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SYNAPTIC MECHANISMS

# Chronic activation of the D2 dopamine autoreceptor inhibits synaptogenesis in mesencephalic dopaminergic neurons *in vitro*

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## Abstract

Chronic blockade or activation of dopamine receptors is critical for the pharmacological treatment of diseases like schizophrenia, Parkinson's or attention deficit and hyperactivity disorder. However, the long-term impact of such treatments on dopamine neurons is unclear. Chronic blockade of the dopamine D2 receptor *in vivo* triggers an increase in the axonal arborization of dopamine neurons [*European Journal of Neuroscience*, 2002, 16, 787–794]. However, the specific involvement of presynaptic (autoreceptors) vs. postsynaptic D2 receptors as well as the molecular mechanisms involved have not been determined. Here, we examined the role of D2 autoreceptors in regulating the ability of mouse dopamine neurons to establish axon terminals. Chronic activation of this receptor with quinpirole, a specific agonist, decreased the number of axon terminals established by isolated dopamine neurons. This effect was accompanied by a decrease in dopamine release and was mediated through inhibition of protein kinase A. The decrease in axon terminal number induced by D2 receptor activation was also occluded when the mammalian Target of Rapamycin pathway of mRNA translation was blocked. Our results suggest that chronic activation of the D2 autoreceptor inhibits synaptogenesis by mesencephalic dopamine neurons through translational regulation of the synthesis of proteins required for synapse formation. This study provides a better understanding of the impact of long-term pharmacological interventions acting through the D2 receptor.

## Introduction

The dopamine (DA) system plays a central role in the control of locomotion and cognition. Mesencephalic dopaminergic neurons are located in the substantia nigra (SN) and ventral tegmental area and send projections through the nigrostriatal, mesocortical and mesolimbic pathways. The death of SN DA neurons accompanies Parkinson's disease, whereas dysfunction of ventral tegmental area DA neurons is thought to be implicated in addiction to drugs of abuse and in neuropsychiatric diseases such as depression, schizophrenia and attention deficit and hyperactivity disorder. Hence, numerous therapies, using antipsychotics or psychostimulants, aim to modulate DA neurotransmission in the central nervous system. Chronic D2 receptor (D2R) blockade represents the standard pharmacotherapeutic treatment of the symptoms of schizophrenia. DA receptor activation is commonly used to treat Parkinson's disease and DA reuptake blockade is the principal strategy used to treat attention deficit and hyperactivity disorder (reviewed in Nieoullon, 2002; Greydanus, 2005). However, the impact of such chronic stimulation or blockade of DA receptors remains poorly understood. Recent work has shown that chronic oral administration of a specific agonist of the D2R to rats and mice for several months triggers a decrease of the size of the axonal arbor of SN pars compacta DA neurons in the striatum. Conversely, pharmacological blockade or knockout of the D2R causes an increase in the axonal arbor size of these neurons (Parish *et al.*, 2002; Tripanichkul *et al.*, 2003). These findings thus suggest that D2R activity plays an inhibitory role in the synaptic development or stability of DA neurons. However, a limitation of these *in-vivo* studies is that they did not make it possible to distinguish between the involvement of a direct (presynaptic) or indirect (postsynaptic) mechanism, or to pinpoint the specific molecular mechanisms involved. Considering, on the one hand, the recent finding that activation of the DA D1 receptor enhances protein translation and transcription (Smith *et al.*, 2005) and, on the other hand, the frequent opposite activation of signalling cascades by the D1 receptor and D2R, we hypothesize that chronic activation of the D2 autoreceptor in DA neurons inhibits synapse formation or stability through inhibition of protein synthesis at the mRNA translation level. Here we developed an *in-vitro* model to directly evaluate the effect of chronic activation of the D2 autoreceptor on axon terminal development in mesencephalic DA neurons.

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## Materials and methods

### Cell cultures

All experiments were performed using primary cultures prepared from postnatal day 0–2 pups of the transgenic mouse line TH-EGFP/21–31. These mice carry the enhanced green fluorescent protein gene under the control of the tyrosine hydroxylase (TH) promoter (Sawamoto *et al.*, 2001; Matsushita *et al.*, 2002). Experimental procedures were conducted in accordance with the guide to the care and use of experimental animals of the Canadian Council on Animal Care. The experimental protocols were approved by the animal ethics committee of the Université de Montréal. Primary cultures of mesencephalic neurons of the SN and ventral tegmental area were prepared according to a previously described protocol (Bourque & Trudeau, 2000; Michel & Trudeau, 2000; Jomphe *et al.*, 2005; Fasano *et al.*, 2008) derived from Cardozo (1993) and Sulzer *et al.* (1998). Brain tissues were obtained from cryoanesthetized mice. Briefly, for microcultures, a mesencephalic cell suspension containing neurons was plated at 80 000 living cells/mL onto astrocyte microislands in order to obtain cultures containing individual isolated DA neurons. Under such conditions, these neurons establish synaptic contacts, called 'autapses', onto their own cell body and dendritic processes. This model makes possible the quantification of the number of morphologically identifiable axon terminals established by individual DA neurons. For standard cultures, dissociated neurons were plated on cortical astrocytes grown on collagen/poly-L-lysine precoated glass coverslips. The cell density was 240 000 living cells/mL, except for the DA release assay and for western blotting experiments for which the density was 350 000 living cells/mL. Under our experimental conditions, each coverslip contained on average 28 ± 2% ($n$ = 10) DA neurons in addition to non-DA neurons and astrocytes. Cultures were incubated at 37°C in a 5% $CO_2$ atmosphere in a Neurobasal-A/B27 medium (Gibco, Logan, UT, USA) supplemented with penicillin/streptomycin, Gluta-MAX-1 (Gibco) and 10% fetal calf serum (Hyclone Laboratories, Logan, UT, USA).

### Chronic drug treatments

Cell cultures were chronically treated by adding drugs to the culture medium on three occasions after 2, 4 and 6 days in culture. Analyses were performed on the seventh day of culture, except for patch-clamping experiments, which were performed from days 8 to 10 in culture. Chronic activation of the D2 autoreceptor was achieved using quinpirole (Sigma, St Louis, MO, USA). The D2R antagonist sulpiride (Sigma) and the protein kinase A (PKA) antagonists Rp-adenosine-3′,5′-cyclic monophosphorothioate (Rp-cAMP) (Sigma) and *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride (H-89; Sigma) were applied 30 min before quinpirole, whereas the translation inhibitor rapamycin (Calbiochem, San Diego, CA, USA) was applied for 1 h prior to quinpirole. Drug stocks were diluted as follows: quinpirole, Rp-cAMP and H-89 in water, sulpiride in ethanol (final ethanol dilution 4/10 000) and rapamycin in methanol (final methanol dilution 1/1500). For control experiments, the volume of drug applied was replaced by the appropriate vehicle. For each drug applied, potential toxic effects were evaluated by counting the total number of isolated DA neurons per coverslip. This analysis, presented in Table 1, indicates that none of the chronic treatments reduced DA neuron survival in microcultures.

TABLE 1. Chronic treatments do not affect the survival of DA neurons in microculture

| Drug | Number of isolated DA neurons per coverslip | | *t*-test *P*-value |
|---|---|---|---|
| | Control group ($n$) | Treated group ($n$) | |
| Quinpirole (1 μM) | 3.98 ± 0.31 (96) | 4.11 ± 0.35 (76) | 0.786 |
| Sulpiride (4 μM) | 2.78 ± 0.32 (27) | 2.86 ± 0.40 (29) | 0.871 |
| Rp-cAMP (50 μM) | 10.16 ± 0.54 (6) | 12.33 ± 0.88 (6) | 0.063 |
| H-89 (1 μM) | 4.00 ± 0.44 (32) | 4.00 ± 0.59 (17) | 1.000 |
| Rapamycin (20 nM) | 3.81 ± 0.60 (31) | 3.38 ± 0.45 (29) | 0.577 |

None of the drugs used for chronic treatment induced a statistically significant difference in the number of isolated DA neurons per coverslip. Quinpirole is a D2R agonist, sulpiride is a D2R antagonist, Rp-cAMP and H-89 are PKA inhibitors and rapamycin is an mTOR inhibitor.

### Immunocytochemistry

In order to count the number of axon terminals, cells were fixed for 25 min in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, permeabilized with 0.1% Triton X-100 (25 min) and nonspecific binding was blocked with 10% normal goat serum (5 min). Cells were incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal anti-TH antibody (1 : 5000; Chemicon, Temecula, CA, USA) and mouse monoclonal anti-synaptic vesicle protein 2 (SV2) antibody (1 : 500; Developmental Studies Hybridoma Bank, IA, USA). The cells were then incubated for 1 h at room temperature (23-25°C) with rabbit Alexa-fluor 488- and mouse Alexa-fluor 647-conjugated secondary antibodies (1 : 200; Molecular Probes Inc., Eugene, OR, USA). Coverslips were finally mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA).

To identify and count axon terminals, immunostaining for the ubiquitous presynaptic marker SV2 was preferred to the alternative approach of colocalizing pre- and postsynaptic markers. This was done to prevent underestimation of axon terminals, considering that up to 70% of axon terminals established by DA neurons are considered to be non-synaptic and involved in diffuse or volume transmission (Descarries *et al.*, 2008). In additional to axonal release, DA neurons also release DA from their dendritic compartment (Fortin *et al.*, 2006). However, SV2 immunostaining labels only release sites associated with axon terminals, as dopaminergic dendrites do not contain large clusters of synaptic vesicles that are associated with extensive accumulation of vesicular proteins such as SV2. For experiments measuring extracellular DA concentrations, only coverslips containing at least 300 DA neurons were kept for analysis. The number of DA neurons per coverslip was counted following immunostaining for TH, as described above.

### Confocal imaging

Quantification of the number of morphologically identifiable axon terminals per single DA neuron was performed by confocal microscopy using single-neuron microcultures. Observations were made with a Nikon 40X objective. A z-series projection of 5–12 images with 1 μm focal plane interval was acquired using a point-scanning confocal microscope from Prairies Technologies LLC (Middleton, WI, USA). The 488 nm line of an argon ion laser and the 633 nm line of a helium neon laser were used to excite the Alexa-fluor 488- and Alexa-fluor 647-conjugated secondary antibodies, respectively. Images were analysed using Metamorph software v4.5 (Universal Imaging Corp., Downington, PA, USA). Axon terminal quantification was not performed on microislands that were excessively small because this precluded spreading of axonal processes and led to excessive

clustering of axon terminals. SV2-immunoreactive punctate structures ranging in size from 0.28 to 5.75 $\mu m^2$ were counted as a single synaptic terminal, according to a previously described approach (Bourque & Trudeau, 2000). Briefly, axon terminals located on the cell body were not included in the counts because these were usually too dense to reliably quantify. The image plane of the *z*-series containing the highest number of terminals was chosen to estimate the relative number of axon terminals per neuron. This number was calculated automatically with Metamorph. Experiments were performed blindly from data acquisition up to data analysis.

### *DA assay*

To measure extracellular DA levels, standard culture coverslips were placed in wells containing 400 μL of a normal saline solution (NSS). Samples (100 μL) were taken every 3 min and replaced with 100 μL of NSS. After 30 min of basal sampling, DA neurons were depleted of their releasable DA stores by replacing NSS with a high potassium solution containing 145 mM of potassium chloride for 3 min, thus increasing the final potassium chloride concentration to 40 mM. Neurons were maintained in this depolarizing medium (high potassium solution containing 40 mM of potassium chloride) up to the end of the sampling period. The NSS contained (in mM): 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 6 sucrose, 10 glucose, 10 HEPES. For high potassium solutions, 145 and 40 mM of potassium chloride, respectively, replaced equimolar NaCl. For all solutions, the osmolarity was 305–310 mOsm and the pH was adjusted to 7.35. In order to prevent DA oxidation, 2 μL of a preservative solution was added to aliquots prior to the experiment. This solution consisted of EGTA (90 mg/mL) and reduced glutathion (60 mg/mL), adjusted to pH 6.5. DA was detected by using a high performance liquid chromatography system coupled to a reverse phase C18 column (CSC-vitesse 3 μm; Chromatography Science Comp., Montreal, QC, Canada) and to a coulometric detector (Coulochem II, ESA, Bedford, USA). The mobile phase used to separate catecholamines consisted of (in mM): 44 $NaH_2PO_4$, 44 $CH_3COONa$, 0.5 sodium dodecyl sulphate (SDS), 0.45 EDTA, adjusted to pH 3.1 with phosphoric acid and completed with 13.5% of acetonitrile, and was delivered at a flow rate of 0.8 μL/min.

### *Electrophysiology*

Standard cultures (8–10 days in culture) were used for electrophysiological recordings. The spontaneous firing activity of DA neurons was recorded at room temperature by using the patch-clamp technique in whole-cell configuration. Only the brightest enhanced green fluorescent protein-expressing neurons were selected for patch-clamp recordings to ensure that the vast majority were indeed dopaminergic as previously shown (Jomphe *et al.*, 2005). Coverslips were transferred to a recording chamber that was placed onto the stage of a Nikon Eclipse TE-200 inverted microscope and superfused with NSS. Spontaneous activity was recorded with a patch-clamp amplifier (PC-505; Warner Instruments Corp., Hamden, CT, USA) using PClamp 9 software (Axon Instruments, Union City, CA, USA). Analysis was performed with Mini Analysis software v.5.6 (Synaptosoft Inc., Leonia, NJ, USA). Borosilicate glass patch pipettes (5–7 MΩ; World Precision Instruments Inc., Sarasota, FL, USA) were filled with a potassium methylsulphate intrapipette solution consisting of (in mM): 145 $KmeSO_4$, 20 KCl, 10 NaCl, 0.1 EGTA, 2 ATP (Mg salt), 0.6 GTP (Tris salt), 10 HEPES, 10 phosphocreatine (Tris salt), pH 7.35 and osmolarity 295–300 mOsm. A neuron was judged to be satisfactorily recorded when its resting membrane potential was at least −39 mV and when it exhibited action potentials overshooting 0 mV. Neurons were also rejected when their spontaneous firing rate failed to recover to at least 50% of the initial firing rate by the end of the quinpirole washout period. Under our conditions, the rate of rejected neurons was similar in all experimental groups.

### *Western blot*

Cells from standard cultures were collected with a phosphate-buffered saline solution containing 5% of trypsin and then centrifuged for 3 min at 300 ***g***. The cells were lysed in 10 mM Tris–HCl (pH 7.5), 158 mM NaCl, 25 mM NaF, 2 mM $Na_3VO_4$, 1 mM phenylmethylsulphonyl fluoride and a complete protease inhibitor cocktail (Roche Applied Science, QC, Canada). A fraction of each sample was used to quantify the proteins using a bicinchoninic acid protein assay kit (Pierce, IL, USA). Protein samples were sonicated and heated at 95°C for 5 min in one-third of the final volume of SDS-polyacrylamide gel electrophoresis sample buffer containing: 75 mM Tris–HCl (pH 6.8), 30% glycerol (v/v), 5% SDS (w/v), 0.5% β-mercaptoethanol (v/v), 0.01% bromophenol blue (w/v). Proteins (30 or 50 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Non-specific interactions were blocked with 2.5% (w/v) milk and 2.5% bovine serum albumin for 1 h at room temperature and the membranes incubated overnight with the indicated concentrations of primary antibodies. Proteins of interest were labelled using a rabbit polyclonal anti-TH antibody (1 : 3000; Chemicon), a mouse anti-β-actin monoclonal antibody (1 : 3000; Sigma), a rabbit monoclonal anti-Thr389-phospho-p70-S6 kinase antibody (1 : 500; Cell Signaling, Danvers, MA, USA) and a rabbit anti-p70-S6 kinase polyclonal antibody (1 :1 000, Cell Signaling). Horseradish peroxidase-conjugated goat anti-mouse (1 : 7000; Jackson Immunoresearch Laboratories, West Grove, PA, USA) or goat anti-rabbit (1 : 3000, Jackson Immunoresearch Laboratories) secondary antibodies were added for 1 h at room temperature. For detection of the total form of p70-S6 kinase, a p70-S6 kinase antibody was used after stripping of the nitrocellulose membrane using a buffer consisting of 0.1 M glycine (pH 2.2) and 1% SDS. Detection and quantification of the immunoreactive bands were performed using the ECL Plus Western Blotting Detection System (Amersham Biosciences, Buckinghamshire, UK) and a Typhoon 9410 gel documentation scanning system (Amersham Biosciences).

### *Statistical analysis*

All values in text and figures are expressed as mean ± SEM. Statistical analysis between groups was performed using a *t*-test or an ANOVA (one-way or two-way) followed by a Tukey *post hoc* test, as appropriate. In western blotting experiments, two outlier results were rejected according to Grubb's method. Values were taken to be statistically different if $P < 0.05$.

## Results

### *Chronic stimulation of the D2 autoreceptor decreases the number of axon terminals established by DA neurons*

In primary neuron microcultures, the number of axon terminals established was quantified in single isolated DA neurons making autapses (Fig. 1A). The dopaminergic phenotype of isolated neurons was determined by TH immunostaining (Fig. 1A and B) and axon terminals were identified by immunostaining for SV2, a ubiquitous presynaptic marker (see Materials and methods; Fig. 1C and D).

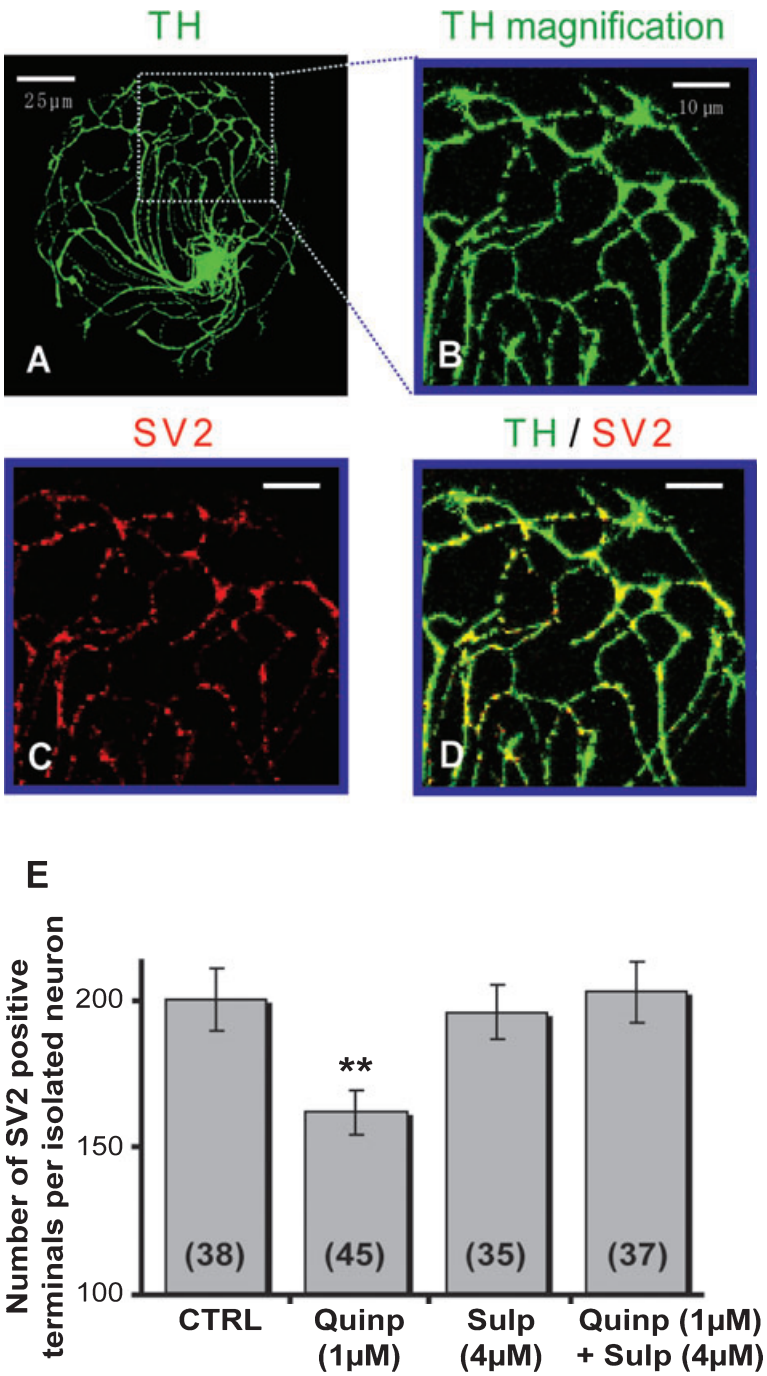


FIG. 1. Chronic activation of the D2 autoreceptor decreases the number of axon terminals established by single DA neurons in microisland culture. (A–D) Identification of axon terminals established by isolated DA neurons in microisland culture. (A) TH immunostaining (green) of an isolated postnatal mesencephalic neuron identifying the cell as dopaminergic. (B) Magnification of the cell presented in A showing some of the axonal varicosities. (C) Immunostaining of the same field shown in B against SV2 (red) identifies the location of presumed axon terminals. (D) Merge of TH and SV2 immunostaining. (E) Summary diagram presenting the quantification of axon terminal number in isolated DA neurons. Chronic activation of the D2R with 1 µM quinpirole (Quinp) significantly decreased the number of morphologically-identifiable axon terminals. The effect of quinpirole was blocked when neurons were pretreated with the D2R antagonist sulpiride (Sulp) (4 µM). Chronic blockade of the D2R with sulpiride did not by itself modify axon terminal number. $^{**}P < 0.01$ compared with the control group (CTRL). Note that the y axis of the histogram is truncated for clarity. Numbers inside histogram bars indicate the number of DA neurons analysed. This presentation format is used for all histograms showing the evaluation of axon terminal number.

Under control conditions, isolated DA neurons established 201 ± 10 SV2-positive axon terminals ($n = 38$, Fig. 1E). In comparison, neurons treated with the D2R agonist quinpirole (1 µM) three times for 5 days established 162 ± 9 SV2-positive terminals ($n = 45$), representing a mean decrease of 19 ± 4% ($P < 0.01$). Moreover, when quinpirole was applied in the presence of sulpiride (4 µM), a D2R antagonist, it failed to induce any change in axon terminal number (203 ± 10 SV2-positive terminals, $n = 37$). Compared with control conditions, there was no statistically significant change in axon terminal number induced by sulpiride, applied alone (196 ± 10 SV2-positive terminals, $n = 35$, $P = 0.758$), thus suggesting that endogenous DA levels accumulating in the medium of microcultures was too

low to cause a basal tone of D2R activation sufficient to decrease the number of autapses. These data demonstrate that chronic activation of the D2 autoreceptor specifically inhibits synaptogenesis in mesencephalic DA neurons.

### *Chronic stimulation of the D2R decreases DA release without decreasing expression of TH*

Next, we measured extracellular DA levels in standard cultures chronically treated with quinpirole to determine whether the reduction in axon terminal number was accompanied by a decrease in DA release. In such cultures, DA neurons spontaneously fire action potentials thus leading to a basal rate of DA release. To ensure a minimal level of DA that was reliably quantifiable, only coverslips containing at least 300 DA neurons were kept for the analysis of extracellular DA levels by high performance liquid chromatography. The control and quinpirole-treated groups contained 517 ± 32 ($n$ = 13) and 517 ± 44 ($n$ = 14) DA neurons per coverslip, respectively. Hence, chronic D2R activation does not change the number of DA neurons, indicating that it does not influence DA neuron survival in our culture conditions. Levels of DA were measured for 12 min in basal conditions and then DA release was evoked by sustained membrane depolarization induced by increasing extracellular levels of potassium to 40 mM (see Materials and methods) until the end of the sampling period (Fig. 2A). In basal conditions (Fig. 2B), extracellular DA levels were 97 ± 5 pg/mL in control cultures and 70 ± 7 pg/mL in cultures chronically treated with 1 μM quinpirole ($P$ < 0.01; 27 ± 7% decrease). Peak depolarization-evoked DA release was 932 ± 75 pg/mL for control cultures (Fig. 2C) and 568 ± 48 pg/mL for quinpirole-treated cultures ($P$ < 0.001; 39 ± 5% average decrease). Finally, during the plateau phase of DA release, extracellular DA levels were 237 ± 23 pg/mL in control cultures and 163 ± 9 pg/mL in quinpirole-treated cultures (Fig. 2D) ($P$ < 0.01; 31 ± 4% average decrease).

Decreased DA release after chronic activation of the D2R could have been the consequence of an inhibition of the expression of TH, leading to a decrease in DA synthesis. Western blot analysis ($n$ = 7, Fig. 2E) demonstrated that TH expression was not affected by chronic treatment with 1 μM quinpirole ($P$ = 0.506, Fig. 2F). Quantification of the ratio of TH to β-actin revealed that cultures chronically treated with quinpirole expressed TH to 92 ± 11% of the level detected in the control group. As expected, no TH was detected in astrocyte cultures containing no neurons, thus confirming the specificity of the signal ($n$ = 3) (Fig. 2E).

### *Inhibition of PKA is required for the decrease in synapse number induced by chronic activation of the D2 autoreceptor*

Considering that inhibition of adenylate cyclase and of the PKA signalling pathway is one of the better known effectors of the D2R, we next evaluated if inhibition of PKA was involved in the reduction of synapse number induced by quinpirole. We reasoned that, if inhibition of this pathway was involved, chronic application of a PKA inhibitor should reduce synapse number and occlude the effect of quinpirole. In this series of experiments, isolated DA neurons in control coverslips established 309 ± 12 SV2-positive terminals ($n$ = 37, Fig. 3), whereas DA neurons treated with quinpirole established 250 ± 16 SV2-positive terminals ($n$ = 51), indicating a statistically significant decrease of 19 ± 5% in the number of synapses ($P$ < 0.01). Following chronic treatment with Rp-cAMP (50 μM), a PKA inhibitor, the neurons established 207 ± 12 SV2-positive terminals ($n$ = 54), indicating a statistically significant decrease of 33 ± 4% ($P$ < 0.001) of the number of axon terminals. The PKA antagonist thus mimicked the effect of quinpirole. Moreover, when applied in the presence of Rp-cAMP, quinpirole failed to induce any additional reduction in synapse number (225 ± 16 SV2-positive terminals, $n$ = 45) ($P$ = 0.362 in comparison to Rp-cAMP alone). The involvement of PKA was confirmed by using another PKA inhibitor, H-89 (1 μM), which produced very similar results (see supplementary Fig. S1). Thus, activation of the D2 autoreceptor failed to decrease synapse number when PKA activity was already blocked, suggesting a role for this kinase in the response to chronic D2 autoreceptor activation.

### *Regulation of synapse number by the D2 autoreceptor involves translation inhibition*

Considering the recent demonstration that activation of the PKA signalling pathway through the DA D1 receptor leads to activation of local mRNA translation in neurons through a rapamycin-sensitive pathway (Smith *et al.*, 2005), we hypothesized that D2 autoreceptor-mediated inhibition of PKA could produce the opposite effect, leading to inhibition of mRNA translation. The ensuing inhibition of protein synthesis could then explain in part the reduction in synapse number. Rapamycin is known to inhibit mammalian Targets of Rapamycin (mTOR), a kinase involved in the activation of the translation machinery and cell growth (Hay & Sonenberg, 2004; Jaworski & Sheng, 2006). To test this hypothesis, we next evaluated the effect of rapamycin on synapse number in isolated DA neurons. Under control conditions, isolated DA neurons established an average of 236 ± 13 SV2-positive axon terminals ($n$ = 39, Fig. 4). Chronic treatment with quinpirole (1 μM) reduced this number to 191 ± 11 SV2-positive terminals ($n$ = 27), thus indicating a statistically significant decrease of 19 ± 5% ($P$ < 0.01). Neurons chronically treated with rapamycin (20 nM) alone had 150 ± 9 SV2-positive terminals ($n$ = 35), thus representing a mean decrease of 36 ± 4% ($P$ < 0.001) of the number of synapses. Finally, in the presence of rapamycin, chronic treatment with quinpirole, either alone or in the presence of the D2 antagonist sulpiride (4 μM), failed to induce any additional reduction in synapse number (164 ± 9 SV2-positive terminals, $n$ = 36, $P$ = 0.365 for quinpirole plus rapamycin; 157 ± 16 SV2-positive terminals, $n$ = 25, $P$ = 0.164 for quinpirole plus sulpiride and rapamycin). This result suggests that the reduction in axon terminal number induced by D2 autoreceptor activation requires inhibition of the mTOR pathway.

As a control for the possibility that the rapamycin treatment simply reduced the expression of the D2R, thus preventing the action of quinpirole, we next investigated if activation of D2R still inhibited the firing rate of DA neurons, a well-known effect of autoreceptor activation (Lacey *et al.*, 1987; Maslowski & Napier, 1991; Fig. 5A). For this set of experiments, we took advantage of enhanced green fluorescent protein expression by DA neurons to identify them for whole-cell patch-clamp recordings in standard cultures. Chronic treatment with rapamycin (20 nM) was found to affect neither the resting membrane potential nor the spontaneous firing rate. Control and rapamycin-treated DA neurons had a mean resting membrane potential of −46 ± 2 and −46 ± 1 mV and a firing rate of 1.4 ± 0.3 and 1.3 ± 0.3 Hz, respectively ($n$ = 13 for both groups). However, rapamycin treatment increased the membrane resistance by 47 ± 14% ($P$ < 0.01, 863 ± 74 and 1266 ± 117 MΩ for control and rapamycin-treated DA neurons, respectively), indicating that rapamycin may decrease the expression of ionic channels such as leak channels. Brief activation of D2R by quinpirole (1 μM for 2 min) decreased the firing rate of DA neurons by 45 ± 7% ($P$ < 0.001, $n$ = 13, Fig. 5B). In DA neurons chronically treated with rapamycin, quinpirole still decreased

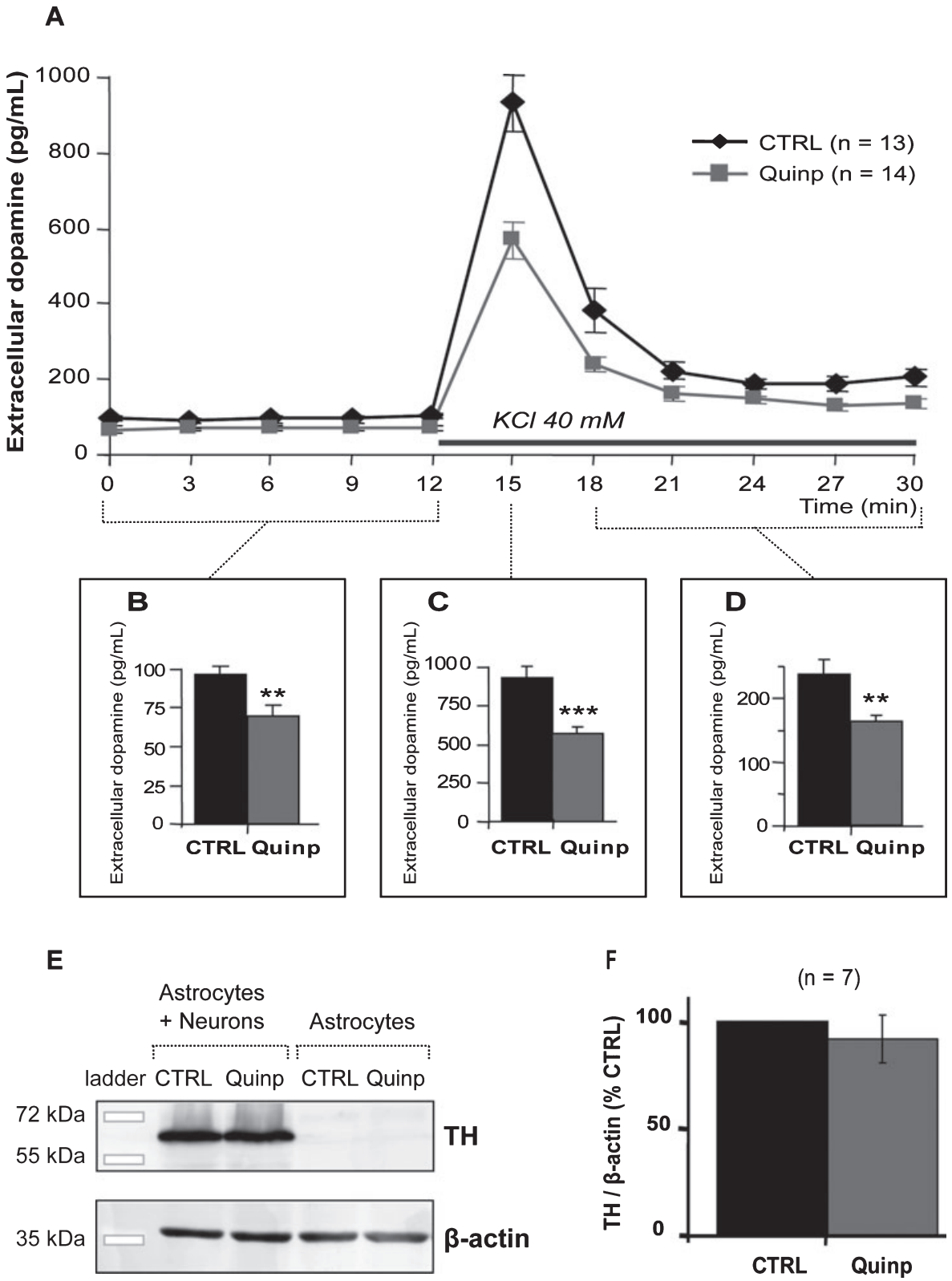


FIG. 2. Chronic activation of the D2R inhibits DA release but not TH expression. (A) Time-course of extracellular DA concentration. Basal extracellular DA levels were recorded from 0 to 12 min, after which DA release was evoked with a high potassium solution that was maintained until the 30th min. Chronic treatment with 1 μM quinpirole (Quinp) decreased both basal and potassium-evoked DA levels. Histograms represent the average levels of extracellular DA during basal conditions (B) and the maximal levels of extracellular DA in response to high potassium depolarisation (C) and during the sustained depolarization period (D). (E) Western blot analysis of the relative TH protein levels expressed by DA neurons grown on an astrocyte monolayer culture with or without chronic treatment with quinpirole. Note that in pure astrocyte cultures no TH signal was detected. (F) Quantification of TH expression in DA neuron cultures by western blot densitometry. Data are expressed as a ratio of TH to β-actin band intensity. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control (CTRL) group.

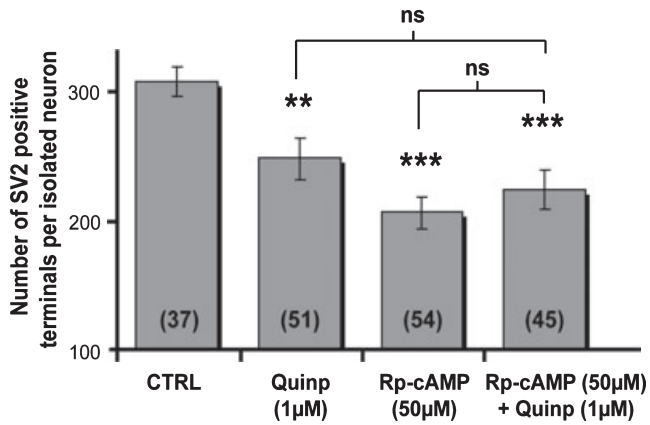


FIG. 3. Role of PKA in D2R-mediated reduction in axon terminal number. Summary diagram presenting the quantification of axon terminal number in isolated DA neurons in microisland culture. Chronic treatment of neurons with quinpirole (Quinp) (1 μM) or the PKA antagonist Rp-cAMP (50 μM) decreased the number of axon terminals established by isolated DA neurons. Chronic treatment with 1 μM quinpirole did not cause any additional decrease in axon terminal number in neurons pretreated with 50 μM Rp-cAMP compared with the neurons treated with Rp-cAMP alone [not statistically different (ns)]. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control (CTRL) group.

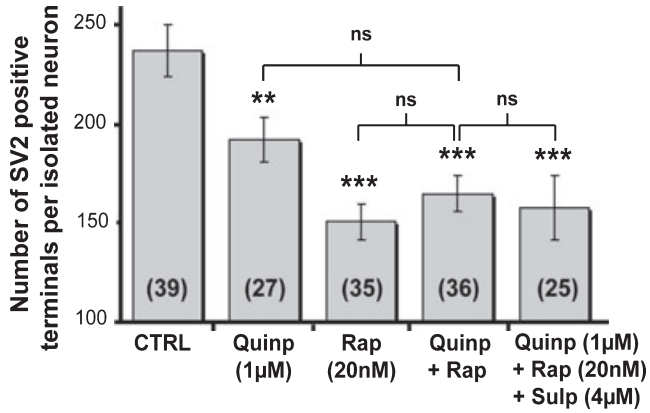


FIG. 4. Role of mTOR-dependent mRNA translation in D2R-mediated reduction in axon terminal number. Summary diagram presenting the quantification of axon terminal number in isolated DA neurons in microisland culture. Chronic treatment of neurons with quinpirole (Quinp) (1 μM) or the mRNA translation inhibitor rapamycin (Rap) (20 nM) decreased the number of axon terminals established by isolated DA neurons. Inhibition of mTOR-dependent translation by rapamycin prevented any further effect of quinpirole on the number of SV2-positive axon terminals compared with the neurons treated with rapamycin alone. $^{**}P < 0.01$, $^{***}P < 0.001$. ns, not statistically different; CTRL, control; Sulp, sulpiride.

the firing rate by 41 ± 6% ($P < 0.001$, $n = 13$, Fig. 5B). There was no statistically significant difference between the effect of the D2R agonist in control and rapamycin-treated neurons ($P = 0.385$, Fig. 5C). This result demonstrates that the chronic treatment of neurons with rapamycin did not suppress the expression of D2R at the membrane and did not affect the ability of this receptor to inhibit firing rate.

If chronic activation of the D2 autoreceptor inhibits synapse number by regulating the mTOR pathway of translation regulation, this should have an impact on the phosphorylation of p70-S6 kinase, a target of mTOR. Rapamycin specifically inhibits the phosphorylation of p70-S6 kinase at its 389 threonine residue, leading to a decrease in its activity and consequently in the activity of the translation machinery. Using western blotting we thus determined the relative level of regulation of p70-S6 kinase by measuring the ratio of phospho(Thr389)-p70-S6 kinase to total p70-S6 kinase in cells from standard primary cultures (Fig. 6A). As a positive control for our ability to detect a decrease in the state of phosphorylation of p70-S6 kinase, we exposed cultures to rapamycin (200 nM) for 30 min (Fig. 6A). This caused a decrease in the phosphorylation levels of p70-S6 kinase of 65 ± 11% ($n = 7$, $P < 0.01$, data not shown). In comparison, chronic activation of D2R with quinpirole (1 μM) decreased the phosphorylation levels of p70-S6 kinase by 27 ± 7% ($n = 5$, $P < 0.05$, Fig. 6B). As a control, we also evaluated S6 kinase phosphorylation in purified astrocyte cultures, cells that do not express the D2R. Quinpirole treatment was without significant effect (112 ± 8% of the level detected in the control group, $n = 3$, $P = 0.245$; Fig. 6B). This result suggests that chronic activation of the D2 autoreceptor leads to the decrease in synapse number through the mTOR pathway and inhibition of p70-S6 kinase activity.

## Discussion

In the present study, we established an *in-vitro* model allowing investigation of the regulation of synapse number in response to chronic activation of the D2 autoreceptor in cultured mouse DA neurons. We found that activation of the D2R reduces synapse number and that this is accompanied by a decrease in DA release but not in TH expression. This phenomenon is mediated through inhibition of the PKA pathway and implicates the mTOR pathway of mRNA translation regulation.

Synaptogenesis in the central nervous system is regulated by numerous mechanisms where both pre- and postsynaptic neurons play active roles through the release of diffusible neurotrophic factors and cell-cell interactions (Garner *et al.*, 2002; Munno & Syed, 2003). Electrical activity and neurotransmitter receptors also play a critical role (Lovell *et al.*, 2002; Sohya *et al.*, 2007). In this context, regulation of synaptogenesis or synapse stability by G-protein-coupled receptors is of significant interest. Previous *in-vivo* studies have suggested a role for the D2R in regulating the establishment of synapses by DA neurons. Chronic oral administration of a specific D2R agonist to rats and mice for several months induces a decrease in the size of the terminal arbor of SN pars compacta DA neurons (Parish *et al.*, 2001, 2002). In an opposite manner, pharmacological blockade or genetic deletion of the D2R induced an increase in the terminal arbor volume (Parish *et al.*, 2002; Tripanichkul *et al.*, 2003). However, manipulating the D1 DA receptor was without effect on the size of the terminal arbor. These initial studies suggested a specific inhibitory role of the D2R in the development or stability of synapses made by SN neurons. However, in these *in-vivo* experiments, it was not possible to readily distinguish between the roles of pre- or postsynaptic D2Rs. In the present study, our use of an *in-vitro* model of isolated DA neurons allowed us to confirm the specific involvement of the D2 autoreceptor in inhibition of synaptic terminal development and/or stability by DA neurons. Despite the fact that, in microcultures, normal postsynaptic targets are absent, isolated DA neurons growing under such conditions establish axon terminals and autapses, which provide a useful index of the ability of DA neurons to establish synaptic contacts. Synaptogenesis under such conditions has been shown to be sensitive to local environmental cues such as growth factors (Bourque & Trudeau, 2000) or other astrocyte-mediated signals (Forget *et al.*, 2006). It should be noted that, in microcultures, DA neurons have been shown to establish a subset of glutamate-containing axon terminals (Sulzer

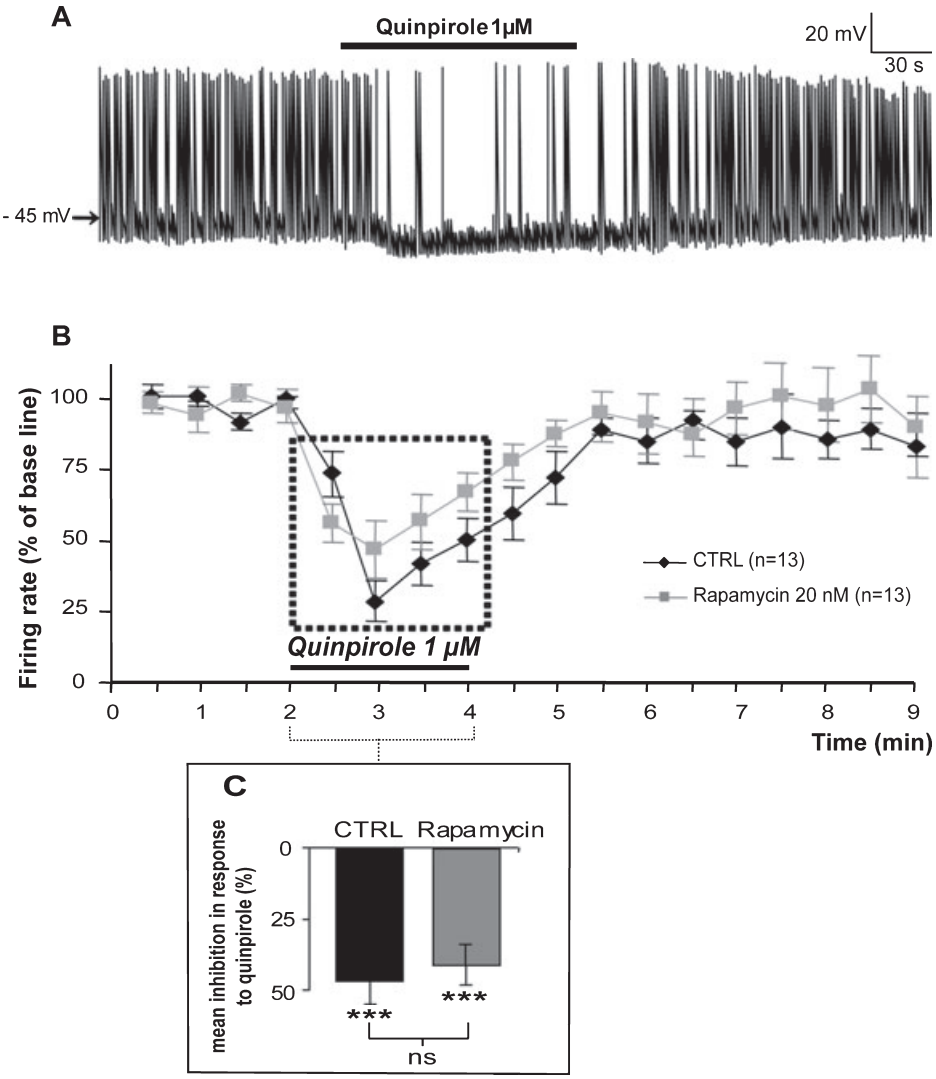


FIG. 5. Chronic treatment with rapamycin fails to affect D2R-mediated inhibition of firing rate in DA neurons. (A) Whole-cell electrophysiological recording from a cultured DA neuron. Superfusion with quinpirole (1 µM) for 2 min produced a transient decrease in firing rate. (B) Average time-course of the firing rate measured in cultured DA neurons. Chronic treatment of neurons with the translation inhibitor rapamycin (20 nM) did not affect the ability of quinpirole to inhibit firing rate. (C) Mean inhibition of the firing rate during the 2 min of superfusion with 1 µM quinpirole in the control (CTRL) and rapamycin-treated groups. ***$P < 0.001$ compared with the baseline. ns, not statistically different.

*et al.*, 1998; Dal Bo *et al.*, 2004), even in the presence of normal target neurons (Joyce & Rayport, 2000). In the present study, we did not distinguish between dopaminergic or glutamatergic axon terminals. However, considering that both DA and glutamate release by DA neurons are regulated by D2 autoreceptors in a similar fashion (Congar *et al.*, 2002), we hypothesize that D2Rs are likely to regulate the growth of both types of terminals. Future studies will also be required to determine whether the inhibition of synapse number was a direct consequence of activating the D2 autoreceptors localized to axon terminals, as opposed to D2 autoreceptors localized in the dendritic arbor of DA neurons, which could also influence synaptogenesis by inhibiting dendritic growth.

In the present study, quinpirole was used to chronically activate D2 autoreceptors. No effect of this chronic treatment on DA neuron survival or health was observed, compatible with the fact that there was no change in the number of dopaminergic neurons in both microcultures and standard cultures. We did not examine the D1 receptor because mesencephalic DA neurons do not express this receptor (Gross *et al.*, 2005). Moreover, pharmacological blockade of D2R with sulpiride completely abolished the effects of quinpirole on SV2-positive terminal establishment by DA neurons, thus demonstrating that the D2 autoreceptor specifically triggers inhibition of synaptogenesis in DA neurons.

The D2R exists as two isoforms produced through alternative splicing: a long isoform, D2L, and a short isoform, D2S. Although both isoforms appear to have the capacity to act as an autoreceptor *in vitro* (Jomphe *et al.*, 2006), genetic deletion studies have shown that the D2S isoform acts as the primary autoreceptor *in vivo* (Usiello *et al.*, 2000) due to its predominant expression in DA neurons. Although DA neurons also express the D3 receptor (Diaz *et al.*, 2000; Du *et al.*, 2005; Van Kampen & Robertson, 2005), the D2R has been shown to mediate most of the rapid autoreceptor functions, including negative regulation of DA release and DA neuron excitability (Lacey *et al.*, 1987; Maslowski & Napier, 1991; Jomphe *et al.*, 2006). Activation of the D2R is well known to inhibit the PKA pathway but it also inhibits Akt kinase (Beaulieu *et al.*, 2007), a kinase otherwise

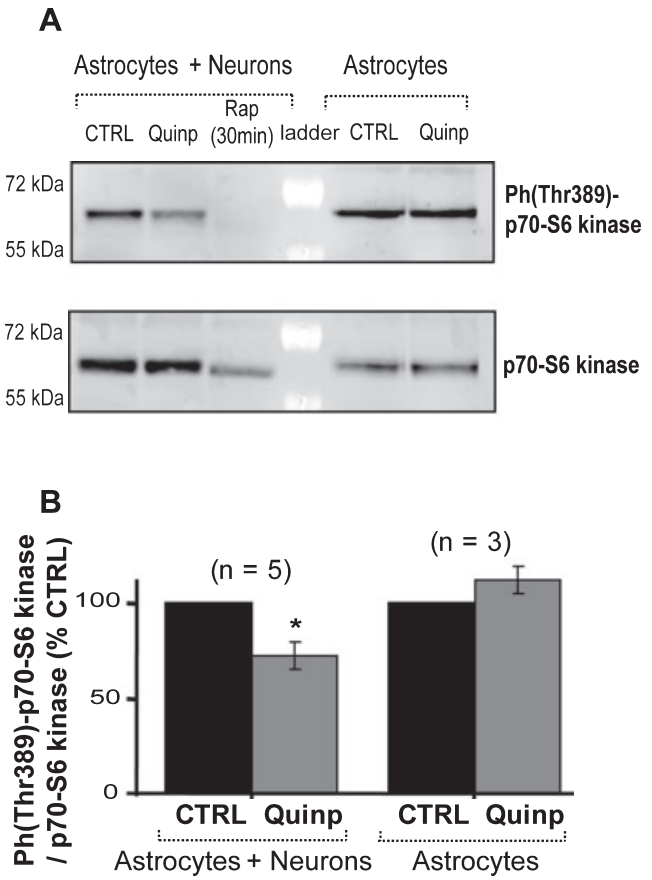


FIG. 6. Chronic activation of D2R decreases the activity of p70-S6 kinase in mesencephalic neurons. (A) Western blot analysis of the phosphorylation state of p70-S6 kinase measured in protein extracts prepared from cultured DA neurons. Chronic activation of the D2R with quinpirole (Quinp) (1 μM) decreased the phosphorylation of p70-S6 kinase at the 389 threonine residue [Ph(Thr389)-p70-S6 kinase] (left). The same chronic activation had no effect on p70-S6 kinase measured in pure astrocyte cultures (right). A 30 min perfusion with rapamycin [Rap (30 min)], used as a positive control (CTRL), strongly reduced p70-S6 kinase phosphorylation. (B) Histogram summarizing western blot densitometry. The data are presented as the ratio of phosphorylated p70-S6 kinase to total p70-S6 kinase. $*P < 0.05$ compared with control.

known to activate mTOR (Hay & Sonenberg, 2004). Through such signalling pathways, D2R activation leads to a wide range of biological effects (Bozzi & Borrelli, 2006). In the present study, we demonstrated that, when PKA was blocked with Rp-cAMP or H-89, chronic activation of the D2R failed to induce any decrease in synapse number. This indicates that PKA inhibition is likely to be the major pathway involved in the decrease in synaptogenesis induced by D2R activation. Although complementary experiments with a PKA activator such as forskolin could also have been performed, an eventual reduction by quinpirole of the ability of forskolin to increase synapse formation could have been mediated by an independent signalling pathway. On the contrary, our use of a PKA antagonist allowed us to show not only that PKA inhibition by itself mimicked the effect of the D2 agonist but also that prior inhibition of PKA occluded the effect of quinpirole, thus suggesting that the PKA cascade is a necessary final common pathway. In agreement with our finding, Tojima *et al.* (2003) demonstrated that treatment with H-89 decreased the number of presynaptic-like varicosities established by NG108-15 cells, a neuron-like cell line. PKA activity is also known to be involved in developmental processes such as growth cone guidance (Song *et al.*, 1997; Ming *et al.*, 2001) and in the regulation of synaptic function and structure (Frey *et al.*, 1993; Bozdagi *et al.*, 2000; Bradshaw *et al.*, 2003). Both PKA activity and protein synthesis are involved in synaptogenesis occurring during the late phase of long-term potentiation in the hippocampus *in vitro* (Pita-Almenar *et al.*, 2006). More recently it has been demonstrated that repeated activation of PKA induces a long-lasting (> 2 weeks) increase in the number of synaptic sites in primary hippocampal neuron cultures (Yamamoto *et al.*, 2005) and in hippocampal slices (Urakubo *et al.*, 2006).

In the present report, we also showed that activation of the D2R leads to a decrease in the phosphorylation levels of S6 kinase (p70-S6 kinase), a target of mTOR. Furthermore, the mTOR inhibitor rapamycin prevented D2R-mediated inhibition of synapse number. These observations suggest that chronic activation of the D2 autoreceptor decreases synapse number through inhibition of mRNA translation. mTOR is known to be regulated by many factors, such as nutrients and growth factors, and is involved in a number of physiological mechanisms including neuronal development, macroautophagy, synaptic plasticity, memory and learning (Larsen & Sulzer, 2002; Hay & Sonenberg, 2004; Parsons *et al.*, 2006; Ravikumar *et al.*, 2006; Swiech *et al.*, 2008). Recent studies have demonstrated that mTOR acts as an effector of brain-derived neurotrophic factor in its regulation of dendrite arborization and dendritic spines in organotypic and primary cultures of hippocampal neurons (Jaworski *et al.*, 2005; Kumar *et al.*, 2005). We found that chronic activation of the D2R induced an inhibition of 27 ± 7% of p70-S6 kinase phosphorylation in standard culture conditions. This represents a strong inhibition considering that our primary cultures contained approximately 30% of D2-responsive DA neurons. A direct regulation of p70-S6 kinase activity by the D2R is the simplest interpretation of our results. However, we cannot exclude an effect of quinpirole on some non-dopaminergic neurons expressing D2Rs present in the culture or the possibility of indirect effects secondary to the decrease in terminal number and DA release. However, we have previously provided evidence showing that GABAergic neurons present in these cultures are not responsive to D2R agonists (Congar *et al.*, 2002), thus making a contribution of this cell type unlikely. Additional studies are required to evaluate possible pathways linking D2R activation to p70-S6 kinase activity. Nevertheless, taken together, our findings of an occlusion of the effect of the D2R agonist on synaptogenesis by rapamycin and of the inhibition of p70-S6 kinase by this same treatment support the hypothesis that D2Rs inhibit synaptogenesis through inhibition of the mTOR-p70-S6 kinase pathway. How the cAMP/PKA and mTOR/p70-S6 kinase pathways converge in DA neurons is a major question that will have to be investigated in the future. Previous data in the literature suggest the existence of cross-talk between these two pathways. It has been demonstrated in Wistar rat thyroid cells, rat Schwann cells, Swiss 3T3 fibroblasts and feline cardiocytes (Cass & Meinkoth, 1998; Cass *et al.*, 1999; Iijima *et al.*, 2002) but not in NIH3T3 and REF52 fibroblasts (Cass & Meinkoth, 1998) that activation of the cAMP/PKA pathway can activate p70-S6 kinase in a rapamycin-dependent manner. Also Zhang *et al.* (2006) demonstrated that PKA inhibition potentially inhibits the enzymatic activity of p70-S6 kinase. Welsh *et al.* (1998) showed that acute D2R activation in transfected Chinese hamster ovary cells increases the phosphorylation state of p70-S6 kinase. The difference between our results and those obtained in Chinese hamster ovary cells could be due to a number of reasons. However, a major difference is that, in our study, the D2R was activated chronically, whereas in the study of Welsh *et al.* (1998), D2R was activated acutely. In addition, it is important to consider that the signalling pathways activated by the D2R in various *in-vitro* cell lines are variable and may be partly

different from those activated by native D2R expressed in DA neurons. For example, activation of the D2R in Chinese hamster ovary cells (Hayes *et al.*, 1992) and in murine Ltk fibroblast cells (Liu *et al.*, 1992) leads to a mobilization of intracellular calcium, whereas D2R activation has an opposite effect in DA neurons (Jomphe *et al.*, 2006). Further experiments will be necessary to identify the specific mechanisms by which the D2R, through PKA and the mTOR pathway, inhibits synapse number in DA neurons. Synapse formation is a developmental mechanism requiring protein synthesis (Schacher & Wu, 2002). Considering that protein translation can happen locally in axons and presynaptic sites (reviewed in Giuditta *et al.*, 2002), and that local protein synthesis in axonal growth cones undergoing regeneration is mTOR dependent (Verma *et al.*, 2005), it might be possible that D2R activation decreases the local translation of proteins critical for growth cone formation or stability. However, a parallel regulation of gene transcription cannot be excluded. The identity of mRNAs whose translation is inhibited in response to D2R activation also remains to be determined. Interestingly, in primary cultures of mouse embryonic midbrain neurons, chronic activation of the D2R decreases the expression of synapsin II, a phosphoprotein involved in both neurotransmitter release and synapse formation (Chong *et al.*, 2006). Whether this is involved in the decrease in synapse number reported here remains to be tested.

The decrease in synapse number is the most likely cause of the decrement in DA release that we observed in response to chronic D2R activation. However, the decrease in DA release could also be explained in part by a reduction in the abundance or activity (Lindgren *et al.*, 2003) of TH, leading to decreased synthesis of DA. Although we did not measure TH enzymatic activity, we found no evidence of a decrease in the abundance of TH protein. A chronic decrease in the firing rate of DA neurons could also be implicated in part but the lack of change in the levels of TH also argues against this possibility, considering the well-established activity dependence of the synthesis of TH in neurons (Guo *et al.*, 1998). Additional studies will be required to further elucidate the multiple changes that are likely to be induced in DA neurons by chronic D2R activation. Nevertheless, the present study represents an important step in this direction.

Chronic activation of the D2R occurs during treatment with psychostimulants such as in the treatment of attention deficit disorders (Greydanus, 2005). Chronic activation is also likely to occur in the context of drug addiction to amphetamines or cocaine. The consequences of such chronic activation of the D2R are not presently established. However, this study suggests that this may lead to structural plasticity of DA neurons, which might mediate some of the adaptive and maladaptive changes that accompany therapeutic drug response as well as drug dependence.

## Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Confirmation of the involvement of PKA in D2R-mediated reduction in axon terminal number.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

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## Abbreviations

D2R, D2 receptor; DA, dopamine; H-89, *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride; mTOR, mammalian Target of Rapamycin; NSS, normal saline solution; PKA, protein kinase A; Rp-cAMP, Rp-adenosine-3′,5′-cyclic monophosphorothioate; SDS, sodium dodecyl sulphate; SN, substantia nigra; SV2, synaptic vesicle protein 2; TH, tyrosine hydroxylase.

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