

Bidirectional regulation of dopamine D2 and neurotensin NTS1 receptors in dopamine neurons

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Abstract

Several lines of evidence suggest a close association between dopamine (DA) and neurotensin (NT) systems in the CNS. Indeed, in the rodent brain, abundant NT-containing fibres are found in DA-rich areas such as the ventral tegmental area and substantia nigra. Moreover, it has been shown *in vivo* that NT, acting through its high-affinity receptor (NTS1), reduces the physiological and behavioural effects of DA D2 receptor (D2R) activation, a critical autoreceptor feedback system regulating DA neurotransmission. However, the mechanism of this interaction is still elusive. The aim of our study was thus to reproduce *in vitro* the interaction between D2R and NTS1, and then to characterize the mechanisms implicated. We used a primary culture model of DA neurons prepared from transgenic mice expressing green fluorescent protein under the control of the tyrosine hydroxylase promoter. In these cultures, DA neurons endogenously express both D2R and NTS1. Using electrophysiological recordings, we show that activation of D2R directly inhibits the firing rate of DA neurons. In addition, we find that NT, acting through a NTS1-like receptor, is able to reduce D2R autoreceptor function independently of its ability to enhance DA neuron firing, and that this interaction occurs through a protein kinase C- and Ca²⁺-dependent mechanism. Furthermore, prior activation of D2R reduces the ability of NTS1 to induce intracellular Ca²⁺ mobilization. Our findings provide evidence for bidirectional interaction between D2R and NTS1 in DA neurons, a regulatory mechanism that could play a key role in the control of the activity of these neurons.

Introduction

Neurotensin (NT) is an endogenous tridecapeptide that is widely distributed throughout the mammalian CNS (Fassio *et al.*, 2000). In the rodent brain, a high density of NT-immunoreactive fibres innervates dopamine (DA)-rich areas such as the ventral tegmental area (VTA) and substantia nigra (SN) (Uhl *et al.*, 1977; Jennes *et al.*, 1982; Quirion, 1983; Binder *et al.*, 2001; Zahm *et al.*, 2001). Furthermore, NT receptors, predominantly the type 1 high-affinity receptor (NTS1), are expressed in SN and VTA DA neurons (Palacios & Kuhar, 1981; Uhl, 1982; Quirion *et al.*, 1985; Moysse *et al.*, 1987; Brouard *et al.*, 1992; Nicot *et al.*, 1995; Fassio *et al.*, 2000). NT acting on NTS1 receptors has an excitatory effect on midbrain DA neurons (Pinnock, 1985; Seutin *et al.*, 1989; Mercuri *et al.*, 1993; Jiang *et al.*, 1994; Farkas *et al.*, 1996). In addition to this excitatory effect, a number of indirect evidence suggests that NT also acts to regulate DA neurons indirectly by modulating the function of the DA D2 receptor (D2R) (Binder *et al.*, 2001).

At the cellular level, NTS1 and D2R are present within some of the same axon terminals and dendrites (Delle Donne *et al.*, 2004). In addition, NT produces a modest decrease in the affinity of the D2R for its agonists (von Euler *et al.*, 1989; Fuxe *et al.*, 1992; Tanganelli *et al.*, 1993; Li *et al.*, 1995). However, the nature and mechanism of the

antagonistic interaction between NTS1 and D2R has not yet been elucidated. Several hypotheses have been proposed. A first hypothesis is that NTS1 and D2R oppositely regulate a similar potassium conductance in DA neurons (Farkas *et al.*, 1997); NT would thus simply prevent D2 agonists from activating the potassium channels by independently inhibiting them. Another plausible hypothesis is that activation of NTS1 leads to second messenger-dependent D2R alterations such as phosphorylation. Accordingly, it has been shown in heterologous cell lines that protein kinase C (PKC) can phosphorylate D2R, resulting in functional desensitization (Rogue *et al.*, 1990; Namkung & Sibley, 2004). Because NTS1 activates the phospholipase C (PLC)/inositol phosphate (IP)₃/PKC pathway (Hermans & Maloteaux, 1998; Vincent *et al.*, 1999), it is thus possible that the functional regulation of D2R exerted by NT results from a heterologous desensitization phenomenon.

In the present study we used primary cultures of mesencephalic neurons from tyrosine hydroxylase (TH)-enhanced green fluorescent protein (EGFP) transgenic mice (Jomphe *et al.*, 2005) to investigate the mechanisms underlying the interaction between D2R and NTS1. In this model, DA neurons can be identified by the expression of enhanced green fluorescent protein (EGFP), and they endogenously express both D2R and NTS1. Using electrophysiological recordings, we evaluated the ability of NT to modulate D2R-mediated inhibition of firing rate. We found that NT, through activation of a NTS1-like receptor, is indeed able to reduce D2 autoreceptor function, and that this interaction occurs through a PKC- and Ca²⁺-dependent inhibition

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of D2R function. In addition, prior activation of D2R similarly reduces the ability of NTS1 to induce intracellular Ca^{2+} mobilization. These results indicate a bidirectional interaction between D2R and NTS1 functions that is involved in the regulation of DA neuron activity.

Materials and methods

Transgenic mice and cell culture

The transgenic mice (TH-GFP/21–31 line) carrying the EGFP gene under the control of the TH promoter were described in recent studies (Sawamoto *et al.*, 2001; Matsushita *et al.*, 2002). Transgenic lines were maintained as heterozygotes by breeding with C57BL/6J inbred mice, and offsprings that carried the transgene were identified by polymerase chain reaction (PCR) on the genomic DNA extracted from tail biopsies. A 475-bp fragment of EGFP DNA was amplified by PCR, using the primers: AAGTTCATCTGCACCACCG and TGCTCAGGTATGGTTGTCG. Primary cultures of postnatal mesencephalic neurons were prepared from postnatal day zero TH-GFP/21–31 transgenic mice as recently described (Jomphe *et al.*, 2005), according to procedures, including anaesthesia, that were approved by the animal ethics committee of the Université de Montréal. Briefly, neonatal (P0) mouse pups were cryoanaesthetized and their brains were rapidly removed and transferred into ice-cold dissociation solution before enzymatic digestion with papain and mechanical trituration. Cells were then plated on astrocyte monolayers and maintained in Neobasal A/B27 medium supplemented with penicillin/streptomycin, GlutaMAX-1 and 10% fetal calf serum (Gibco, Logan, UT, USA).

Electrophysiology

Electrophysiological recordings were performed at room temperature on EGFP-expressing living neurons maintained for 10–18 days in culture. Cultures were transferred to a recording chamber that was fixed to the stage of an inverted Nikon Eclipse TE-200 microscope. The coverslip was constantly perfused with physiological saline solution using a gravity flow system (2.5–3 mL/min) with a standard extracellular bathing solution containing (in mM): NaCl, 140; KCl, 5; MgCl_2 , 2; CaCl_2 , 2; HEPES, 10; sucrose, 6; glucose, 10; pH 7.35, ~300 mOsm. Drugs were bath applied, with a delay between valve opening and onset of drug action of approximately 15 s. Whole-cell or perforated patch-clamp (using the antibiotic amphotericin B, 150–200 $\mu\text{g}/\text{mL}$) current-clamp recordings were performed using a Warner PC-505 patch-clamp amplifier (Warner Instruments, Hamden, CT, USA). Signals were filtered at 1 kHz, digitized at 10 kHz, and recorded and analysed using Pclamp7 software (Axon Instruments, Foster City, CA, USA) and Mini Analysis software (version 5.6) (Synaptosoft, Leonia, NJ, USA), respectively. Action potentials were recorded with borosilicate glass patch pipettes (5–7 M Ω) filled with a potassium methylsulphate intrapipette solution containing (in mM): KMeSO_4 , 145; KCl, 20; NaCl, 10; EGTA, 0.1; ATP (Mg salt), 2; GTP (Tris salt), 0.6; HEPES, 10; phosphocreatine (Tris salt), 10 (pH 7.35) or, when required, a BAPTA intrapipette solution containing (in mM): KMeSO_4 , 100; KCl, 20; NaCl, 5; EGTA, 0.1; ATP (Mg salt), 2; GTP (Tris salt), 0.5; HEPES, 10; phosphocreatine (Tris salt), 10; BAPTA (tetraacetic acid tetrapotassium salt), 10 (pH 7.35). In all experiments, the firing frequency was normalized to the average frequency recorded during the baseline period (2 min before drug application) and expressed as a percentage. The effect of a drug on firing rate was determined as the ratio of mean firing frequency at the peak of the effect (usually 1 min for the effect of the

D2 agonist quinpirole) to the average frequency measured during baseline and washout periods. This calculation method takes into account any rundown in firing rate that could occur during long recording periods. *Post hoc* immunocytochemistry against TH was performed systematically to confirm dopaminergic phenotype. The proportion of EGFP-expressing neurons that expressed TH was approximately 80%. Neurons not expressing TH were excluded from further analysis.

Calcium imaging

Changes in cytosolic intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) were measured with fura-2. Briefly, cells grown on 15-mm coverslips were loaded with fura-2 by incubating in saline (pH 7.4) containing 5 μM fura-2-AM and 0.02% pluronic acid (Molecular Probes, Eugene, OR, USA) for 45 min at room temperature. For $[\text{Ca}^{2+}]_i$ measurements, the coverslip was mounted in a recording chamber that was fixed to the stage of an inverted Nikon Eclipse TE-200 microscope. The coverslip was constantly perfused with physiological saline solution (containing in mM: NaCl, 140; KCl, 5; MgCl_2 , 2; CaCl_2 , 2; HEPES, 10; glucose, 10; sucrose, 6; at a pH of 7.35) added with 5 μM tetrodotoxin (Alomone Laboratories, Jerusalem, Israël) to prevent Ca^{2+} variations due to action potentials. $[\text{Ca}^{2+}]_i$ was measured by digital fluorescence imaging using Ratiotool software (Inovision Corporation, Raleigh, NC, USA). Fluorescence excitation at 340/380 nm was controlled by a DG4 xenon lamp (Sutter Instruments, Novato, CA, USA). Fluorescence was collected after passing through a 495-nm long-pass filter. Excitation image pairs (340/380 nm) were acquired every 5 s with an Orca-II digital cooled-CCD camera (Hamamatsu, Bridgewater, NJ, USA). $[\text{Ca}^{2+}]_i$ was calculated using an *in situ* calibration protocol and Grynkiewicz's equation (Grynkiewicz *et al.*, 1985). In experiments evaluating the effect of NT(8–13) on $[\text{Ca}^{2+}]_i$, neurons were considered responsive if they showed an increase in $[\text{Ca}^{2+}]_i$ of more than twice the standard deviation of $[\text{Ca}^{2+}]_i$ measured during the baseline period. The amplitude of the effect of NT was calculated as the difference between the maximal $[\text{Ca}^{2+}]_i$ increase at the peak (obtained 2 min after the beginning of the application of NT) and the $[\text{Ca}^{2+}]_i$ measured during a 1-min baseline period prior to the application. Two responses to NT were successively measured in the same cell, separated by an interval of 20 min. Drug effects were determined by comparing the amplitude of the initial NT-evoked $[\text{Ca}^{2+}]_i$ elevation to that of the second response measured in the presence of different drugs (SR48692, quinpirole).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) and incubated with a monoclonal anti-TH antibody (TH-2 clone) (Sigma-Aldrich, Oakville, Ontario, Canada) to confirm DA phenotype. The primary antibody was visualized using an Alexa-546-labelled secondary antibody (Molecular Probes). Coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and observed by epifluorescence microscopy on a Nikon Eclipse TE-200 inverted microscope.

Statistical analysis

The results are expressed as arithmetic means \pm SEM, with each observation (*n*) corresponding to a single recording made on neurons from different coverslips. For statistical analysis, two-factor ANOVA and *t*-tests were used as indicated.

Results

Quinpirole-induced inhibition of DA neuron firing rate is dose dependent and does not desensitize

In a previous study, we showed that cultured DA neurons prepared from TH-EGFP mice possess the well-known physiological and pharmacological properties of DA neurons (Jomphe *et al.*, 2005). Indeed, these neurons possess functional D2 autoreceptors and show an inhibition of their firing rate in response to a D2 agonist. Using electrophysiological recordings, we first characterized the inhibition of firing rate induced by quinpirole, a D2R-selective agonist. Whole-cell perforated patch-clamp recordings were obtained from EGFP-expressing DA neurons, identified by epifluorescence prior to recording. As expected, quinpirole (0.1 and 1 μM) reduced the spontaneous firing rate in a dose-dependent manner (Fig. 1A, B and D), with the highest concentration producing a significant effect. Activation of D2R with 1 μM quinpirole induced a $59.7 \pm 14.1\%$ reduction in firing frequency ($n = 6$, Student's *t*-test for paired observations, $P = 0.03$), while this inhibition was limited to $20.1 \pm 6.9\%$ with 0.1 μM ($n = 6$, Student's *t*-test for paired observations, $P = 0.1$). In addition, we confirmed that the effect of quinpirole (1 μM) on firing rate was the result of D2 autoreceptor activation as sulpiride (5 μM), a D2R antagonist, totally prevented this effect ($n = 4$, Student's *t*-test for paired observations, $P = 0.93$). Therefore all subsequent experiments were performed with 1 μM quinpirole (Fig. 1D).

Because subsequent experiments involved repeated applications of quinpirole, we next verified whether two successive applications of the D2 agonist caused a reproducible response. We found that when spaced by a 6-min interval, two applications of quinpirole (1 μM , for 2 min) induced a similar inhibition of the firing rate of cultured DA neurons ($Q1 = 59.7 \pm 14.1\%$, $Q2 = 56.1 \pm 12.1\%$; $n = 6$, Student's *t*-test for paired observations, $P = 0.8$) (Fig. 1C and E). This suggests that the response mediated by D2 autoreceptor activation does not show acute desensitization.

Activation of NT receptor reduces D2 autoreceptor function

We next aimed to reproduce *in vitro* the interaction previously seen *in vivo* between the D2R and NT receptors. We assessed the ability of the active fragment of NT [NT(8–13)] to modulate the effect of the D2 agonist quinpirole on the firing rate of cultured DA neurons. During each recording, we first evaluated the effect of quinpirole alone ($Q1$, 1 μM), and then a second application of quinpirole was done in the presence of NT(8–13) (1 nM). It should be noted that NT(8–13) by itself often caused an increase in firing rate, followed, in some cells, by brief periods of inactivity. Only neurons displaying stable firing in the presence of NT(8–13) were considered for further analysis. NT(8–13) was applied for 10 min starting 3 min before the second application of quinpirole ($Q2$). We found that the effect of the second application of quinpirole, in the presence of NT(8–13), was significantly reduced relative to the first one (Fig. 2A and C). In the presence of NT(8–13), the inhibition of firing rate induced by quinpirole was limited to $35.0 \pm 5.1\%$, in comparison to $78.9 \pm 7.9\%$ when applied alone ($n = 5$, Student's *t*-test for paired observations, $P = 0.008$).

We next used SR48692 (1 μM) and SR142948A (100 nM), a selective NTS1 and a mixed NTS1/NTS2 receptor antagonist, respectively, to evaluate the implication of these receptors in the modulatory effect of NT(8–13) on D2 autoreceptor function. Each antagonist was present during the complete duration of these experiments. Our results show that SR48692 as well as SR142948A blocked the modulatory effect of NT(8–13) on D2R function (Fig. 2B and D), while they did not affect the ability of the D2R agonist to reduce the firing rate of DA neurons. Indeed, in the presence of

SR48692, the first application of quinpirole induced a decrease of $72.0 \pm 6.0\%$ in the firing rate, while the second application performed in the presence of NT(8–13) produced a decrease of $58.3 \pm 10.1\%$. The two responses were not statistically different ($n = 7$, Student's *t*-test for paired observations, $P = 0.08$). Similarly, the first application of quinpirole produced an inhibition of firing rate of $64.3 \pm 6.7\%$ in the presence of SR142948A, while the second application of quinpirole performed in the presence of NT(8–13) reduced firing by $54.8 \pm 6.5\%$ ($n = 6$, Student's *t*-test for paired observations, $P = 0.18$). Because the mixed NTS1/NTS2 antagonist did not block the modulatory effect of NT(8–13) to a greater extent than the selective NTS1 antagonist and because NTS1 is expressed at a high level in DA neurons, we conclude that the effect of NT(8–13) on D2R function is mainly mediated through the high-affinity NTS1 receptor.

Modulation of D2R function by NT is independent of its ability to increase the firing rate of DA neurons

The excitatory effect of NT on DA neurons is well established (Palacios & Kuhar, 1981; Pinnock, 1985; Seutin *et al.*, 1989; St-Gelais *et al.*, 2004; Jomphe *et al.*, 2005). One possibility is that the interaction between D2R and NTS1 is simply the consequence of their opposite effect on DA neurons excitability. However, this explanation is unlikely as the excitatory effect of NT(8–13) was variable and the receptor interaction could be detected even in experiments where the excitatory effect of NT(8–13) was modest (Fig. 2A). Nonetheless, we tested this possibility by evaluating the ability of NT to inhibit D2R function under conditions where we interfered with the excitatory effect of the peptide. In cultured DA neurons from rat midbrain, we have previously shown that blocking IP₃ receptors using heparin prevented the excitatory effect of NT (St-Gelais *et al.*, 2004). We first reproduced this finding by showing that inclusion of heparin (1 mg/mL) in the intrapipette solution blocks the excitatory effect of NT(8–13) (Fig. 3A, B and D). Indeed, in the presence of heparin, NT(8–13) increased the firing rate by only $37.6 \pm 16.4\%$ over baseline, compared with $293.9 \pm 105.5\%$ when applied alone ($n = 7$, Student's *t*-test, $P = 0.04$). However, under such conditions, NT was still able to negatively regulate D2 autoreceptor function: when DA neurons were exposed to NT(8–13) in the presence of heparin, the inhibition of firing rate induced by quinpirole was still smaller ($41.3 \pm 10.5\%$) in comparison to the first application of quinpirole in the absence of NT(8–13) ($69.9 \pm 5.1\%$) ($n = 9$, Student's *t*-test for paired observations, $P = 0.03$; Fig. 3C and E). Therefore, we can conclude that the excitatory effect of NT is not necessary for the interaction seen *in vitro* between D2R and NTS1.

The functional interaction between D2R and NTS1 requires calcium

NTS1 is functionally coupled to the PLC IP signalling cascade (Hermans & Maloteaux, 1998; Vincent *et al.*, 1999), leading to Ca²⁺ elevation in DA neurons, mostly through extracellular Ca²⁺ influx, with a minor contribution of the IP₃ pathway (Borges *et al.*, 1996; St-Gelais *et al.*, 2004; Jomphe *et al.*, 2005). Although IP₃-dependent intracellular Ca²⁺ store mobilization is not apparently required for the D2R–NTS1 interaction (Fig. 3), a role for Ca²⁺ influx remains possible. To verify this hypothesis, we recorded cultured DA neurons using an intrapipette solution containing the Ca²⁺ chelator BAPTA (10 mM). In the presence of BAPTA, the two successive applications of quinpirole, in the absence or presence of NT, produced a similar inhibition of firing rate (BAPTA + Quinpirole: $72.2 \pm 9.1\%$; BAPTA + Quinpirole + NT: $73.8 \pm 10.6\%$, $n = 6$, Student's *t*-test for

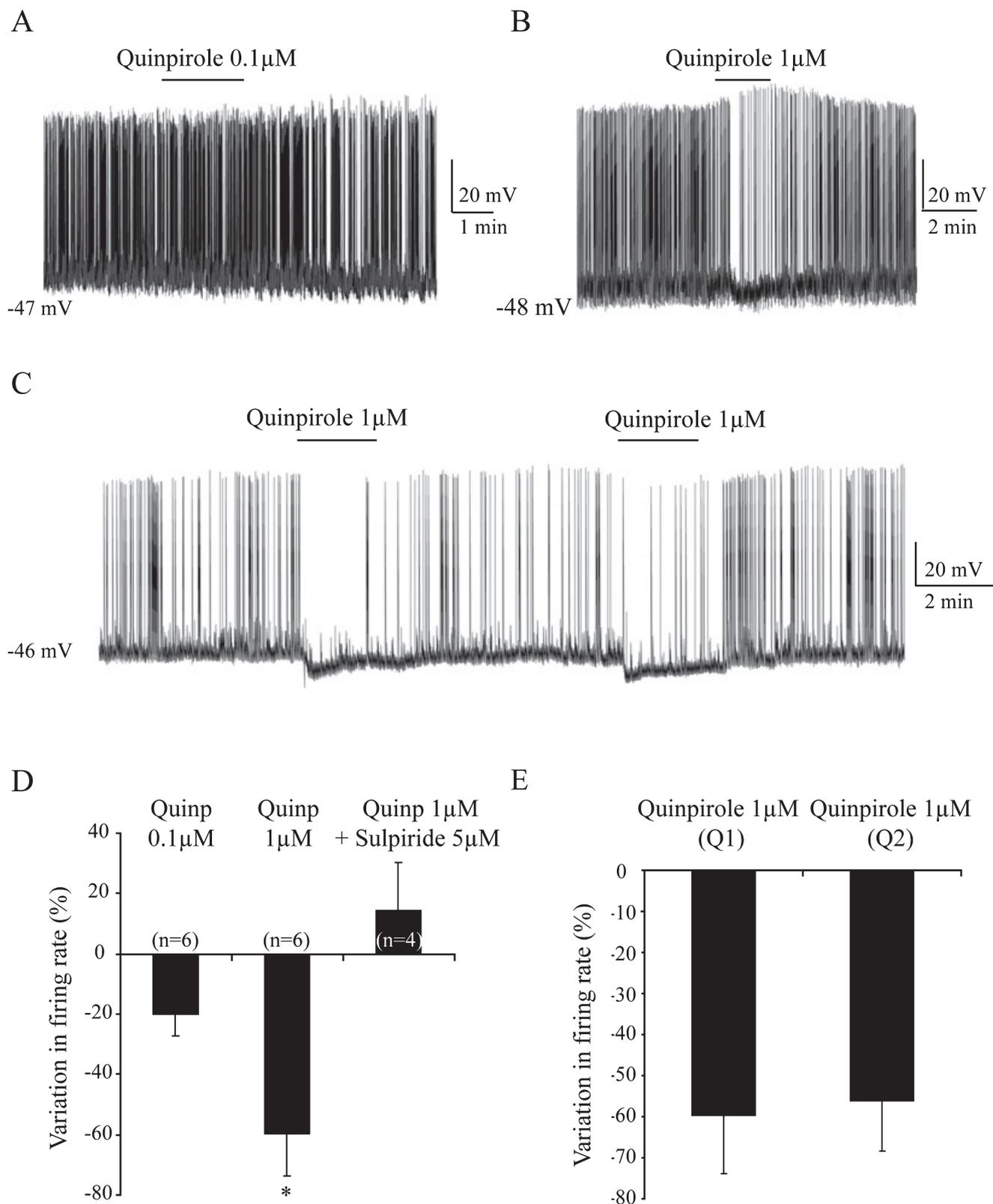


FIG. 1. Effect of quinpirole on the spontaneous firing of DA neurons. (A, B) Representative examples of patch-clamp recordings of spontaneous action potentials in cultured midbrain DA neurons from TH-GFP mice. The D2R agonist quinpirole induced a dose-dependent inhibition of the firing rate. Quinpirole was applied for 2 min at 0.1 μM (A) or 1 μM (B). At the highest dose, quinpirole caused a strong reduction in firing rate. (C) Representative recording of spontaneous action potentials in a DA neuron exposed to two successive applications of quinpirole (1 μM for 2 min). (D) Summary graph of the effect of quinpirole (1 μM) on the firing rate of DA neurons. Quinpirole produced a significant inhibition of the firing rate that was totally blocked by the selective D2 antagonist sulpiride (5 μM). (E) Summary graph showing the mean inhibition of firing rate induced by two successive applications of quinpirole (Q1 and Q2) ($n = 6$). The second application produced an inhibition similar to the first one. Data are expressed as mean \pm SEM. * $P < 0.05$.

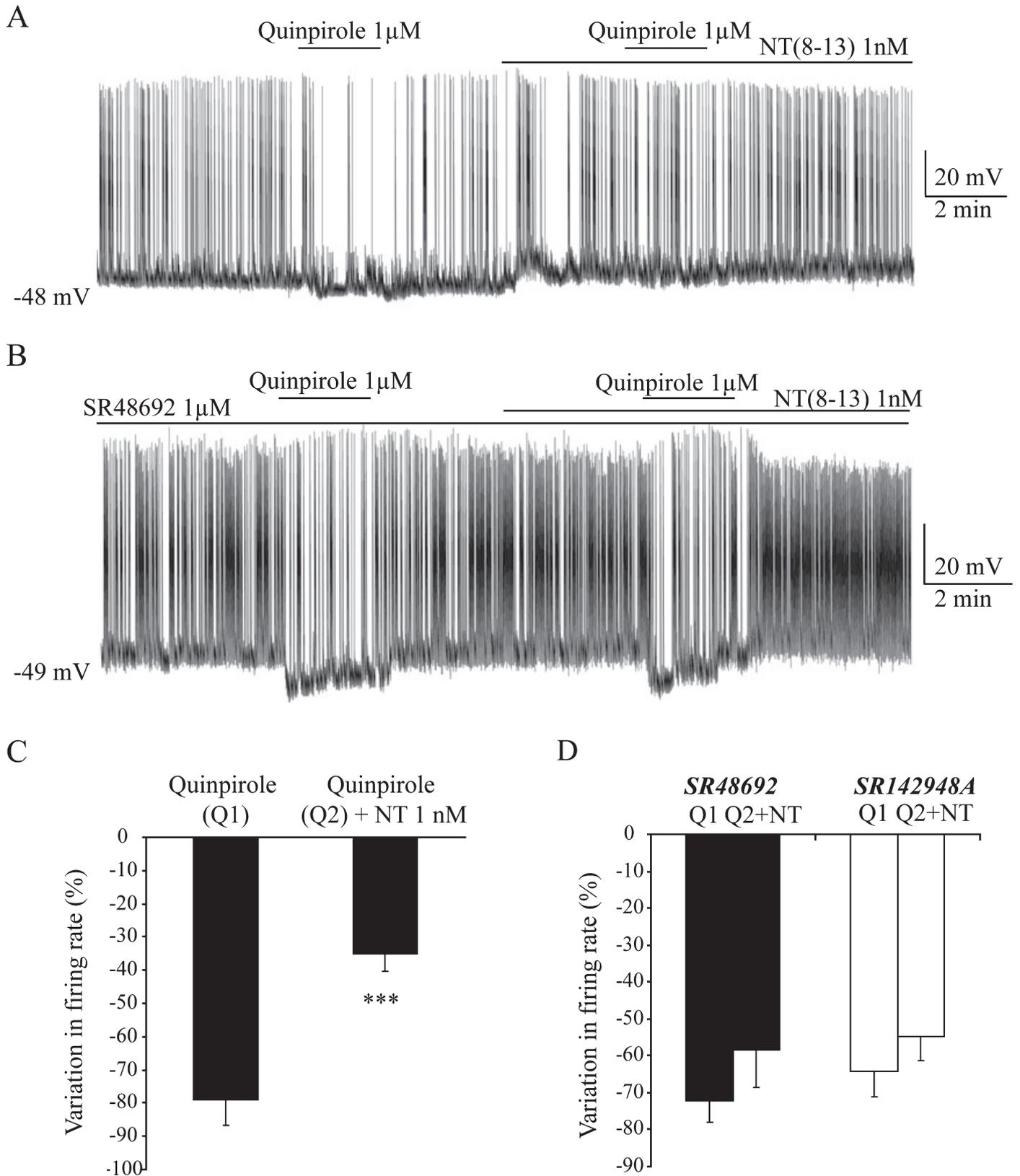


FIG. 2. Activation of NTS1 reduces the effect of quinpirole on firing rate. (A, B) Representative examples of spontaneous action potentials recorded from cultured DA neurons. The second application of quinpirole was made in the presence of 1 nM neurotensin (NT(8–13)) alone (A) or together with the selective NTS1 antagonist SR48692 (1 μ M) (B). (C) Graph summarizing the effect of NT(8–13) on the quinpirole-induced inhibition of firing rate ($n = 5$). (D) Summary graph of the effect of the selective NTS1 antagonist SR48692 (1 μ M) ($n = 7$) and the NTS1/NTS2 mixed antagonist SR142948A (100 nM) ($n = 6$) on the ability of NT(8–13) to inhibit the second response to quinpirole. Data are expressed as mean \pm SEM. *** $P < 0.001$.

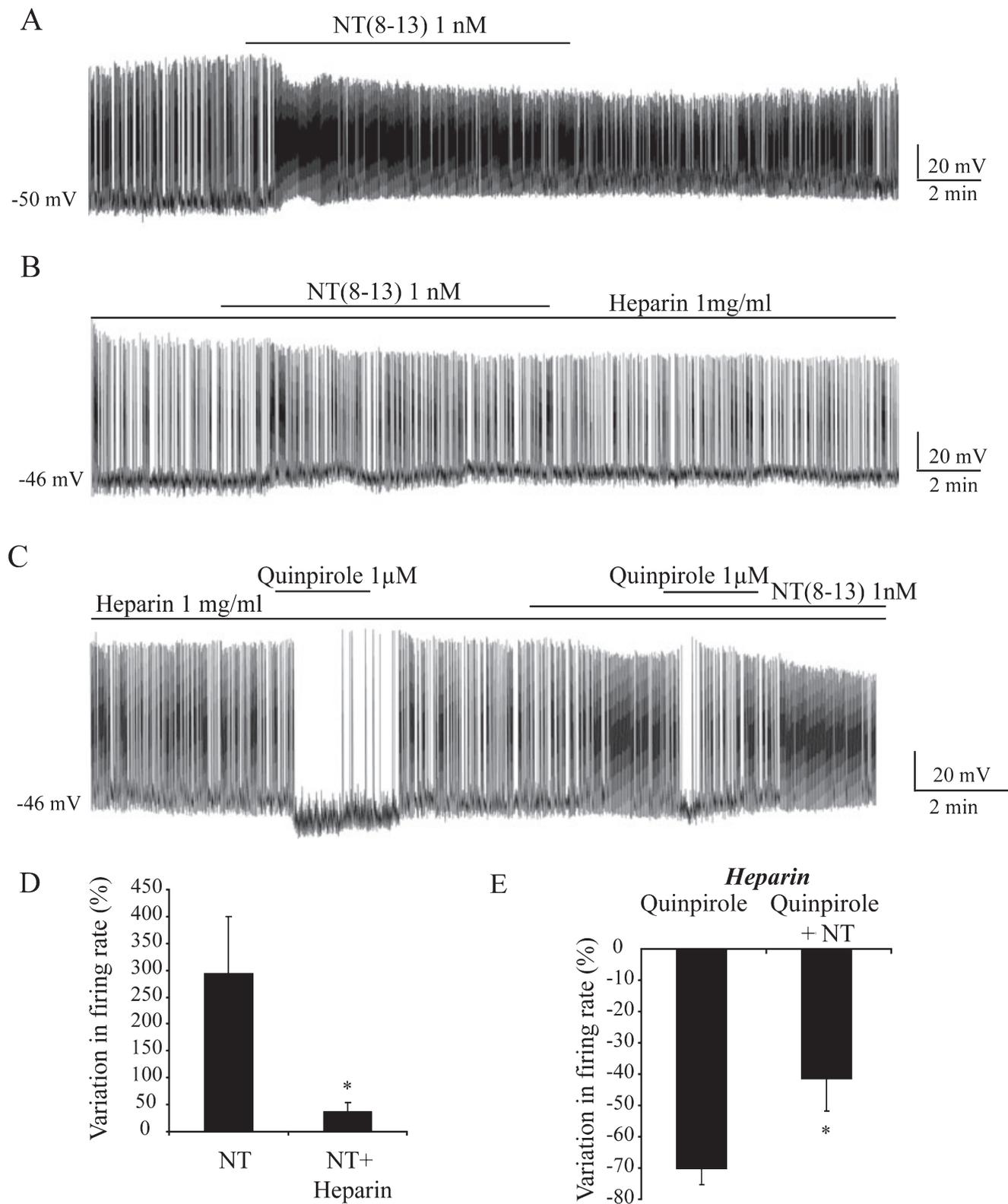


FIG. 3. The excitatory effect of NTS1 is not required for the functional regulation of D2R function. (A, B) Representative recording of the effect of neurotensin (NT(8-13)) (1 nM, for 10 min) on DA neurons. NT(8-13) produced a strong increase in the firing rate of DA neurons (B), an effect that was blocked by heparin (1 mg/mL) (B). (C) Example of a patch-clamp recording experiment measuring spontaneous action potentials in DA neurons exposed to two successive applications of quinpirole, the second of which was performed in the presence of NT(8-13). The IP₃ receptor antagonist heparin (1 mg/mL), included in the patch pipette, failed to prevent the ability of NT(8-13) to inhibit the effect of quinpirole. (D) Graph summarizing the effect of heparin on NT(8-13)-induced increase in firing ($n = 7$) in comparison to the effect of NT(8-13) alone ($n = 8$). Note that heparin blocked the excitatory effect of NT(8-13). (E) Summary graph showing the effect of NT(8-13) on quinpirole-evoked inhibition of firing, in the presence of heparin ($n = 9$). Note that heparin failed to prevent the interaction. Data are expressed as mean \pm SEM. * $P < 0.05$.

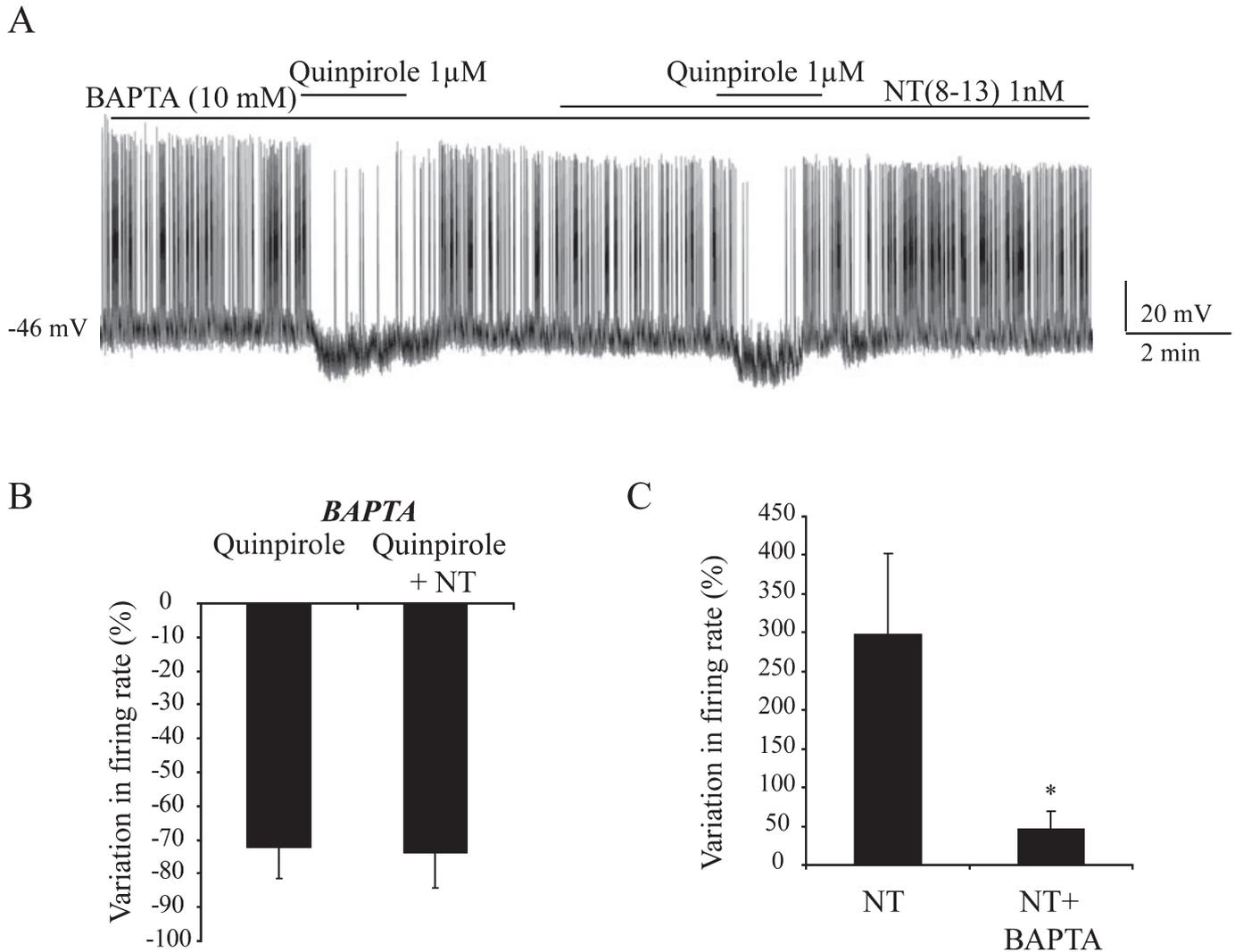


FIG. 4. The functional interaction between D2R and NTS1 requires calcium. (A) Representative patch-clamp recording of a spontaneously firing cultured DA neuron exposed twice to quinpirole, the second of which was performed in the presence of neurotensin (NT(8–13)). The calcium chelator BAPTA (10 mM) was included in the intrapipette solution. (B) Summary graph showing the effect of BAPTA on the response of DA neurons to quinpirole in the presence or absence of NT(8–13) ($n = 6$). Note that BAPTA completely prevented the interaction. (C) Graph summarizing the effect of BAPTA on NT(8–13)-induced increase in firing rate ($n = 7$) in comparison to the effect of NT(8–13) alone ($n = 8$). Note that BAPTA blocked the excitatory effect of NT(8–13). Data are expressed as mean \pm SEM. * $P < 0.05$.

paired observations, $P = 0.80$; Fig. 4A and B), showing that BAPTA totally abolished the functional regulation of D2R seen after NTS1 activation. In addition to blocking this receptor interaction, intrapipette BAPTA, like the IP_3 receptors antagonist heparin, blocked the increase in firing rate induced by NT (Fig. 4C). The increase in firing rate was $45.9 \pm 24.2\%$ in BAPTA-loaded neurons, while it was $297.6 \pm 105.2\%$ under normal recording conditions (NT, $n = 8$; NT + BAPTA, $n = 7$; Student's t -test, $P = 0.047$). We can thus conclude that although the increase in intracellular Ca^{2+} following NTS1 activation is required both for the increase in the firing rate and for the functional interaction between D2R and NTS1, the two phenomena are apparently not directly linked.

PKC is involved in the functional regulation of D2R by NTS1

It has been shown, in heterologous expression systems, that D2R signalling is reduced by PKC (Rogue *et al.*, 1990; Namkung & Sibley, 2004). Because stimulation of NTS1 leads to PLC and PKC activation (Hermans & Maloteaux, 1998; Vincent *et al.*, 1999), we assessed the

implication of this kinase in the interaction between D2R and NTS1 by including PKC(19–36), a selective PKC peptide inhibitor, in the patch pipette. We observed that in the presence of PKC(19–36) (10 μ M), the ability of NT(8–13) to reduce the effect of quinpirole on firing frequency was blocked (Fig. 5). The first application of quinpirole produced a $65.2 \pm 8.6\%$ decrease in the spontaneous firing, while the second application, made after NTS1 receptor activation, caused an inhibition of $52.7 \pm 4.5\%$, which was not statistically different from the first response ($n = 7$, Student's t -test for paired observations, $P = 0.24$; Fig. 5B). Blocking PKC activity is thus sufficient to counteract the functional regulation of the D2R by NTS1.

Activation of D2R reduces the ability of NT to increase intracellular calcium in DA neurons

If NTS1 and D2R functionally interact, this interaction could perhaps be bidirectional, with D2R also regulating NTS1 function. To answer this question we performed intracellular Ca^{2+} imaging experiments, taking advantage of the fact that activation of NTS1 produces an

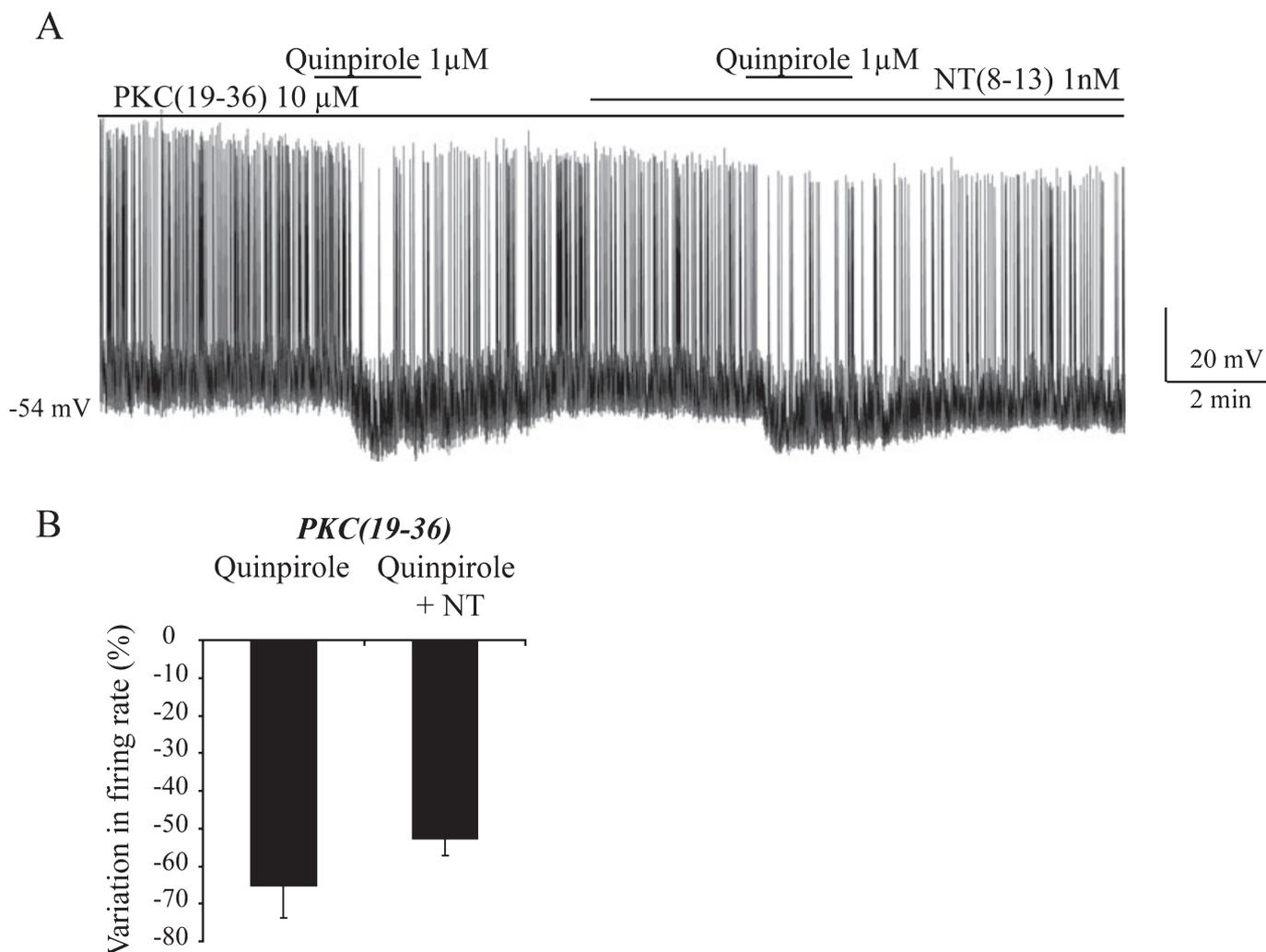


FIG. 5. The functional interaction between D2R and NTS1 is protein kinase C (PKC)-dependent. (A) Representative patch-clamp recording experiment where PKC(19–36) (10 μ M), a PKC inhibitor, was included in the patch pipette. The recorded DA neuron was first exposed to quinpirole alone and then quinpirole in the presence of neurotensin (NT(8–13)). (B) Summary graph showing that PKC(19–36) prevented the ability of NT(8–13) to negatively regulate the response to quinpirole ($n = 7$). Data are expressed as mean \pm SEM.

increase in $[Ca^{2+}]_i$ in both rat and mouse DA neurons (St-Gelais *et al.*, 2004; Jomphe *et al.*, 2005). Cultured neurons were loaded with fura-2-AM, a fluorescent Ca^{2+} indicator, and changes in $[Ca^{2+}]_i$ were measured with fura-2 ratio fluorescence in EGFP-expressing DA neurons, identified by epifluorescence prior to the experiment. As expected, application of NT(8–13) (10 nM, for 2 min) produced a large increase in $[Ca^{2+}]_i$ (Fig. 6A), an effect that showed only limited desensitization (Fig. 6B). The first application of NT(8–13) caused an average increase of 794.2 ± 95.6 nM, over the baseline level, while the second application, done 20 min after the first, led to an average increase of 664.0 ± 74.0 nM ($n = 17$, Student's *t*-test for paired observations, $P = 0.01$). Calculated on a cell by cell basis, the second response to NT was on average $10.6 \pm 5.5\%$ smaller than the first (Fig. 6D). The first application of NT also did not lead to any long-lasting change in baseline levels of $[Ca^{2+}]_i$, as the first baseline value was on average 152.6 ± 18.4 nM and the second baseline value, measured just before the second application of NT, was 152.2 ± 16.5 nM ($n = 17$, Student's *t*-test for paired observations, $P = 0.97$, results not shown). To evaluate the contribution of NTS1, the sensitivity of this Ca^{2+} mobilization response to SR48692 was evaluated. We found that SR48692 totally abolished NT(8–13)-

induced Ca^{2+} elevation in DA neurons (not shown). In the presence of SR48692, the increase in $[Ca^{2+}]_i$ induced by NT(8–13) was limited to 14.4 ± 9.8 nM, compared with 644.8 ± 89.0 nM when NT(8–13) was applied alone ($n = 8$, Student's *t*-test for paired observations, $P < 0.001$), corresponding to a $97.2 \pm 1.7\%$ inhibition.

Finally, to verify if D2R functionally regulates NTS1 signalling, we stimulated D2R with quinpirole (1 μ M) 2 min prior to the second NT(8–13) application. In the absence of quinpirole, activation of NTS1 receptors leads to a large increase in $[Ca^{2+}]_i$ (940.7 ± 111.7 nM). In response to quinpirole, a small decrease in basal $[Ca^{2+}]_i$ was first noted (from 201.1 ± 22.2 nM to 89.2 ± 6.6 nM in the presence of quinpirole) ($n = 18$, Student's *t*-test for paired observations, $P < 0.001$; Fig. 6C). In the presence of quinpirole, the second response to NT(8–13) was on average 623.6 ± 91.7 nM ($n = 8$), which, calculated on a cell by cell basis, corresponds to a decrease of $37.3 \pm 7.2\%$ (Student's *t*-test for paired observations, $P < 0.001$; Fig. 6C), a value that was much higher than the 10.6% decrement noted in control experiments. Based on these observations, we conclude that the D2R negatively regulates NTS1 function. The interaction seen *in vitro* between D2R and NTS1 is thus bidirectional.

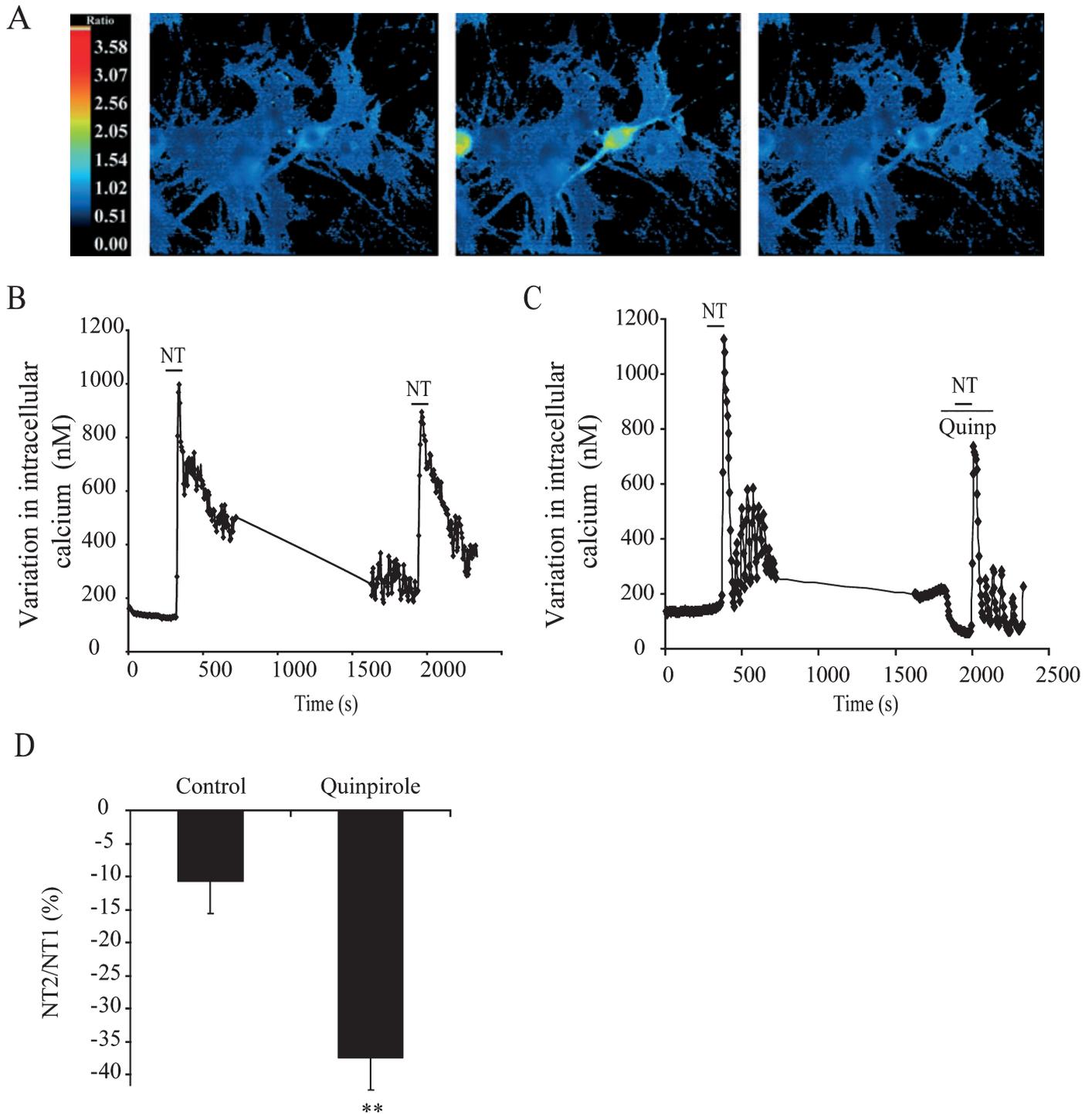


FIG. 6. D2R inhibits the ability of NTS1 to increase intracellular calcium in DA neurons. (A) Ratio images from a representative fura-2 Ca^{2+} imaging experiment. DA neurons were identified prior to the experiment by EGFP epifluorescence (not shown). (B and C) Graphs showing the variation in intracellular Ca^{2+} concentration as a function of time in representative experiments. Neurotensin (NT(8–13)) (10 nM) was applied twice for 2 min, separated by a 25-min washout period. On the second occasion, it was applied either alone (B) or in the presence of quinpirole (1 μM) (C). (D) Summary graph showing the amplitude of the second response to NT relative to the first. The second application of NT was preceded by saline in control experiments (control $n = 17$) or by quinpirole ($n = 18$). Data are expressed as mean \pm SEM. $**P < 0.01$.

Discussion

Several lines of evidence suggest a close association between DA and NT systems in the CNS. It has been demonstrated that in addition to its direct excitatory effects on the excitability of DA neurons, NT through its high-affinity receptor, also modulates D2 autoreceptor function (Shi & Bunney, 1991; Rostene *et al.*, 1997; Werkman *et al.*, 2000).

However, the mechanism underlying this interaction is still elusive. The present work provides novel information on the mechanism by which NT induces functional inhibition of D2R function. Using electrophysiological recordings, we made three major observations. First, we showed that NT, through the activation of a NTS1-like receptor, is effectively able to reduce D2 autoreceptor function in

cultured DA neurons and that this effect is independent of the direct excitatory effect of this peptide. Second, we found that the functional interaction between D2R and NTS1 receptors requires Ca^{2+} and PKC activation. Finally, we demonstrated that the cross-talk between NTS1 and D2R is bidirectional.

Because midbrain DA neurons are excited by NT and inhibited by DA, it has been suggested that the interaction between D2R and NTS1 functions results from an opposite regulation of a similar potassium conductance (Farkas *et al.*, 1997). In the present set of experiments, we did not directly measure the activity of ionic channels influencing the excitability of DA neurons. Nonetheless, our results allow us to discount this earlier hypothesis. We had previously shown that the excitatory effect of NT on cultured rat DA neurons is Ca^{2+} -dependent and implicates IP_3 receptors (St-Gelais *et al.*, 2004). In the present work, we similarly demonstrated that the excitatory effect of NT on cultured DA neurons from TH-GFP mice is blocked by BAPTA, a Ca^{2+} chelator, and by heparin, an IP_3 receptor antagonist. Moreover, we found that including BAPTA in the intrapipette solution effectively blocked the functional regulation of the D2R by NTS1, while heparin was without effect on this interaction. These results suggest on the one hand that an increase in intracellular Ca^{2+} is required for the interaction to occur, and on the other that the major source of this Ca^{2+} is the extracellular medium, a finding compatible with our previous report showing that NT elevates intracellular Ca^{2+} in DA neurons mainly by promoting Ca^{2+} influx, with only a minor contribution from intracellular Ca^{2+} stores (St-Gelais *et al.*, 2004). As it was possible to block the excitatory effect of NT without blocking the receptor interaction, we therefore conclude that the two mechanisms are independent. This conclusion is further supported by the previous demonstrations that NT is able to reduce the inhibitory effect of D2R agonists, even at concentrations of NT that do not increase firing by themselves (Shi & Bunney, 1990, 1992; Nalivaiko *et al.*, 1998; Werkman *et al.*, 2000). Also in agreement with this conclusion, we found that although the excitatory effect of NT on firing was quite variable, the peptide always caused a robust inhibition of D2R function, thus arguing in favour of independent mechanisms.

The functional interaction between D2R and NTS1 leads to a reduction of D2 autoreceptor function. We have not in the present work demonstrated direct receptor or signalling interactions between D2R and NTS1. The cross-talk between these two receptor systems could indeed involve a number of intermediate steps. However, the functional interaction we have described can nonetheless be considered a form of functional desensitization and could be caused by a loss of receptors from the cell surface, an uncoupling of receptors from their signal transduction pathways, or an alteration of distal components of these pathways. Our results with heparin argue against the possibility of an opposite regulation of a common effector. We thus favour the hypothesis that second-messenger pathways activated through NTS1 lead to a cascade of events promoting uncoupling of the D2 receptor from its signalling pathways. Because NTS1 is functionally coupled to the PLC, IP_3 metabolism and PKC signalling cascades (Hermans & Maloteaux, 1998; Vincent *et al.*, 1999), and because PKC activation leads to phosphorylation and desensitization of many Gi and Gq-linked GPCRs (Haga *et al.*, 1996; Oppermann *et al.*, 1996; Diviani *et al.*, 1997; Liang *et al.*, 1998; Tang *et al.*, 1998; Hipkin *et al.*, 2000), we investigated the role of PKC in the D2R–NTS1 receptor interaction. We found that blocking PKC with the selective peptide inhibitor PKC(19–36) prevents the functional desensitization of D2R induced by NTS1. The D2R possesses PKC phosphorylation sites within its third intracellular loop, a region that plays a central role in the coupling of GPCRs to their effectors. In addition, in heterologous

cell lines, PKC can mediate phosphorylation of D2R to promote desensitization and internalization (Rogue *et al.*, 1990; Namkung & Sibley, 2004). Although additional data will be required to demonstrate this, it is therefore possible that the reduction in D2R function induced by NTS1 results from a direct phosphorylation of the D2R by PKC. However, the processes underlying heterologous desensitization are still not well understood, and it is likely that multiple mechanisms, in addition to phosphorylation of the D2R by PKC, can contribute to this process. For example, it is possible that PKC acts through the phosphorylation of a GRK thus promoting homologous desensitization. Compatible with this possibility, it has been shown that GRK2, thought to be the major GRK involved in D2R internalization (Ito *et al.*, 1999; Iwata *et al.*, 1999; Kim *et al.*, 2001), can be directly phosphorylated by PKC (Chuang *et al.*, 1995) or that PKC increases its ability to interact with the receptor (Mundell *et al.*, 2004), leading to an increase in its ability to promote desensitization (Chuang *et al.*, 1996).

An alternate but not necessarily exclusive explanation of a D2R–NTS1 interaction is that the two receptors physically interact. Indeed, an allosteric interaction between D2R and NTS1 has been previously proposed based on indirect data (Tanganelli *et al.*, 1989; von Euler *et al.*, 1991). It is now widely accepted that GPCRs form homo- and hetero-oligomers, the formation of which can influence their ligand-binding pharmacology, intracellular signal transduction and trafficking (Bouvier, 2001). Although the mechanistic details remain unclear, heteroreceptor-induced desensitization and internalization could result from direct protein–protein interactions. It has been previously shown that D2R can form heterodimers with the adenosine A2A receptor (Fuxe *et al.*, 1998; Hillion *et al.*, 2002; Canals *et al.*, 2003; Kamiya *et al.*, 2003), as well as the somatostatin SSTR5 receptor (Rocheville *et al.*, 2000) and, more recently, with the cannabinoid CB1 receptor (Kearn *et al.*, 2005). Moreover, there is compelling evidence in heterologous cell lines that the heterodimerization between D2R and the A2A receptor is responsible for the heterologous desensitization of D2R following A2A receptor stimulation (Yang *et al.*, 1995; Salim *et al.*, 2000; Hillion *et al.*, 2002). Although there is no evidence yet supporting a direct interaction between D2R and NTS1, we cannot presently exclude this possibility.

Finally, we demonstrated that the D2R can reduce the ability of NTS1 to induce Ca^{2+} mobilization in cultured DA neurons. Although a previous study has shown a similar phenomenon in rat pituitary cells (Memo *et al.*, 1985), this is the first report of a bidirectional antagonistic interaction of D2 and NTS1 receptors. The mechanism of this reverse interaction is presently unexplored. The concept of bidirectional interactions between GPCRs is widespread, as highlighted by the recent observation of a similar relationship between μ -opioid and chemokine receptors (Rogers *et al.*, 2000), cannabinoid CB1 and δ -opioid receptors (Shapira *et al.*, 2003), and for angiotensin II and alpha 1 adrenergic receptors (Jerez *et al.*, 2004).

The D2R acts as an autoreceptor and feedback regulator of DA neurons (Lacey *et al.*, 1987; Chiodo & Kapatos, 1992; Mercuri *et al.*, 1997). It is otherwise known to exhibit relatively weak homologous desensitization (Sibley & Neve, 1997). Its functional regulation by NT, through NTS1, could thus be a critical mechanism to regulate the activity of DA neurons. The consequences of the regulation of NTS1 receptor signalling by the D2R have been less explored. However, this action would act as an ideal feedback system to dampen any excessive activity of DA neurons as an accumulation of extracellular DA would not only directly activate the D2 autoreceptor and its associated potassium conductances, but it would at the same time lead to partial inhibition of an excitatory peptidergic system.

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Abbreviations

DA, dopamine; EGFP, enhanced green fluorescent protein; IP, inositol phosphate; NT, neurotensin; PCR, polymerase chain reaction; PKC, protein kinase C; PLC, phospholipase C; SN, substantia nigra; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

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