801 (100 μM), plus an AMPA receptor antagonist, CNQX (20 μM), followed by the addition of nicotine

(100 μM) for 3 min. The levels of phospho-Thr75 DARPP-32 were quantified by densitometry, and the data were normalized to values obtained from untreated slices. Data represent means ± SEM for 7-8 experiments. *p<0.05, **p<0.01 compared with no addition; $^{\#\#}p$<0.01 compared with raclopride alone, $^{\dagger}p$<0.05 compared with α-BgTx alone, $^{\S}p$<0.05 compared with MK801 plus CNQX; analysis of variance and Newman-Keuls test.

Fig. 4. Role of dopamine receptors, nAChRs and NMDA/AMPA receptors in nicotine-induced increase in DARPP-32 Ser97 phosphorylation. (A) Neostriatal slices were pre-incubated with TTX (1 μM), SCH23390 (1 μM), or raclopride (1 μM) for 10 min, followed by the addition of nicotine (100 μM) for 30 s. (B) Neostriatal slices were pre-incubated for 60 min with mecamylamine (10 μM), DHβE (50 μM), or α-bungarotoxin (10 nM), or for 10 min with MK801 (100 μM) plus CNQX (20 μM), followed by the addition of nicotine (100 μM) for 30 s. The levels of phospho-Ser97 DARPP-32 were quantified by densitometry, and the data were normalized to values obtained from untreated slices. Data represent means ± SEM for 4-12 experiments. *p<0.05, **p<0.01 compared with no addition; analysis of variance and Newman-Keuls test.

Fig. 5. Role of dopamine receptors, nAChRs and NMDA/AMPA receptors in nicotine-induced increase in DARPP-32 Ser130 phosphorylation. (A) Neostriatal slices were pre-incubated with TTX (1 μM), SCH23390 (1 μM), or raclopride (1 μM) for 10 min, followed by the addition of nicotine (100 μM) for 30 s. (B) Neostriatal slices were pre-incubated for 60 min with mecamylamine (10 μM), DHβE (50 μM), or α-bungarotoxin (10 nM), or for 10 min with MK801 (100 μM) plus CNQX (20 μM), followed by the addition of nicotine (100 μM) for 30 s. The levels of phospho-Ser130 DARPP-32 were quantified by densitometry, and the data were normalized to values obtained from untreated slices. Data represent means ± SEM for 7-12 experiments. *p<0.05, **p<0.01 compared with no addition; analysis of variance and Newman-Keuls test.

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Table 1. Regulation of DARPP-32 phosphorylation at multiple sites by nicotine (100 μM) in neostriatal neurons.

	T34	**T75**	**S97**	**S130**
Effect	↑	↓	↑	↑
time of peak effect	fast (30 s)	slow (3 min)	fast (30 s)	fast (30 s)
(antagonist)				
D1 receptor	block	block	block	block
D2 receptor	*no effect*	*no effect*	block	block
α4β2 nAChR	block	block	block	block
α7 nAChR	block	*no effect*	block	block
NMDA/AMPA receptor	block	*no effect*	block	block
functional consequences of nicotine	pro-dopaminergic: inhibition of PP1	pro-dopaminergic: dis-inhibition of PKA	pro-dopaminergic: Thr34 becomes a better substrate for PKA.	pro-dopaminergic: P-Thr34 becomes resistant to PP-2B dephosphorylation.

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Fig. 1

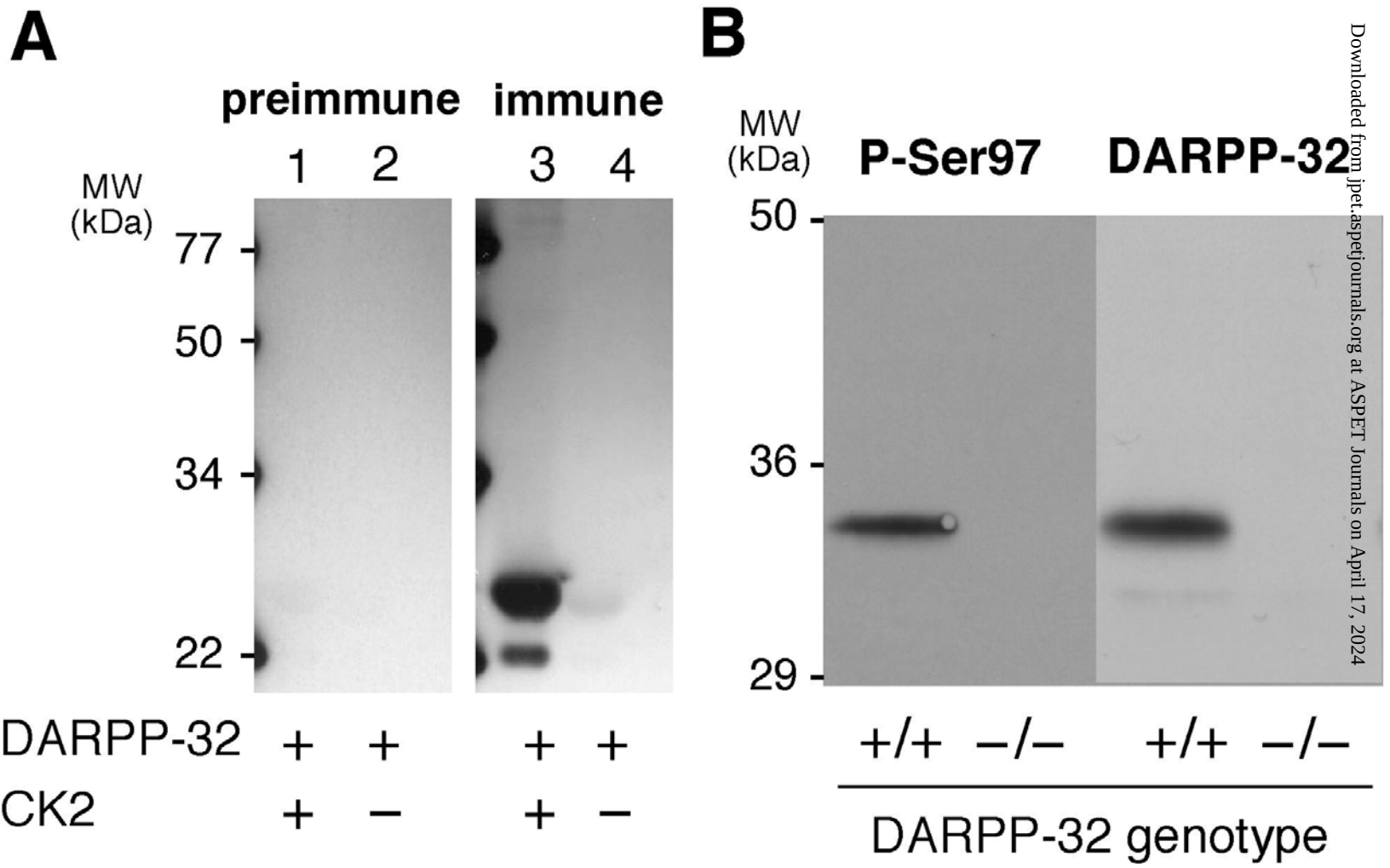

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Fig. 2

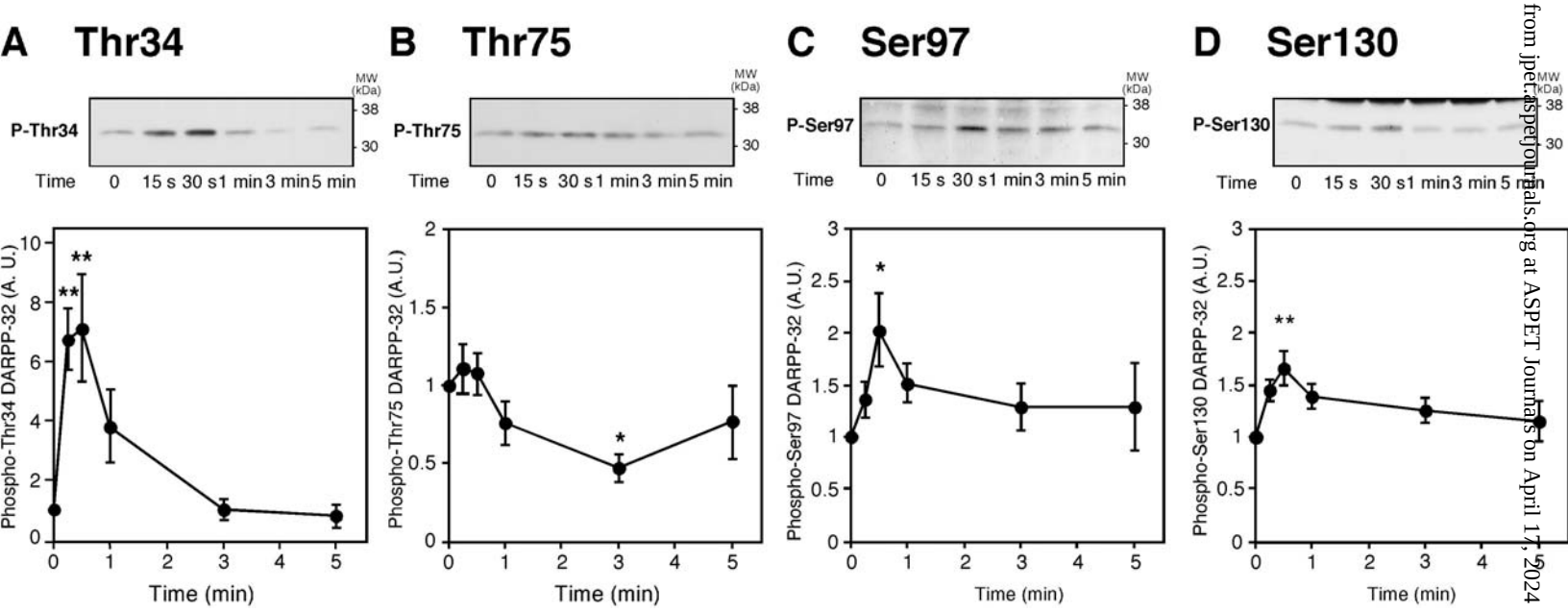

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Fig. 3

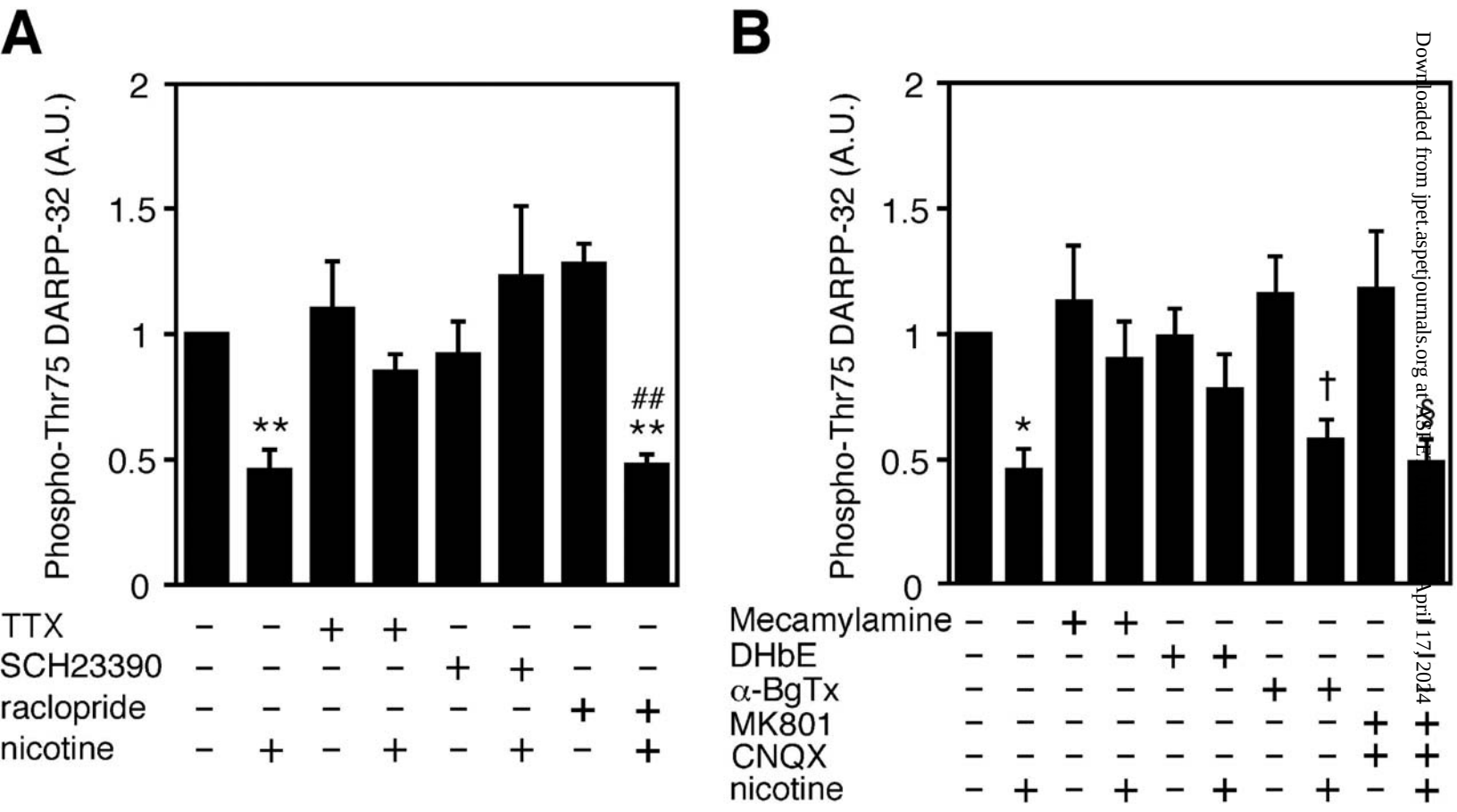

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Fig. 4

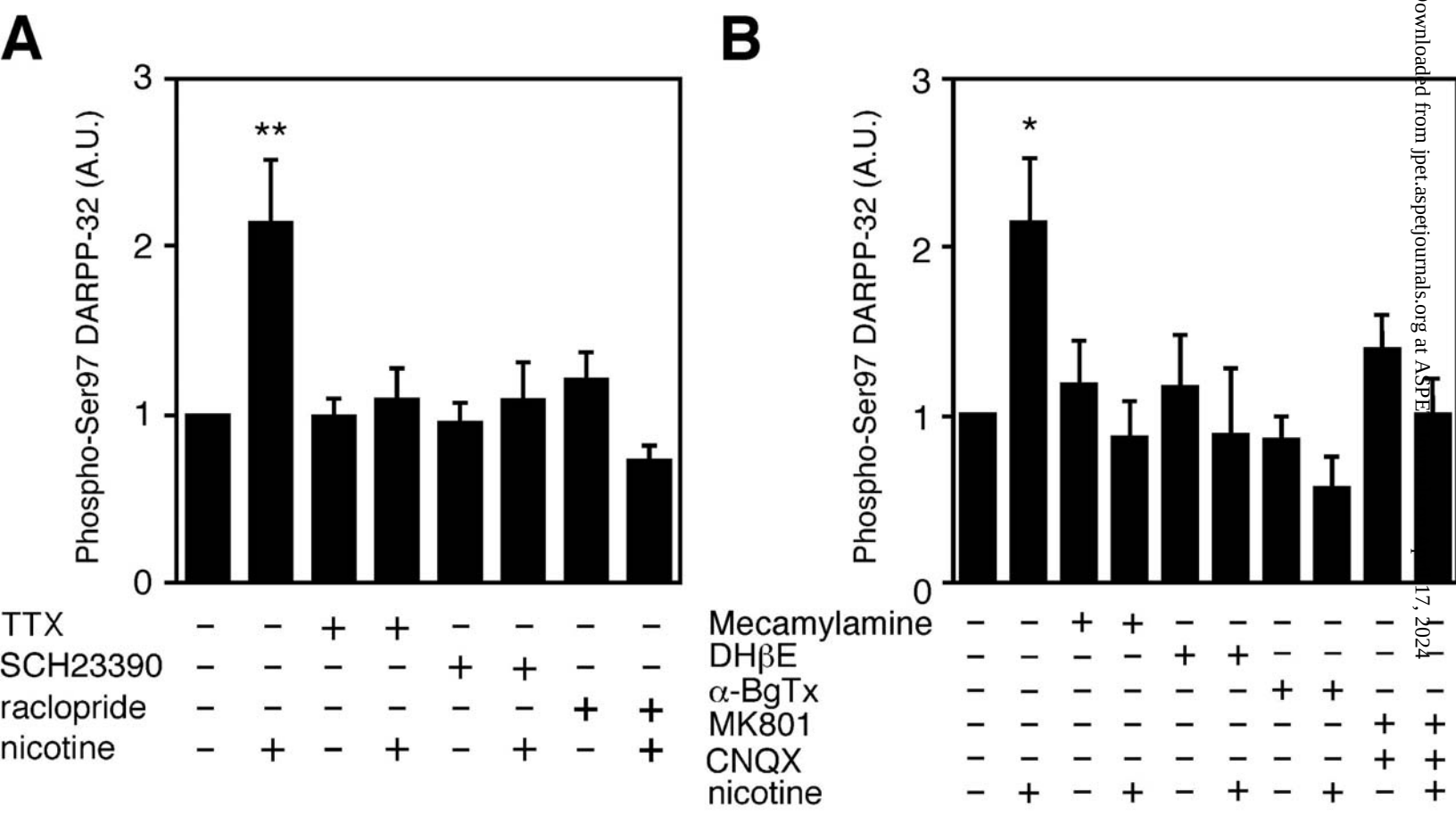

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Fig. 5