

# Neurotensin Triggers Dopamine D2 Receptor Desensitization through a Protein Kinase C and $\beta$ -Arrestin1-dependent Mechanism\*

Received for publication, July 20, 2010, and in revised form, January 12, 2011 Published, JBC Papers in Press, January 13, 2011, DOI 10.1074/jbc.M110.166454

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The peptide neurotensin (NT) is known to exert a potent excitatory effect on the dopaminergic system by inhibiting D2 dopamine (DA) receptor (D2R) function. This regulation is dependent on activation of PKC, a well known effector of the type 1 NT receptor (NTR1). Because PKC phosphorylation of the D2R has recently been shown to induce its internalization, we hypothesized that NT acts to reduce D2R function through heterologous desensitization of the D2R. In the present study, we first used HEK-293 cells to demonstrate that NT induces PKC-dependent D2R internalization. Furthermore, internalization displayed faster kinetics in cells expressing the D2R short isoform, known to act as an autoreceptor in DA neurons, than in cells expressing the long isoform, known to act as a postsynaptic D2R. In patch clamp experiments on cultured DA neurons, overexpression of a mutant D2S lacking three key PKC phosphorylation sites abrogated the ability of NT to reduce D2R-mediated cell firing inhibition. Short interfering RNA-mediated inhibition of  $\beta$ -arrestin1 and dynamin2, proteins important for receptor desensitization, reduced agonist-induced desensitization of D2R function, but only the inhibition of  $\beta$ -arrestin1 reduced the effect of NT on D2R function. Taken together, our data suggest that NT acutely regulates D2 autoreceptor function and DA neuron excitability through PKC-mediated phosphorylation of the D2R, leading to heterologous receptor desensitization.

Since its discovery in the 1970s, NT<sup>2</sup> has been well established as a potent modulator of the DA system (1, 2). A vast majority of DA neurons in the substantia nigra and the ventral tegmental area express NTR1, the high affinity NT receptor (3, 4). Activation of these  $G\alpha_q$ -coupled receptors has been shown

to increase DA neuron excitability via distinct mechanisms, one of which consists in a reduction of presynaptic D2 autoreceptor function (5–8). Activation of these D2Rs by DA normally causes cell hyperpolarization, which leads to a decrease in both spontaneous firing and DA release (9–13). D2R regulation is an important area of interest for modern neuropharmacology because all clinically effective antipsychotic drugs (APDs) act at least in part as D2R antagonists. Moreover, because a growing amount of data from both human and animal studies provides evidence for the implication of NT in the pathophysiology of schizophrenia and in the action of APDs (14–22), a better understanding of the mechanism mediating the NTR1–D2R interaction seems necessary.

The major second messenger cascade triggered by NTR1 signaling involves activation of phospholipase C and the subsequent increase in intracellular calcium concentration, inositol-triphosphate production, and PKC activity, all of which have been linked to physiological effects of NT (7, 23–26). We have previously demonstrated that the regulation of D2R function by NT in cultured DA neurons requires PKC activity (27). Interestingly, it was also shown that the D2R can be directly phosphorylated by PKC, leading to agonist-independent functional desensitization and internalization of the membrane D2R (28, 29). The goal of the present study was to assess whether NTR1 activation leads to PKC-induced D2R desensitization and to establish whether a mechanism of agonist-independent desensitization of the D2R could have any functional significance for the physiological effects of NT on DA neurons. Our results show that NTR1 activation can trigger PKC-dependent internalization of both short and long isoforms of D2R (D2S and D2L) in a transfected HEK-293 cell line. We also demonstrate that the effect of NT on D2R function in cultured DA neurons is strongly reduced by overexpression of a D2S receptor mutated on PKC phosphorylation sites that are critical for D2R desensitization and by siRNA-mediated down-regulation of  $\beta$ -arrestin1.

## EXPERIMENTAL PROCEDURES

**HEK-293 Cell Culture and Transfection**—HEK-293 cells were maintained in DMEM (Invitrogen) enriched with 1% penicillin/streptomycin, 0.5% Glutamax, and 10% fetal bovine serum (Invitrogen). Upon reaching ~80% confluence, the enriched medium was replaced with basic DMEM, and the cells

\* This work was supported by Canadian Institutes of Health Research Grant MOP-49591 (to L.-E. T.) and a graduate studentship (to D. T.), an Ontario Mental Health Foundation grant (to P. R. A.), and an infrastructure grant from the Fonds de la Recherche en Santé du Québec to the Groupe de Recherche sur le Système Nerveux Central.

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<sup>2</sup> The abbreviations used are: NT, neurotensin; NTR1, type 1 NT receptor; DA, dopamine; D2R, D2 dopamine receptor; APD, antipsychotic drug; D2S, short isoform of D2R; D2L, long isoform of D2R; ANOVA, analysis of variance; Quin, quinpirole; CHE, chelerythrine; EGFP, enhanced GFP; Scr, scrambled siRNA sequences.

were transiently transfected with pcDNA3.1 plasmids containing the sequences of HA-tagged NTR1 (Missouri S&T cDNA Resource Center) and FLAG-tagged D2L or D2S using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen).

**Internalization Assay by Immunofluorescence**—24 h after transfection, HEK-293 cells were reseeded in enriched DMEM onto glass coverslips precoated with poly-L-lysine (Invitrogen). The next day, the enriched medium was removed, and cells were incubated with basic DMEM containing a rabbit polyclonal anti-FLAG antibody (1:1000, Sigma-Aldrich) for 30 min to allow binding to surface receptors, before being treated for 10 min with basic DMEM (vehicle), 10  $\mu$ M quinpirole (Quin, specific D2R agonist, Sigma-Aldrich) or 10 nM NT (NT 8-13, active fragment, Sigma-Aldrich). After fixation, cells were incubated with a first secondary antibody (1:500, Alexa Fluor 633 anti-rabbit, Molecular Probes, Eugene, OR) for 30 min, which labeled surface receptors (membrane signal). After washout of the first secondary antibody, cells were permeabilized in a 0.01% Triton X-100 solution and incubated with a second secondary antibody (1:500, Alexa Fluor 488 anti-rabbit, Molecular Probes) for 30 min, which labeled both surface and internalized receptors (total signal = membrane + internalized). Images were acquired with a point-scanning confocal microscope (Prairie Technologies) equipped with argon and helium/neon lasers. Internalization was then quantified cell by cell from images processed with NIH ImageJ software by subtracting the membrane signal from the total signal and expressed as a percentage of the total signal of each individual transfected cell. Statistical analysis was performed with a one-way ANOVA followed with Tukey's multiple comparison tests.

**D2S Colocalization with Rab5**—HEK-293 cells transfected with the D2S, NTR1, and Rab5-GFP plasmids were handled identically to the internalization assay protocol described above, with a labeling of surface D2S on live cells and a subsequent 10-min drug treatment. After fixation and permeabilization, cells were incubated with a goat polyclonal anti-GFP antibody (1:1000, Novus Biologicals, Littleton, CO) and then with secondary antibodies (Alexa Fluor 488 anti-goat and Alexa Fluor 546 anti-rabbit, 1:500 each, Molecular Probes). Images were acquired with a laser scanning confocal microscope (FV1000 MPE, Olympus) equipped with multi-argon and helium/neon lasers. D2S-Rab5 colocalization was measured with the "Mander's coefficient" plug-in of the ImageJ software. The Pearson correlation coefficient obtained for each image from the different treatment groups were averaged and statistically analyzed with a one-way ANOVA followed with Tukey's multiple comparison tests.

**Internalization Assay by ELISA**—24 h after transfection, HEK-293 cells were resuspended in enriched medium and reseeded into 24-well plates precoated with poly-L-lysine. After an overnight incubation, the enriched medium was removed, and the cells were washed with basic DMEM (vehicle) or 10  $\mu$ M chelerythrine (CHE, Sigma-Aldrich), a wide spectrum PKC inhibitor, for 30 min. The cells were then treated with 10  $\mu$ M quinpirole and/or 10 nM NT for different time periods. Receptor trafficking was stopped by addition of ice-cold PBS for 5 min, following which cells were rinsed and fixed in 3% PFA for

20 min. Membrane immunoreactivity of the D2R was revealed by incubation for one hour with an anti-FLAG primary antibody (rabbit polyclonal, 1:1000, Sigma-Aldrich) and for 30 min with HRP-coupled secondary antibody (1:5000, Amersham Biosciences). After antibody incubations and PBS washes, the HRP substrate *o*-phenylenediamine dihydrochloride (SIGMAFAST OPD, Sigma-Aldrich) was added to each well, and the reaction was stopped after 15 min by addition of 3 N HCl. 200  $\mu$ l from each well was then transferred to a 96-well plate for subsequent absorbance readings at 490 nm on a Wallac Victor2 1420 multilabel plate reader (PerkinElmer Life Sciences). Each experiment had two different internal controls consisting of untreated transfected cells (100% membrane signal value, set as time 0) and untreated nontransfected cells (nonspecific labeling). Specific membrane immunoreactivity value was calculated by subtracting the intensity level of wells seeded with nontransfected cells, and the signal from treated transfected cells was expressed as a percentage of the intensity level of wells containing untreated transfected cells. Values were always obtained and averaged in triplicate, and statistical analyses were performed by two-way repeated measure ANOVAs followed with Bonferroni multiple comparison tests to compare the membrane signal measured at different time points following vehicle or CHE pretreatments.

**DA Neuron Culture and Plasmid Transfections**—Mesencephalic DA neuron primary culture was prepared according to a protocol described in earlier publications (27, 30–34). Briefly, dissociated neurons from the isolated mesencephalon of newborn transgenic mice expressing the enhanced GFP (EGFP) under the control of the tyrosine hydroxylase promoter (TH-EGFP/21–31) (35, 36) were seeded on a monolayer of cortical astrocytes coming from the same animals and grown on glass coverslips. These cultures were maintained in Neurobasal medium enriched with 1% penicillin/streptomycin, 1% Glutamax, 2% B-27 supplement, and 10% fetal bovine serum (Invitrogen). For plasmid transfections, 3-day-old neuron cultures were co-transfected with pcDNA3 plasmids containing sequences for the DsRed protein and either the D2S-FLAG (D2S-WT) or a D2S-mutant (triple mutant T225A,S228G,S229G) (29) for a total of 4  $\mu$ g of DNA per coverslip by means of calcium phosphate co-precipitation.

**Knockdown of mRNAs by siRNA Transfection**—RT-PCR was first performed to verify the presence of each RNA of interest in DA neuron cultures at the appropriate time (7–12 days *in vitro*). In all cases, total mRNA was extracted from neuronal culture coverslips using TRIzol (Invitrogen) according to the manufacturer's protocol and dissolved in RNase-free water. cDNA synthesis was performed with 0.5 mM deoxynucleotide triphosphate mix (Qiagen, Valencia, CA), 2.5  $\mu$ M random hexamers (Applied Biosystems, Foster City, CA), 500 ng of total RNA, 10 mM DTT, 40 units of RNaseOUT, 200 units of Moloney-murine leukemia virus reverse transcriptase (Invitrogen), 50 mM Tris-HCl, 75 mM KCl, and 3 mM MgCl<sub>2</sub>, pH 8.3. For transfections with siRNA, 7-day-old neuron cultures were transfected with a total of 40 or 60 pmol of siRNAs per coverslip using Lipofectamine 2000 according to the manufacturer's guidelines (Invitrogen). Controls consisted in cultures transfected with a Cy-3-labeled scramble siRNA sequence (Ambion, Austin, TX).

Knockdown of target mRNA was then assessed by RT-PCR on whole coverslip extracts at 24, 48, and 72 h post-transfection (8–10 days *in vitro*). Primers and oligonucleotides (synthesized by Alpha DNA, Montreal, Quebec, Canada) were designed to amplify each specific sequence by RT-PCR and to synthesize siRNAs, respectively, using a kit (Ambion). Sequences for  $\beta$ -arrestin1 ( $\beta$ -arr1),  $\beta$ -arrestin2 ( $\beta$ -arr2), dynamin1 (Dyn1), and dynamin2 (Dyn2) mRNAs were obtained from the Entrez Nucleotides mouse database. PCR primers were as follows (left primers followed by right primers):  $\beta$ -arr1, 5'-gagtctacgtgacactgacctg-3' and 5'-ctcaggggcatactgaacctt-3';  $\beta$ -arr2, 5'-aagtcgagccctaactgcaa-3' and 5'-cagctctttgtccaggaag-3'; Dyn1, 5'-ttgtgctgactgctgagaatc-3' and 5'-ctccttcagtcgctgtgtaca-3'; and Dyn2, 5'-gccataccaatcagggggaga-3' and 5'-cccacagcgcataggaatcagggga-3'. The oligonucleotides sequences for siRNA synthesis were as follows (sense oligonucleotides followed by antisense oligonucleotides):  $\beta$ -arr1, 5'-aagcactgtactgagctgtgcctgtctc-3' and 5'-aacacagctcagtagaagtcctgtctc-3'; Dyn1, 5'-aaatcctgtaccagagacagctgtctc-3' and 5'-aatctgtcctgtacaagatcctgtctc-3'; and Dyn2, 5'-aaacaactctagtgtgtctacctgtctc-3' and 5'-aagtagacacactagagttgtctgtctc-3'.

**Patch Clamp Recordings**—Neuron culture coverslips of 7 days (plasmid transfection) or 9–11 days (siRNA transfection) in culture were positioned in a recording chamber affixed to the stage of an inverted Nikon Eclipse TE-200 fluorescent microscope and gravitationally perfused with a physiological saline solution composed of the following: 140 mM NaCl, 5 mM KCl, 2 mM  $MgCl_2$ , 2 mM  $CaCl_2$ , 10 mM HEPES, 6 mM sucrose, 10 mM glucose. Whole-cell patch clamp recordings were performed on GFP-expressing neurons using borosilicate pipettes (5.5–6.5 megohms) filled with an intrapipette solution composed of the following: 145 mM  $KMeSO_4$ , 20 mM KCl, 10 mM NaCl, 0.1 mM EGTA, 2 mM ATP (magnesium salt), 0.6 mM GTP (Tris salt), 10 mM HEPES, 10 mM phosphocreatine, pH 7.35. The signal was amplified by a Warner PC-505 amplifier, filtered at 1 kHz, digitized at 10 kHz, and analyzed with pClamp8 software (Molecular Devices, Sunnyvale, CA). The spontaneous firing rate was recorded and analyzed off-line using MiniAnalysis software (Synaptosoft, Fort Lee, NJ). The threshold for spike detection was set to 30 mV. In every case, the maximal effect of Quin (1  $\mu$ M) on the spontaneous firing rate of DA neurons was calculated by comparing the number of spikes during the minute of maximal effect with the 2-min baseline period. To measure the desensitization of D2R function, patched DA neurons were treated with Quin for a prolonged period of eight consecutive minutes. The mean maximal effect of Quin on firing rate was compared with the average rate of minutes 4 to 6 after the beginning of treatment. A rejection criterion was set as to keep for further analysis only neurons that showed a minimum of 50% reduction in firing in response to Quin. To measure the effect NT on D2R function, patched DA neurons were first exposed to Quin for 2 min to measure D2R-mediated firing rate inhibition (and to exclude nonresponsive neurons), washed for 6 min, treated with NT (1 nM) for 3 min, and finally exposed again to Quin in the presence of NT (Quin+NT). A second rejection criterion was set to exclude unstable neurons that did not show recovery of at least 50% of their initial firing rate by the end of the second Quin washout period. Neurons from control,

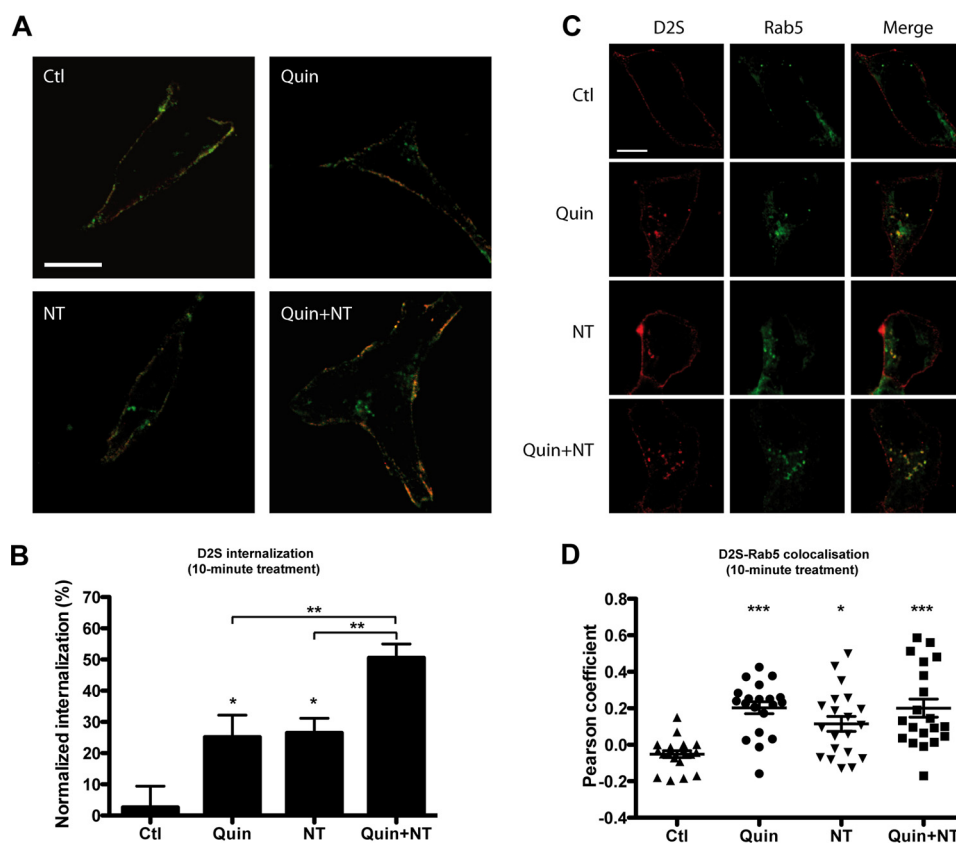
plasmid, and siRNA transfection groups all displayed similar basal firing rate and spike amplitude (data not shown). Typical membrane resistance of recorded DA neurons ranged from ~150 to 400 megohms. Statistical analysis was performed by a two-way repeated-measure ANOVAs followed by Bonferroni multiple comparison tests.

## RESULTS

**NT Induces D2S Internalization in Transfected HEK-293 Cells**—We first used HEK-293 cells transiently transfected with both NTR1 and FLAG-tagged short isoform D2R (D2S-WT) receptors to test whether NTR1 activation triggers D2R internalization. As a first approach, we detected D2R internalization by immunofluorescence using a strategy involving membrane labeling of the D2S prior to treatment and incubation of cells with different secondary antibodies before and after cell permeabilization (Fig. 1A). Cells treated with Quin (10  $\mu$ M) or NT (10 nM) alone both showed equivalent D2S internalization after 10 min ( $28.9 \pm 5.0$  and  $26.5 \pm 3.5\%$ , respectively;  $n = 29$  cells for Quin and 37 for NT, Fig. 1B). On the other hand, co-application of Quin and NT (Quin+NT) produced a significantly greater internalization ( $50.6 \pm 3.4\%$ ;  $n = 36$ ) compared with Quin or NT alone, suggesting an additive effect of the drugs after 10 min of treatment. To confirm that the D2S was readily internalized through a typical endocytotic route, we performed a similar membrane labeling experiment in HEK-293 cells transiently transfected with Rab5-GFP, an early endosomal protein (37), in addition to the NTR1 and D2S-WT receptors (Fig. 1C). Our analysis showed a significant increase in the Pearson correlation coefficient of the Rab5 and D2S signals, compared with control following a 10-min treatment with Quin (10  $\mu$ M), NT (10 nM), or both; this can be interpreted as an increase in Rab5-D2S colocalization ( $-0.052 \pm 0.019$  for control,  $0.203 \pm 0.032$  for Quin,  $0.115 \pm 0.041$  for NT, and  $0.201 \pm 0.049$  for Quin+NT). Thus, NTR1 activation seems to trigger D2S internalization by endocytosis in HEK-293 cells.

**NT-induced D2S Internalization in Transfected HEK-293 Cells Is PKC-dependent**—As a second strategy to confirm our initial findings and to gain insight into the time course of receptor internalization, we used an ELISA approach quantifying the decrease in membrane immunoreactivity induced by drug treatment at different time points. Because PKC is a major component of the NTR1 signaling cascade and was shown to induce D2R internalization via direct receptor phosphorylation (28), we used CHE, a wide-spectrum PKC inhibitor, to test the hypothesis that NT-induced D2R internalization requires PKC activity. Compared with controls, set to a membrane signal value of 100%, the D2S membrane signal decreased to a value of  $80.2 \pm 2.1\%$  ( $n = 5$  experiments) after 5 min of treatment with Quin following a 30-min pretreatment with vehicle (Fig. 2A, black curve). Cells that were treated with Quin for 5 min after pretreatment with CHE (10  $\mu$ M) displayed a similar level of receptor internalization ( $80.1 \pm 1.4\%$  of control;  $n = 4$ ), showing that agonist-induced homologous D2S internalization is PKC-independent. In both cases, Quin-induced reduction in membrane signal reached a plateau value after 5 min and remained constant during the entire treatment period. In cells pretreated with vehicle, NT induced more extensive internal-



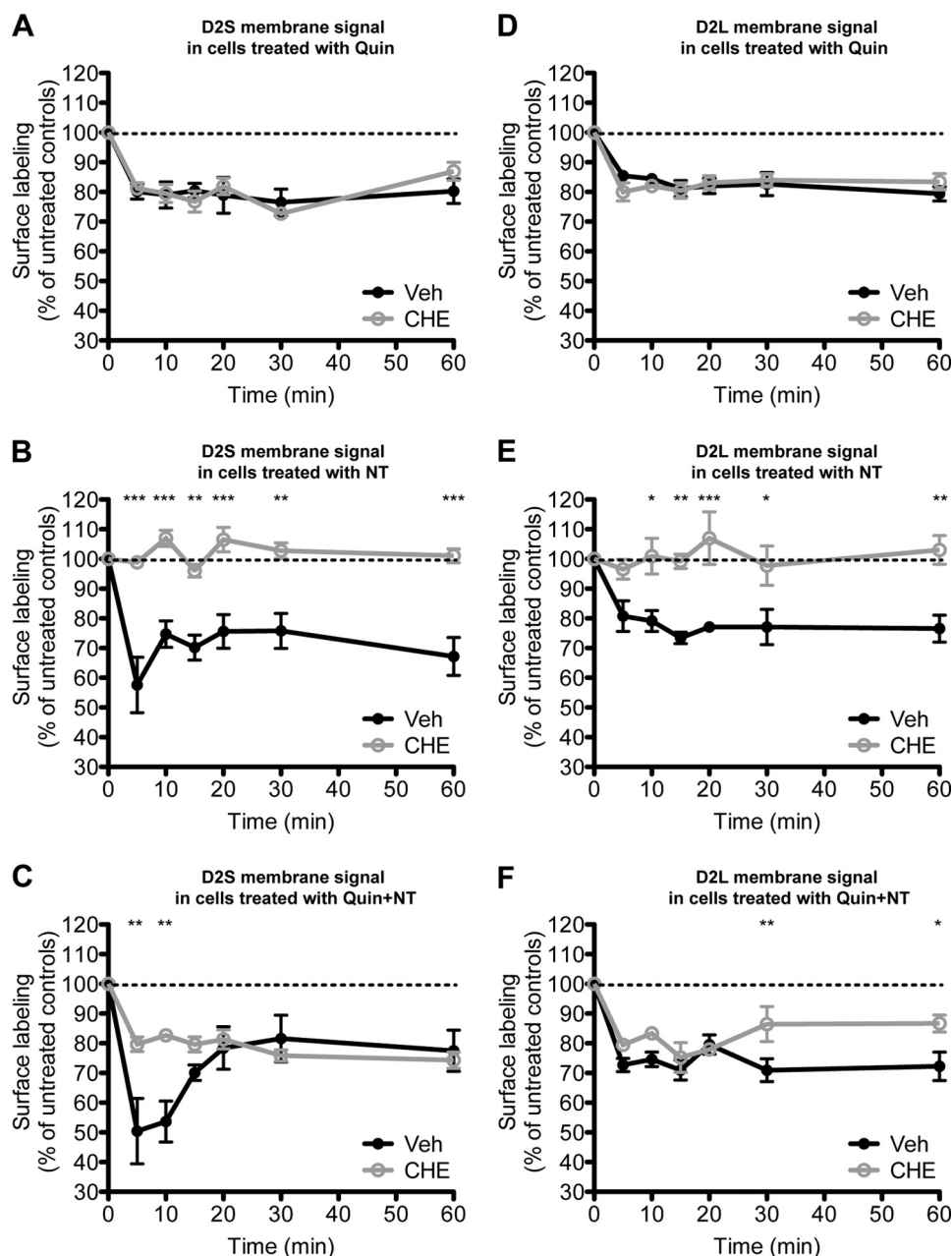


**FIGURE 1. NT induces D2S internalization in transfected HEK-293 cells.** HEK-293 cells transfected with D2S and NTR1 were exposed to receptor agonists (10  $\mu$ M Quin and/or 10 nM NT) for 10 min to measure membrane-labeled D2R internalization and colocalization with Rab5 by immunofluorescence. *A*, immunolabeling against the FLAG epitope of membrane D2S-FLAG using two different secondary antibodies before and after permeabilization, such that the internalized signal appears in pure green, and the membrane signal appears in red and yellow. Scale bar, 15  $\mu$ m. *B*, quantification of D2S internalization treatment measured by immunofluorescence and normalized against the total signal of each cell ( $n = 26$  for control (Ctl), 29 for Quin, 37 for NT, and 36 for Quin+NT; results from three separate experiments). *C*, immunolabeling against the FLAG epitope of membrane D2S-FLAG (red) and GFP from cotransfected endosomal protein Rab5-GFP (green). Scale bar, 10  $\mu$ m. *D*, colocalization of D2S and Rab5 signals analyzed by the Pearson correlation coefficient following treatment ( $n = 20$  for every group). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ , one-way ANOVA with Tukey's multiple comparison tests.

ization of D2S, with cell surface signal reaching  $57.6 \pm 6.8\%$  of control ( $n = 5$ ) after 5 min (Fig. 2*B*, black curve); the signal came back to  $74.7 \pm 2.4\%$  of control ( $n = 5$ ) at 10 min. In cells pretreated with CHE, NT failed to induce any change in membrane D2S signal (Fig. 2*B*, gray curve), which was significantly different from the NT group with vehicle pretreatment (Fig. 2*B*, black curve;  $p = 0.0003$ ); all time points were statistically different between the two conditions ( $p < 0.01$  or lower, Bonferroni post-test), clearly showing that NT-induced D2S internalization is PKC-dependent. Co-treatment with both Quin and NT together (Quin+NT, Fig. 2*C*, black curve) produced a large reduction in membrane signal at 5 min ( $50.5 \pm 8.8\%$  of control;  $n = 5$ ), which persisted after 10 min ( $53.7 \pm 3.8\%$ ) and rose back to  $70.2 \pm 3.0\%$  of control ( $n = 5$ ) after 15 min. Following CHE pretreatment, Quin and NT co-application still caused a decrease in membrane D2S signal (Fig. 2*C*, gray curve), but with a pattern of internalization that was similar to that induced by Quin alone. Membrane signal from the Quin+NT-treated cells after vehicle or CHE pretreatment were significantly different overall ( $p = 0.0069$ ), but post hoc tests confirmed that only the 5- and 10-min time points differed ( $p < 0.01$  for both). Overall, these results show that although PKC is not required for agonist-dependent D2S internalization, this kinase is necessary for NT-induced D2S internalization through a mechanism that

seems additive to Quin-induced internalization only within the first 10 min of treatment in transfected HEK-293.

**NT Induces PKC-dependent D2L Internalization in Transfected HEK-293 Cells**—Because prior studies have reported that rapid (2 min) uncoupling of D2L is less sensitive to PKC phosphorylation than D2S (29, 38), we also examined the ability of NT to regulate D2L internalization in ELISA experiments with transfected HEK-293 cells. Similar to our results with D2S, pretreatment with the PKC inhibitor CHE failed to interfere with Quin-induced D2L internalization (Fig. 2*D*;  $p = 0.8645$ ) but prevented NT-induced D2L internalization (Fig. 2*E*;  $p = 0.0003$ ). Interestingly, the amplitude and kinetics of D2L internalization in response to NT or Quin+NT co-application were different than for D2S (Fig. 2, *E* and *F*): the peak reduction in membrane D2S seen after 5 min of NT was not present for the D2L, reaching only  $80.8 \pm 3.9\%$  of control in response to NT and  $72.7 \pm 1.7\%$  of control in response to Quin+NT. Pretreatment with CHE also altered the response to Quin+NT co-application (Fig. 2*F*;  $p = 0.0267$ ), but post hoc analysis revealed that this was only due to a difference at 30 and 60 min ( $p < 0.01$  and  $0.05$ , respectively), where Quin and NT seem to produce additive effects. Taken together, these experiments demonstrate that activation of NTR1 by NT can trigger D2S and D2L

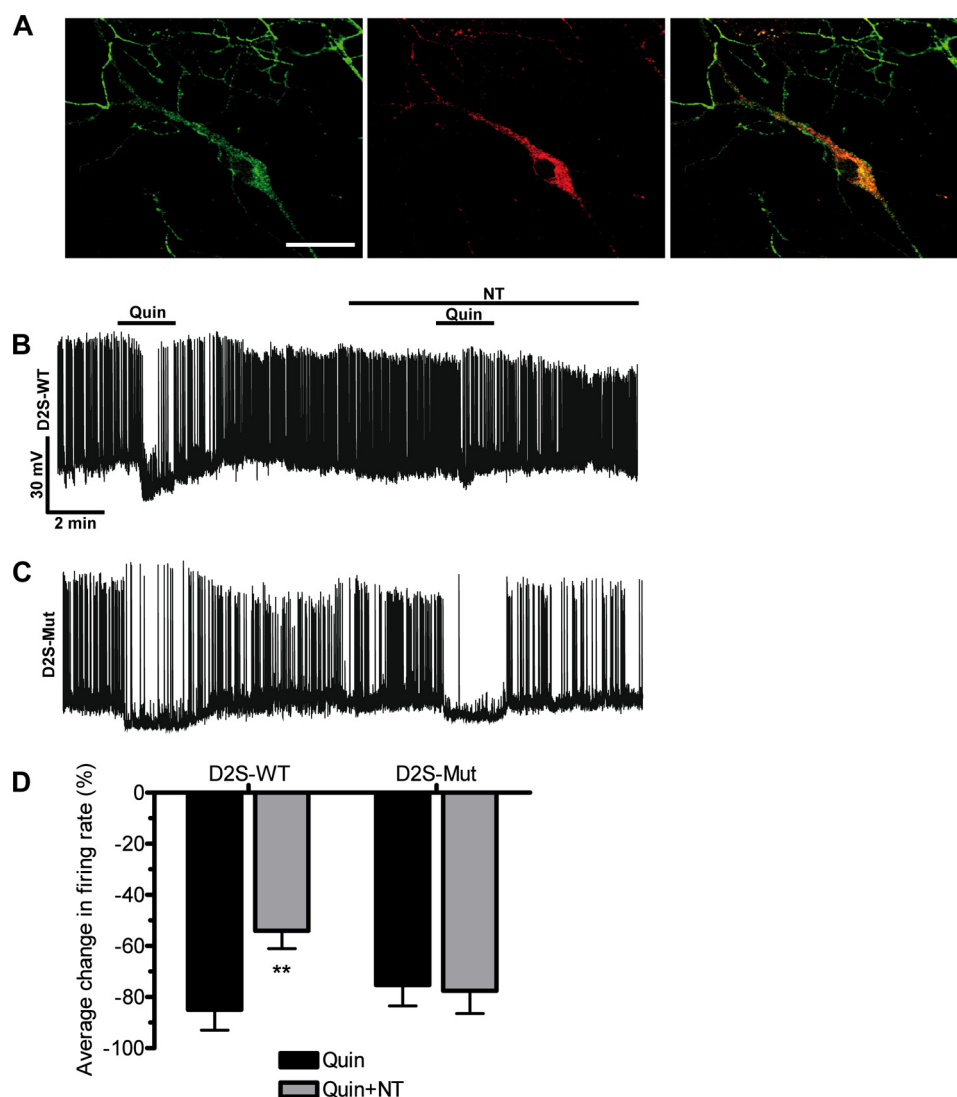


**FIGURE 2. NT-induced D2R internalization in transfected HEK-293 cells is PKC-dependent.** Time course of D2R internalization measured by ELISA as the loss of membrane signal. *Black curves*, vehicle pretreatment (*Veh*). *Gray curves*, chelerythrine pretreatment (*CHE*). *A–C*, internalization profile of the D2S in response to Quin, NT, and Quin+NT treatments, respectively. *D–F*, internalization profile of the D2L in response to Quin, NT, and Quin+NT treatments, respectively. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; two-way ANOVA with Bonferroni multiple comparison tests ( $n = 4–7$  experiments).

internalization in a PKC-dependent manner in transfected HEK-293 cells, although the kinetics of the PKC-dependent response of D2S and D2L in the combined agonist treatments were partially different.

**Mutation of D2S PKC Phosphorylation Sites Prevents NT-mediated Reduction in D2R Function in Cultured DA Neurons—** We next examined cultured DA neurons to extend our conclusions to a cellular context in which NTR1 and D2R are normally co-expressed. For these experiments, primary postnatal cultures were prepared from TH-GFP transgenic mice, thus facilitating identification of DA neurons for patch clamp recordings. Considering that PKC has been shown to phosphorylate D2R directly, leading to its functional desensitization and inter-

nalization (28, 29) and that PKC activity is necessary for the ability of NT to reduce D2R in DA neurons (27), we hypothesized that mutation of D2R PKC phosphorylation sites should prevent the ability of NT to reduce D2R-mediated firing rate inhibition. To test this hypothesis, we used a previously characterized D2S construct in which three key PKC phosphorylation sites specifically required for PKC-induced internalization of the D2R have been mutated (D2S triple mutant T225A,S228G,S229G) (28, 29). Our results show that overexpression of this construct in DA neurons, acting as a dominant negative to compete with endogenous D2Rs (Fig. 3A), produced a complete blockade of the ability of NT to reduce D2R agonist (Quin)-mediated inhibition of cell firing (Fig. 3D). Indeed, in



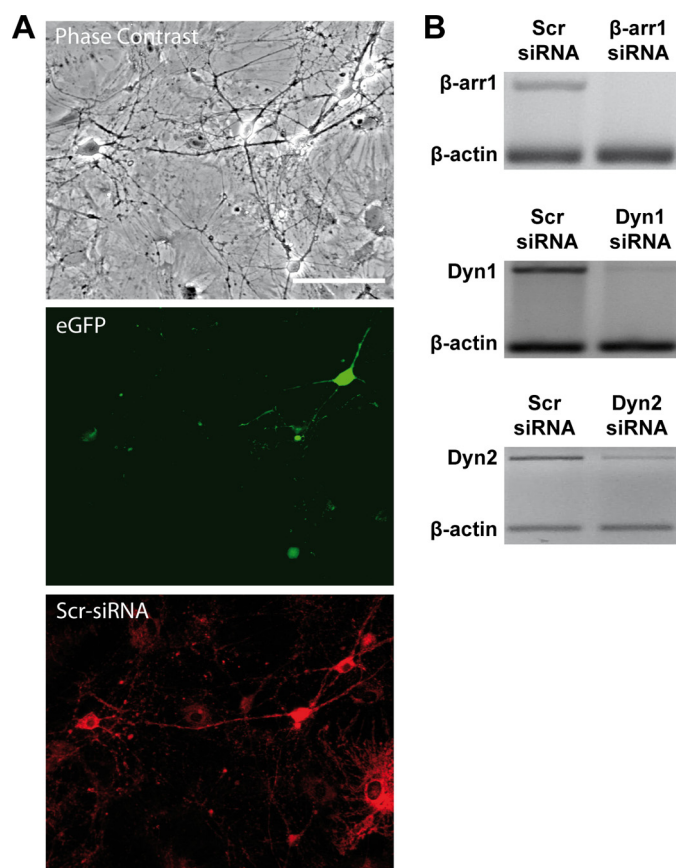
**FIGURE 3. Mutation of D2S PKC phosphorylation sites prevents NT-mediated reduction in D2R function in cultured DA neurons.** Patch clamp whole-cell recordings of DA neurons in culture transfected with either a normal D2S receptor (D2S-WT) or a version in which three PKC phosphorylation sites (T225A, S228G, and S229G) have been mutated (D2S-Mut). *A*, example of a transfected cultured DA neuron identified by immunostaining against the DA transporter (green) and the FLAG epitope of D2S-WT (red). Scale bar, 25  $\mu$ m. *B*, example of a patch clamp recording in a DA neuron transfected with D2S-WT following a typical NT treatment protocol. *C*, same as in *B* but in a neuron transfected with D2S-mut. *D*, average effect of the D2 agonist Quin on firing rate in the absence (Quin) or presence of NT preapplication (Quin+NT) in the two transfection conditions of *B* and *C*. \*\*,  $p < 0.01$ ; two-way ANOVA with Bonferroni multiple comparison tests ( $n = 7$  for D2S-WT and 5 for D2S-Mut).

control cultures transfected with wild-type D2S (D2S-WT), NT strongly reduced the ability of Quin to inhibit spontaneous firing ( $84.9 \pm 8.0\%$  when applied alone, compared with a  $54.1 \pm 6.9\%$  reduction after NT; Fig. 3*B*;  $p < 0.01$ ; two-way ANOVA with Bonferroni post-test;  $n = 7$ ). In contrast, in neurons transfected with mutant D2S, NT failed to reduce the effect of Quin ( $75.3 \pm 8.2\%$  reduction with Quin alone compared with  $77.6 \pm 8.9\%$  average maximal reduction of firing rate in the presence of NT; Fig. 3*C*;  $p = 0.74$ ,  $n = 5$ ). These findings suggest that direct phosphorylation of the D2R by PKC, which can mediate D2R internalization, is required for the effect of NT on D2R function.

**Knockdown of  $\beta$ -arr1 and Dyn2 but Not Dyn1 Prevents Agonist-induced Desensitization of D2R Function in Cultured DA Neurons**—Many proteins, including arrestins and dynamins, mediate the normal agonist dependent desensitization of G protein-coupled receptors (39). Interestingly, PKC-mediated agonist-independent desensitization and sequestration of D2R

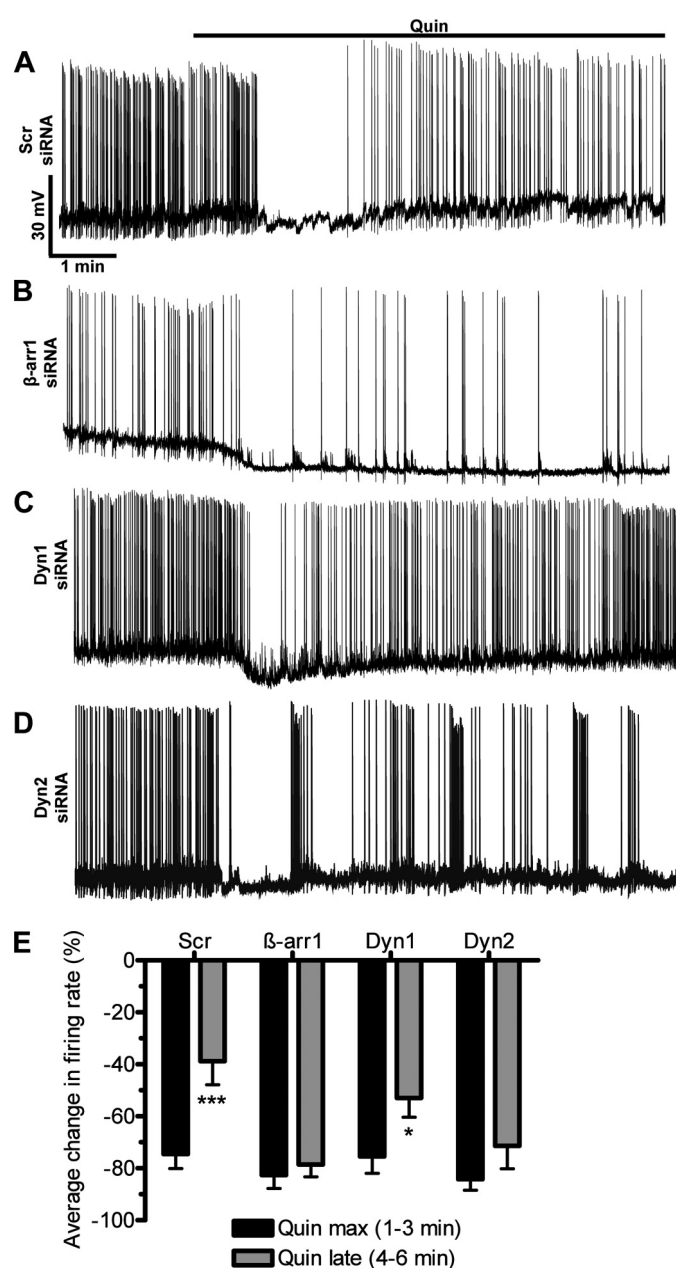
was also shown to depend both on arrestins and dynamins (28). In light of our results showing that NT triggered D2R internalization in transfected HEK-293 cells and our demonstration that mutation of phosphorylation sites important for PKC-dependent D2R internalization prevented the NTR1-D2R interaction in cultured DA neurons, we next evaluated whether siRNA-mediated down-regulation of desensitization-related proteins (Fig. 4, *A* and *B*) would also alter the physiological effect of NT on D2R function. We first examined the presence of  $\beta$ -arr1,  $\beta$ -arr2, Dyn1, and Dyn2 mRNA in mouse DA neurons in a primary culture by RT-PCR and found that all could be detected (Fig. 4*B*) except for  $\beta$ -arr2 (data not shown). Hence, we designed siRNA sequences targeting  $\beta$ -arr1, Dyn1, and Dyn2 mRNA and optimized conditions to ensure efficient transfection of cultured neurons (Fig. 4*A*) and maximal knockdown at the moment of the electrophysiological recordings (Fig. 4*B*).





**FIGURE 4. Knockdown of  $\beta$ -arr1, Dyn1, and Dyn2 in cultured DA neurons.** *A*, top panel: phase contrast image of a DA neuron culture. Middle panel, same field as *A* but showing eGFP expressed endogenously by DA neurons from transgenic TH-eGFP mice. Bottom panel, same field as *A* and *B* but showing transfection with fluorescently scrambled siRNA molecules used for control (Scr). Scale bar, 100  $\mu$ m. *B*, agarose gels showing the product of mRNA amplification by RT-PCR after RNA extraction from DA neuron cultures transfected with Scr and either  $\beta$ -arr1- (top) Dyn1- (middle), or Dyn2-targeting siRNA molecules (bottom). In every example,  $\beta$ -actin was also amplified to normalize the results of the knockdown.

We next determined the consequence of these knockdowns on D2R function and agonist-induced desensitization by measuring Quin-induced firing rate inhibition. This also served the purpose of providing a functional control for our ability to inhibit proteins important for receptor desensitization. Neurons transfected with scrambled siRNA sequences (Scr, Fig. 5*A*) responded to Quin (1  $\mu$ M) with an average maximal decrease in firing rate of  $74.6 \pm 5.5\%$ . As typically found, continuous perfusion with Quin produced a gradual reduction of the D2R agonist effect, as seen by the average decrease in firing rate of  $38.8 \pm 9.1\%$  during the period covering minutes 4 to 6 after the beginning of the treatment, which was significantly different from the maximal effect (Fig. 5*E*;  $p < 0.001$ ; two-way ANOVA with Bonferroni post-test;  $n = 16$ ). In contrast, although the maximal effect of Quin was not significantly different in neurons transfected with siRNAs targeting  $\beta$ -arr1 ( $80.5 \pm 5.3\%$  reduction; Fig. 5*B*), no significant desensitization of this response was observed; the firing inhibition remained at  $78.5 \pm 4.8\%$  of the baseline 4–6 min after the beginning of Quin perfusion, which was statistically identical to the initial effect (Fig. 5*E*;  $p = 0.16$ ;  $n = 9$ ). Neurons transfected with siRNAs targeting Dyn1 (Fig. 5*C*) still showed significant desensitization, with an



**FIGURE 5. Knockdown of  $\beta$ -arr1 and Dyn2 but not Dyn1 prevents Quin-mediated reduction in D2R function in cultured DA neurons.** Patch clamp whole-cell recordings of DA neurons in culture transfected with siRNA molecules. *A–D*, examples of prolonged Quin treatment protocols on recorded DA neurons transfected with Scr,  $\beta$ -arr1-, Dyn1-, or Dyn2-targeting siRNA molecules, respectively. *E*, comparison between the maximal initial effect of Quin (Quin max, 1–3 min) and the late effects (Quin late, 4–6 min) of this D2R agonist on spontaneous firing rate as a measure of D2R functional desensitization. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; two-way ANOVA with Bonferroni multiple comparison tests ( $n = 16$  for Scr,  $n = 9$  for  $\beta$ -arr1,  $n = 8$  for Dyn1, and  $n = 9$  for Dyn2).

average maximal Quin effect of  $75.5 \pm 6.5\%$  compared with  $52.9 \pm 7.4\%$  4–6 min after the beginning of Quin (Fig. 5*E*;  $p < 0.05$ ;  $n = 8$ ). Finally, physiological responses to Quin showed no significant desensitization in neurons transfected with siRNAs targeting Dyn2 (Fig. 5*D*) ( $84.2 \pm 4.3\%$  versus  $71.4 \pm 8.8\%$  after 4–6 min) (Fig. 5*E*;  $p = 0.14$ ;  $n = 9$ ). Hence, our results show that under these conditions,  $\beta$ -arr1 and Dyn2, but not Dyn1, are important for agonist-induced functional desensitization of native D2R expressed in cultured DA neurons.

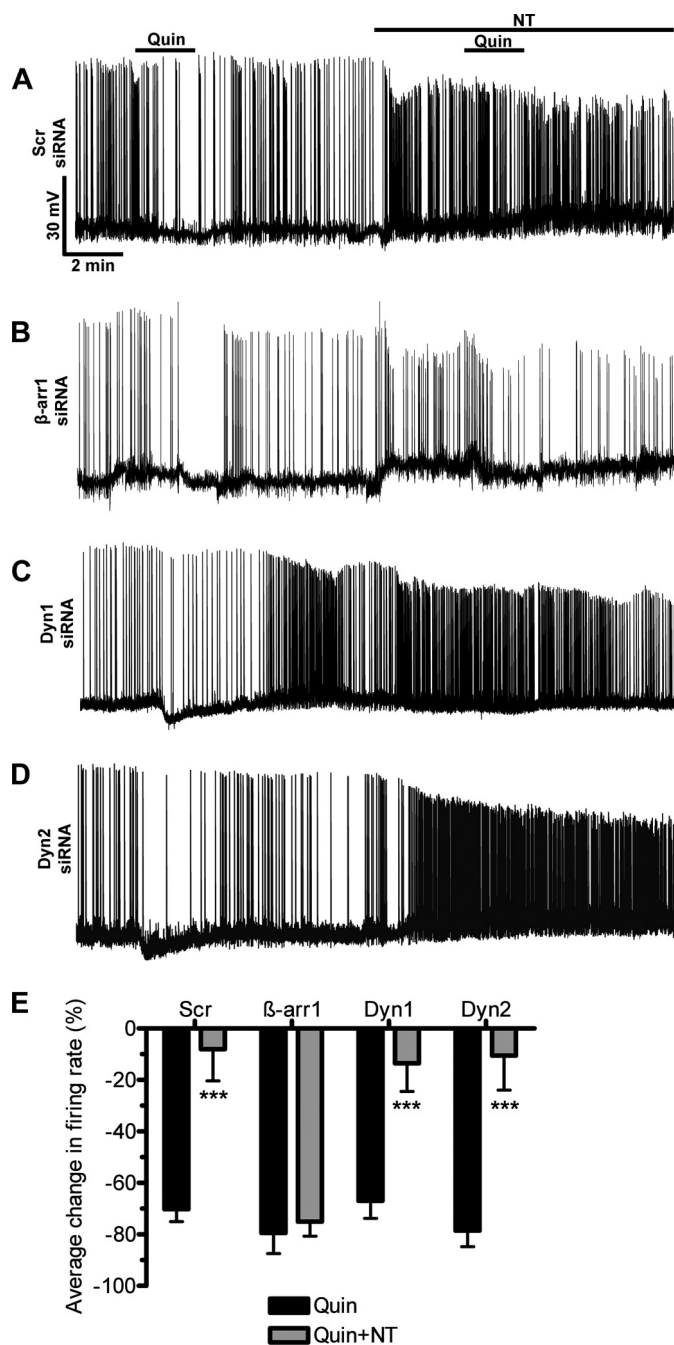
**Knockdown of  $\beta$ -arr1 but Not Dyn2 or Dyn1 Prevents NT-mediated Reduction of D2R Function in Cultured DA Neurons**—To test the hypothesis that NT affects D2R function by causing its desensitization, we measured the effect NT in cultured DA neurons following siRNA knockdown of  $\beta$ -arr1, Dyn1, and Dyn2. We found that in control neurons transfected with Scr sequences, NT displayed its normal ability to reduce D2R-mediated firing rate inhibition (Fig. 6A) ( $70.2 \pm 4.9\%$  under control conditions in comparison with  $8.1 \pm 12.3\%$  in the presence of NT (Fig. 6E; two-way ANOVA with Bonferroni post-test;  $p < 0.001$ ,  $n = 5$ ). In contrast, in DA neurons transfected with siRNAs targeting  $\beta$ -arr1 (Fig. 6B), the ability of NT to reduce D2R function was blocked ( $79.5 \pm 8.2\%$  under control conditions in comparison to  $75.0 \pm 5.0\%$  in the presence of NT; Fig. 6E;  $p = 0.47$ ,  $n = 7$ ). However, neurons transfected with siRNAs targeting Dyn1 (Fig. 6C) or Dyn2 (Fig. 6D), responded to NT in a way that was similar to the Scr control group (Dyn1;  $67.0 \pm 6.8\%$  inhibition of firing rate in response to Quin under control conditions *versus*  $15.5 \pm 11.0\%$  in presence of NT; Fig. 6E;  $p < 0.001$ ,  $n = 5$ ) (Dyn2;  $78.6 \pm 6.3\%$  under control conditions *versus*  $10.5 \pm 13.5\%$  in presence of NT; Fig. 6E;  $p < 0.001$ ,  $n = 6$ ). Thus, we conclude from these electrophysiological experiments that the ability of NT to reduce D2R function in DA neurons is dependent on  $\beta$ -arr1, but not Dyn1 or Dyn2.

## DISCUSSION

In this study, we provided evidence linking the well known ability of the neuropeptide NT to reduce D2R function to a mechanism of heterologous desensitization. Our first primary finding is that activation of NTR1 induces PKC-dependent internalization of D2S and D2L in transfected HEK-293 cells; the wide-spectrum PKC inhibitor CHE completely abolished NT-induced internalization of both isoforms without affecting internalization induced by the D2R agonist Quin. This finding that PKC is not required for homologous internalization of D2R is in line with a previous demonstration that inhibition of PKC activity does not alter agonist-induced phosphorylation of the D2L receptor in HEK-293 cells (28).

Our second major finding is that NT differentially regulates D2S and D2L internalization. Although NT induced a biphasic internalization of D2S, consisting of a large amplitude rapid phase at 5 min and a more modest but protracted phase lasting up to 60 min, D2L showed a comparable slow phase but a blunted fast phase. The difference between the rapid phase of NT-induced D2S and D2L internalization is compatible with the previously reported property of the D2L receptor to be more resistant to phosphorylation by PKC compared with D2S; the 29-amino acid sequence present only in the D2L acts as a pseudo-substrate site to decrease interaction between the kinase and the third intracellular loop of the receptor (29, 38). The transient nature of the observed D2S internalization peak could perhaps be related to the fact that the NTR1 receptor internalizes massively and rapidly upon activation (40, 41).<sup>3</sup>

Although an initial evaluation of D2R internalization by immunolabeling suggested a potentially additive effect of combined NTR1 and D2R activation at a 10-min time point (Fig. 1,



**FIGURE 6. Knockdown of  $\beta$ -arr1 but not Dyn2 or Dyn1 prevents the ability of NT to reduce D2R function in cultured DA neurons.** Patch clamp whole-cell recordings of cultured DA neurons transfected with siRNA molecules. A–D, examples of NT treatment protocols on recorded DA neurons transfected with Scr,  $\beta$ -arr1-, Dyn1-, or Dyn2-targeting siRNA molecules respectively. E, summary diagram showing the ability of NT to reduce Quin-induced firing rate inhibition. The black bars show the initial effect of Quin alone (Quin), whereas the gray bars show the effect of Quin after pretreatment with NT (Quin+NT). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; two-way ANOVA with Bonferroni multiple comparison tests ( $n = 5$  for Scr, 7 for  $\beta$ -arr1, 5 for Dyn1, and 6 for Dyn2).

A and B), an evaluation of the time course of D2R cell surface expression using more quantitative ELISA assay failed to detect an additive effect of a combined stimulation of NTR1 and D2R at most time points examined (Fig. 2). Although not a direct, quantitative measure of internalization, our evaluation of D2S/Rab5 colocalization (Fig. 1, C and D) also argues against an

<sup>3</sup> D. Thibault and L. E. Trudeau, unpublished observations.



additive effect. These findings together suggest that the mechanism of D2R homologous internalization acts as an apparent repressor of PKC-dependent heterologous internalization. We also found that the receptor internalization triggered by co-application of NT and Quin was to a large extent PKC-independent, which is not what would be expected if a purely additive effect of the two agonists occurred. Although for D2S, only the initial fast phase of internalization was reduced by CHE in cells treated with Quin + NT (Fig. 2C), for D2L, only late phases were reduced by the PKC blocker (Fig. 2F). Because D2R agonist-dependent internalization is believed to be primarily mediated by various G protein-related kinase-dependent mechanisms (42–46), activation of D2Rs in the co-treatment condition with NT may recruit G protein-related kinases to the D2R and perhaps reduce the accessibility of PKC phosphorylation sites. It is also possible that stimulation of the D2R may itself lead to the inhibition of NTR1 function and reduced PKC activation, as suggested by our prior work showing that Quin reduces the ability of NT to mobilize intracellular calcium in DA neurons (27). This model may explain why NT and Quin do not lead to an additive or synergistic enhancement of D2R internalization, but further investigation will be necessary to understand the different behaviors of D2S and D2L in this particular context.

We provide evidence that overexpression of a D2S bearing mutation on critical PKC phosphorylation sites (T225A, S228G, and S229G) in cultured DA neurons prevents negative regulation of D2R function by NT. Considering that D2R activation in DA neurons induces the opening of potassium conductances (9, 12) and that NTR1 can negatively regulate inward rectifying potassium currents via PKC (7, 25), an alternate mechanism for NTR1-D2R interaction was that NTR1 inhibits the function of potassium channels that are normally activated by the D2R. Based on our present findings, this alternative is not likely to play a major role in the interaction mechanism. A more likely model is that activation of NTR1 leads to PKC-mediated phosphorylation of D2R, leading to its internalization or functional desensitization. Compatible with this hypothesis, and in particular for the implication of receptor internalization, prior work has shown that mutations of Ser<sup>228</sup> and Ser<sup>229</sup> strongly inhibit PKC-induced sequestration of D2L from the membrane of HEK-293 cells without affecting functional desensitization (28). Other PKC phosphorylation sites on the D2R (Thr<sup>352</sup>, Thr<sup>354</sup>, and Ser<sup>355</sup>) have been identified and characterized as modulators of sequestration and functional desensitization (28) but were not examined in the present study. Although these residues could also be phosphorylated upon NTR1 activation, it is unlikely that they would play a necessary role in the NTR1-D2R interaction because the T225A, S228G, and S229G mutations were sufficient to abolish the effect of NT on D2R function.

Our recordings of DA neuron firing changes in response to prolonged Quin treatment, combined with siRNA knockdown of  $\beta$ -arr1, Dyn1, or Dyn2, confirm previous results showing that agonist-dependent homologous desensitization of D2R is regulated by  $\beta$ -arr1 and Dyn2, but not Dyn1 (44, 47–49; see Ref. 50). Importantly, our results showing that siRNA knockdown of  $\beta$ -arr1 prevents the ability of NT to reduce D2R function in DA neurons also argue in favor of heterologous D2R desensitiza-

tion as the primary mechanism of NTR1-D2R interaction in DA neurons. We did not examine the implication of  $\beta$ -arr2 because it was not detected in our cultured DA neuron model, compatible with anatomical examinations of midbrain DA nuclei (51). Nonetheless, conclusive evidence has been provided for the implication of  $\beta$ -arr2 in D2R internalization and signaling in striatal neurons (52, 53). This distinction argues in favor of the hypothesis that pre- and postsynaptic D2R signaling is partially different, as suggested by previous work (54, 55).

Despite the fact that Dyn2 appears to be implicated in agonist-dependent desensitization of D2R function in DA neurons (Fig. 5D), its knockdown failed to prevent NT-mediated inhibition of D2R function (Fig. 6). This finding may be considered surprising in light of the prior observation that in HEK-293 cells, heterologous D2L internalization induced by PKC phosphorylation depends on both arrestins and dynamins, as shown by the expression of dominant-negative proteins (28). However, a number of differences between the two experimental settings should be considered, including receptor isoform (D2L *versus* D2S), experimental model (HEK-293 *versus* primary cultured DA neurons), and time course of PKC activation (2 h of PMA *versus* 3 min of NT). It is likely that our recordings highlighted acute, early effects of NT, which could differ in mechanism from longer lasting effects mediated by prolonged stimulation of this signaling cascade. Although our results in HEK-293 cells identify D2R internalization as a possible mechanism of NTR1-D2R interaction, our functional data obtained in DA neurons could be mediated by either internalization or functional uncoupling of the D2R, both resulting from direct PKC phosphorylation: following NTR1 activation, arrestin-dependent functional uncoupling of D2R could occur as a first, necessary mechanism, followed later by dynamin-dependent internalization if NTR1 or other PKC-recruiting pathways are activated for more prolonged periods.

Taken together, our results suggest a novel mechanism by which the regulatory peptide NT acutely shuts down D2 autoreceptor function in DA neurons; we propose that NTR1 activation leads to PKC-mediated phosphorylation of D2S, triggering  $\beta$ -arr1 recruitment and receptor functional uncoupling or heterologous internalization. For many years, NT has been examined in relation to the physiopathology of schizophrenia and the action of APDs. Indeed, subsets of schizophrenic patients have lower NT concentrations in their cerebrospinal fluid, and treatment with APDs normalizes these concentrations in parallel with symptom improvement (14, 56, 57). Most interestingly, viral overexpression of NTR1 in rat nucleus accumbens reduces amphetamine- and dizocilpine-induced deficits in prepulse inhibition and hyperlocomotion (16), two behavioral responses associated with schizophrenia symptoms. In recent studies, NTR1 knock-out animals have also been shown to exhibit increased basal and amphetamine-induced DA release in the striatum and decreased responsiveness to a NT analog to improve prepulse inhibition and reduce acoustic startle responses (21, 22). NT receptor ligands have attracted much attention as possible new antipsychotic drug candidates (58–60). It has been argued that antipsychotic-like effects of NT could be caused specifically by a reduction in D2R-mediated neurotransmission in the accumbens through a combina-

tion of pre- and postsynaptic effects (1, 59, 61). Our finding of a differential ability of NT to regulate D2S and D2L internalization is most relevant in this context because it provides a new framework to explain how NT may differentially regulate presynaptic and postsynaptic D2Rs. Indeed, anatomical and functional experiments suggest that the D2L acts mainly as a postsynaptic receptor, whereas the D2S is generally considered as the primary fast-acting autoreceptor in DA neurons (54, 55, 62). Our electrophysiological data suggest that NT causes acute heterologous desensitization of presynaptic D2R through a PKC- and  $\beta$ -arr1-dependent mechanism. Numerous reports have shown that NT can also negatively regulate postsynaptic D2R function in the striatum and other brain regions (55, 63–65). As suggested by our present results, it is possible that NT regulates pre- and postsynaptic D2Rs via a similar heterologous desensitization mechanism, but with different magnitudes and time courses. These differential properties could perhaps prove to be useful in the development of new treatments against schizophrenia.

*Acknowledgment—We are grateful for the technical assistance provided by Marie-Josée Bourque.*

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