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MOLECULAR AND DEVELOPMENTAL NEUROSCIENCE

Chronic activation of the D2 autoreceptor inhibits both glutamate and dopamine synapse formation and alters the intrinsic properties of mesencephalic dopamine neurons *in vitro*

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Abstract

Dysfunctional dopamine (DA)-mediated signaling is implicated in several diseases including Parkinson's disease, schizophrenia and attention deficit and hyperactivity disorder. Chronic treatment with DA receptor agonists or antagonists is often used in pharmacotherapy, but the consequences of these treatments on DA neuron function are unclear. It was recently demonstrated that chronic D2 autoreceptor (D2R) activation in DA neurons decreases DA release and inhibits synapse formation. Given that DA neurons can establish synapses that release glutamate in addition to DA, we evaluated the synapse specificity of the functional and structural plasticity induced by chronic D2R activation. We show that chronic activation of the D2R with quinpirole *in vitro* caused a parallel decrease in the number of dopaminergic and glutamatergic axon terminals. The capacity of DA neurons to synthesize DA was not altered, as indicated by the lack of change in protein kinase A-mediated Ser(40) phosphorylation of tyrosine hydroxylase. However, the spontaneous firing rate of DA neurons was decreased and was associated with altered intrinsic properties as revealed by a prolonged latency to first spike after release from hyperpolarization. Moreover, D2R function was decreased after its chronic activation. Our results demonstrate that chronic activation of the D2R induces a complex neuronal reorganization involving the inhibition of both DA and glutamate synapse formation and an alteration in electrical activity, but not in DA synthesis. A better understanding of D2R-induced morphological and functional long-term plasticity may lead to improved pharmacotherapy of DA-related neurological and psychiatric disorders.

Introduction

Synapse formation occurs during development and its regulation throughout life determines the function of neural circuits and behavior. A dysfunction of dopamine (DA) signaling is thought to be associated with a number of neurological and psychiatric diseases including Parkinson's disease, schizophrenia and attention deficit and hyperactivity disorder (ADHD). Therapies using antipsychotics and psychostimulants aim to modulate DA concentration in the brain in order to re-equilibrate its neurotransmission. For example, to treat ADHD, the principal strategy consists of inhibiting catecholamine reuptake with low-dose psychostimulants, leading to an increase in the extracellular concentration of DA and norepinephrine (reviewed in Wilens, 2008; Heal *et al.*, 2009) and also to an increase of catecholaminergic receptor activation (reviewed in Quinn, 2008; Dopheide & Pliszka, 2009). Although acute D2 autoreceptor (D2R) activation leads to a well-known decrease in electrically evoked DA release (L'hirondel *et al.*, 1998; Benoit-Marand *et al.*, 2001; Schmitz *et al.*, 2002), the impact on DA neurons of chronic DA receptor stimulation remains poorly understood. *In vivo* studies in rats and mice have shown that the chronic blockade of D2R regulates the size of the terminal arbor in DA neurons of the substantia nigra (Parish *et al.*, 2002; Tripanichkul *et al.*, 2003). Using an *in vitro* microculture model in which the axon terminals of single DA neurons can be examined, we recently demonstrated that chronic activation of the D2R in DA neurons inhibits synapse formation and decreases DA release without inhibiting the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme of DA synthesis (Fasano *et al.*, 2008a). Hence, chronic D2R activation directly induces morphological and functional changes in DA neurons.

It is now well established that mesencephalic DA neurons use glutamate as a co-transmitter (see reviews by Trudeau & Gutierrez, 2007; Descarries *et al.*, 2008). They express the vesicular glutamate transporter 2 (VGLUT2) *in vivo* (Dal Bo *et al.*, 2008; Mendez *et al.*, 2008; Birgner *et al.*, 2010) as well as *in vitro* (Rayport, 2001; Dal Bo *et al.*, 2004; Mendez *et al.*, 2008) in a subset of their axon terminals. Because prior work (Fasano *et al.*, 2008a) quantified synapse number based on the expression of the ubiquitous synaptic vesicle protein 2 (SV2) (Yao & Bajjalieh, 2008; Chang & Sudhof,

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2009), the synapse-specificity of D2R-mediated inhibition of synaptogenesis in DA neurons remains undetermined. It would thus be critical to determine specifically whether chronic activation of the D2R causes a parallel decrease in the number of dopaminergic and glutamatergic axon terminals.

In the present study, we thus investigated whether chronic activation of the D2R causes a parallel decrease in the number of both dopaminergic and glutamatergic axon terminals *in vitro*. We also evaluated the impact of this treatment on DA neuron function by investigating the regulation of TH phosphorylation and the electrical activity of DA neurons.

Methods

Primary cultures of mesencephalic dopamine neurons

All experiments were performed in accordance with the Université de Montréal animal ethics committee guidelines. Primary cultures prepared from postnatal day 0 to postnatal day 2 pups of the transgenic mouse line TH-EGFP/21-31 carrying the enhanced green fluorescent protein (eGFP) gene under the control of the TH promoter (kindly provided by Dr K. Kobayashi; see Sawamoto *et al.*, 2001; Matsushita *et al.*, 2002; Roberts *et al.*, 2009). In this transgenic mouse line, DA neurons expressing eGFP present the same electrophysiological properties as wild-type DA neurons (Jomphe *et al.*, 2005). Primary cultures of substantia nigra and ventral tegmental area neurons were prepared as standard mass cultures (see Fig. S1A and B) or as microcultures in which single isolated DA neurons can be studied (Fig. S1C and D) (Fasano *et al.*, 2008a,b).

After cryoanesthesia, brains of the mice were delicately harvested. A mesencephalic slice was cut by hand and a piece of tissue containing the substantia nigra and ventral tegmental area was isolated. Tissue was digested with papain and cells were mechanically dissociated. Briefly, for microcultures, the mesencephalic cell suspension containing neurons was plated at 80 000 living cells/mL onto astrocyte micro-islands grown on collagen/poly-L-ornithine pre-coated glass coverslips (Ted Pella Inc., Redding, CA, USA). Under these conditions, individual isolated DA neurons establish synaptic contacts, called 'autapses', onto their own cell body and dendritic processes allowing for quantification of morphologically identifiable axon terminals established by individual DA neurons. For standard cultures, a mesencephalic cell suspension at 240 000 living cells/mL was plated on cortical astrocytes grown on collagen/poly-L-lysine pre-coated glass coverslips. 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). A detailed protocol for both types of cultures is available in Fasano *et al.* (2008b).

Pharmacological treatments

Over a period of 10 days, young DA neurons in development were treated chronically (every 2 days) with a specific D2R agonist, quinpirole (1 μM), or with vehicle (water). Analyses were performed on the day 11. The effect of such a chronic treatment on DA neuron survival was evaluated by counting the total number of DA neurons per coverslip in standard cultures and microcultures. In standard cultures, coverslips contained 530 ± 99 and 506 ± 84 DA neurons in control and quinpirole-treated groups, respectively ($n = 9$ in each groups, $t = 0.183$, $P = 0.852$, Fig. S1E). In microcultures, coverslips contained 9.8 ± 1.9 and 11.2 ± 1.7 DA neurons in control and quinpirole treated groups, respectively ($n = 10$ in each groups, $t = 0.536$, $P = 0.598$, Fig. S1F). This analysis indicated that chronic D2R activation did not affect DA neuron survival in either culture system.

Immunocytochemistry

Cells were fixed for 20 min in 4% paraformaldehyde in PBS (pH 7.4), permeabilized with 0.1% Triton X-100 (25 min) and nonspecific binding was blocked with a solution containing 5% normal goat serum and 0.5% bovine serum albumin (5 min). Cells were incubated overnight at 4 °C with primary antibodies: rabbit polyclonal anti-TH (1 : 5000, Chemicon, Temecula, CA, USA, cat. no. AB152), mouse monoclonal anti-TH (1 : 5000, Sigma-Aldrich, St Louis, MO, USA, cat. no. T1299), rabbit monoclonal Ser(40)-phospho-TH (1 : 100, Cell Signaling, Danvers, MA, USA, cat. no. 2791), mouse monoclonal anti-vesicular glutamate transporter 2 (anti-VGluT2, 1 : 1000, Synaptic Systems, USA, cat. no. 135611) or rabbit polyclonal anti-vesicular monoamine transporter 2 (anti-VMAT2, 1 : 1000, Chemicon, cat. no. AB1767). The cells were then incubated for 1 h at room temperature with mouse Alexa-fluor 488- or 647-conjugated secondary antibodies (1 : 200, Molecular Probes Inc., Eugene, OR, USA, cat. no. A-11001 and A-21235, respectively) and rabbit Alexa-fluor 488-, 546- or 647-conjugated secondary antibodies (1 : 200, Molecular Probes Inc., cat. no. A-11008, A-11010 and A-21244, respectively), according to each experimental design. Coverslips were finally mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA).

Imaging

Confocal microscopy was used to quantify morphologically identifiable axon terminals per single DA neuron in microcultures. Observations were made with a 40×, 0.75 NA objective mounted on an inverted Olympus IX50 microscope (Olympus Canada Inc., Markham, ON, Canada). A z-series projection of 5–10 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 performed according to previously described criteria (Fasano *et al.*, 2008a).

The relative level of TH activity was estimated by quantifying immunofluorescence images acquired with an inverted Nikon Eclipse TE-200 fluorescence microscope (Nikon Canada, Montréal, QC, Canada). Images were captured using a Hamamatsu Orca-II digital-cooled CCD camera (Hamamatsu, Bridgewater, NJ, USA) and an Olympus workstation using the Image-Pro Plus 6.2 software suite (Olympus Canada Inc.). Fluorescence excitation was controlled through the ScopePRO module of Image-Pro Plus and a DG4 xenon lamp (Sutter Instruments, Novato, CA, USA). Fluorescence was collected after passing through 510/550-nm and 575/645-nm band-pass emission filters. Pictures were analysed using Image J software (National Institutes of Health, NIH, Bethesda, MD, USA): total intensity of signals from Ser(40)-phospho-TH and total TH were measured and the ratio of Ser(40)-phospho-TH to total TH intensity was calculated. In microcultures, images of each isolated DA neurons were acquired, whereas in standard cultures, nine randomly chosen fields were averaged for each coverslip.

All experiments were performed blindly from data acquisition up to data analysis.

Electrophysiology

Standard cultures 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 eGFP-expressing neurons were selected for patch-clamp recordings to ensure that the vast majority were indeed dopaminergic (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 saline solution consisting of (in mM): NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, sucrose 6, glucose 10, HEPES 10 (305–310 mOsm and pH adjusted to 7.35). Spontaneous activity was recorded with a Warner PC-505 patch-clamp amplifier (Warner Instruments Corp., Hamden, CT, USA) using PClamp 9 software (Axon Instruments, Union City, CA, USA). Borosilicate glass patch pipettes (5–7 MΩ; World Precision Instruments Inc., Sarasota, FL, USA) were filled with a potassium methylsulfate intrapipette solution consisting of (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 and osmolarity 295–300 mOsm. A neuron was judged to be satisfactorily recorded when its resting membrane potential was at or below −39 mV and when it exhibited action potentials above 0 mV. Membrane resistance was evaluated by injection of a 50-pA negative current through the recording pipette: the hyperpolarization observed after the negative step current was measured and the membrane resistance was calculated according to Ohm's law. It should be noted that the membrane resistance values observed in the present experiments, in the range 900–1000 MΩ, are notably higher than values typically observed in DA neurons in brain slices; the reason for this discrepancy is unknown, but could be due in part to the impact of culture conditions. The intrinsic properties of DA neurons were evaluated by current-clamp recordings: neurons were clamped at −80 mV and when the clamp was released, the neurons were free to return to their membrane resting potential and to fire action potentials. Two parameters that are known to provide an index of A-type potassium current function were measured: the latency of reappearance of the first action potential and the delay between the first and the second action potentials (interspike interval) (Hahn *et al.*, 2006). These measurements were taken with Clampfit 9.0 software (Axon Instruments). Analysis of the firing rate was performed with Mini Analysis software v.5.6 (Synaptosoft Inc., Leonia, NJ, USA). D2R sensitivity was analysed by quantifying the ability of the D2R agonist quinpirole (1 μM) to transiently decrease neuron firing rate. For this series of experiments, 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. Using these criteria, the number of rejected neurons was similar in all experimental groups (three of 18 for the control group and five of 20 for the quinpirole-treated group). It should be noted that for all experiments evaluating the impact of chronic treatment with quinpirole, neurons were removed from the quinpirole-containing medium prior to the recordings (or other measurements), resulting in a state of acute withdrawal from chronic quinpirole treatment.

Statistical analysis

All values in the text and figures are expressed as mean ± SEM. Statistical analysis between groups was performed by using a *t*-test or ANOVA (one- or two-way), followed by a Tukey post-hoc test, as appropriate. Values were taken to be significantly different at $P < 0.05$.

Results

Chronic D2R activation reduces the number of both dopaminergic and glutamatergic axon terminals

In primary neuron microcultures, the number of dopaminergic and glutamatergic axon terminals established was quantified in single isolated DA neurons making autapses. The dopaminergic phenotype of isolated neurons was determined by TH immunostaining (Figs 1A and 2A). Dopaminergic and glutamatergic axon terminals were identified by immunostaining for VMAT2 (Fig. 1C and D) and VGLUT2 (Fig. 2C and D), respectively. Under control conditions, isolated DA neurons established 285 ± 32 VMAT2-positive axon terminals ($n = 18$, Fig. 1E). In comparison, neurons treated with the D2R agonist quinpirole (1 μM) five times, every second day for 10 days, established 219 ± 21 VMAT2-positive terminals ($n = 33$), representing a mean decrease of 23 ± 7% ($t = 2.570$, $P < 0.05$). Under control conditions, isolated DA neurons established 186 ± 24 VGLUT2-positive axon terminals ($n = 23$, Fig. 2E). In comparison, neurons treated with the D2R agonist quinpirole (1 μM) five times, every second day for 10 days, established 130 ± 17 VGLUT2-positive terminals ($n = 15$), representing a mean decrease of 30 ± 9% ($t = 4.269$, $P < 0.05$).

These data demonstrate that chronic activation of the D2R inhibits synaptogenesis of both dopaminergic and glutamatergic terminals in mesencephalic DA neurons.

Chronic D2R activation does not lead to a long-term decrease in TH activity

We have shown that the inhibition of axon terminal number induced by chronic D2R activation was mediated through inhibition of the protein kinase A (PKA) pathway, a common intracellular target of D2R (Greengard *et al.*, 1999; Lee *et al.*, 2004). PKA is known to phosphorylate TH, the rate-limiting enzyme of DA synthesis at its Ser(40) residue (Harada *et al.*, 1996; Jedynak *et al.*, 2002), leading to a decrease in its catalytic activity and in the synthesis of DA. Hence, a long-term decrease in TH activity could participate in the previously reported decrease of extracellular DA after chronic D2R activation (Fasano *et al.*, 2008a) and could also regulate the electrical activity of DA neurons through the negative feedback induced by the activation of D2Rs (Jomphe *et al.*, 2005).

Although TH activity is commonly measured by Western blot analysis and the quantification of Ser(40)-TH phosphorylation (Fulton *et al.*, 2006; Salvatore *et al.*, 2009), protein levels are not high enough for this approach in microcultures. We therefore used an immunofluorescence approach and quantified the ratio of Ser(40)-phospho-TH to total TH immunoreactivity. We first validated the suitability of this method in standard cultures by testing the effect of drugs known to change the state of phosphorylation of TH at its Ser(40) residue. We found a significant effect of treatments ($F_{3,20} = 94\,017$, $P < 0.001$, one-way ANOVA): whereas activation of PKA with forskolin (100 μM for 30 min) significantly increased the ratio of Ser(40)-phospho-TH to total TH to 291 ± 51% of control ($n = 6$ coverslips, $P < 0.001$), inhibition of PKA using (R)-Adenosine, cyclic 3′,5′-(hydrogenphosphorothioate) triethylammonium (Rp-AMPc) (50 μM, for 30 min) or indirectly with an acute application of quinpirole (1 μM for 15 min) significantly decreased this ratio to 41 ± 9% of control ($n = 6$ coverslips, $P < 0.05$) and 39 ± 4% of control ($n = 8$ coverslips,

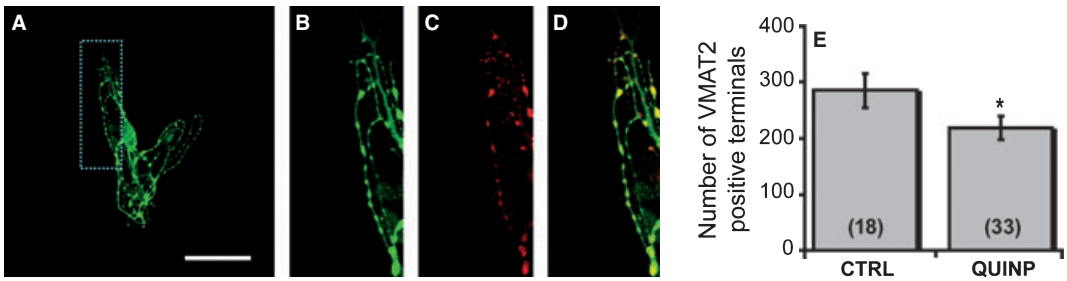

FIG. 1. Chronic activation of the D2 autoreceptor decreases the number of dopaminergic axon terminals established by DA neurons. (A–D) Identification of dopaminergic terminals established by isolated DA neurons in micro-island culture. Scale bar = 50 μm. (A) TH immunostaining (green) of an isolated postnatal 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 VMAT2 (red) identifies the location of dopaminergic terminals. (D) Merged image of TH and VMAT2 immunostaining. (E) Quantification of dopaminergic terminals in isolated DA neurons. Chronic activation of the D2R with 1 μM quinpirole (QUINP) significantly decreased the number of morphologically identifiable dopaminergic terminals. *$P < 0.05$; numbers inside histogram bars indicate the number of DA neurons analysed. This presentation format is used for all histograms presenting an analysis of single DA neurons in microculture.

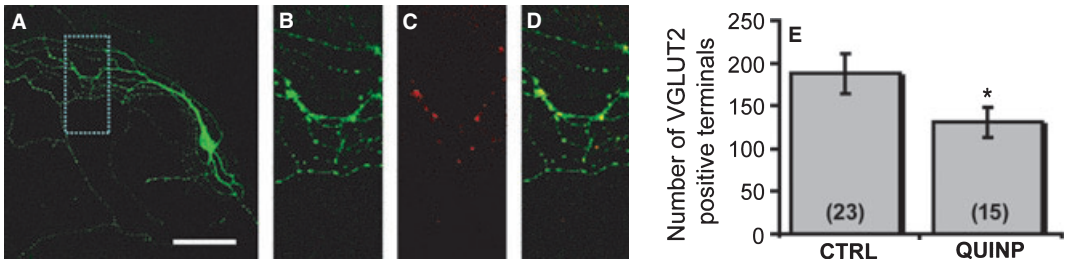

FIG. 2. Chronic activation of the D2 autoreceptor decreases the number of glutamatergic terminals established by DA neurons. (A–D) Identification of glutamatergic terminals established by isolated DA neurons in micro-island culture. Scale bar = 50 μm. (A) TH immunostaining (green) of an isolated postnatal mesencephalic neuron identifying the neuron 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 VGLUT2 (red) identifies the location of glutamatergic terminals. (D) Merged image of TH and VGLUT2 immunostaining. (E) Number of glutamatergic terminals in isolated DA neurons. Chronic activation of the D2R with 1 μM quinpirole significantly decreased the number of morphologically identifiable glutamatergic terminals. *$P < 0.05$.

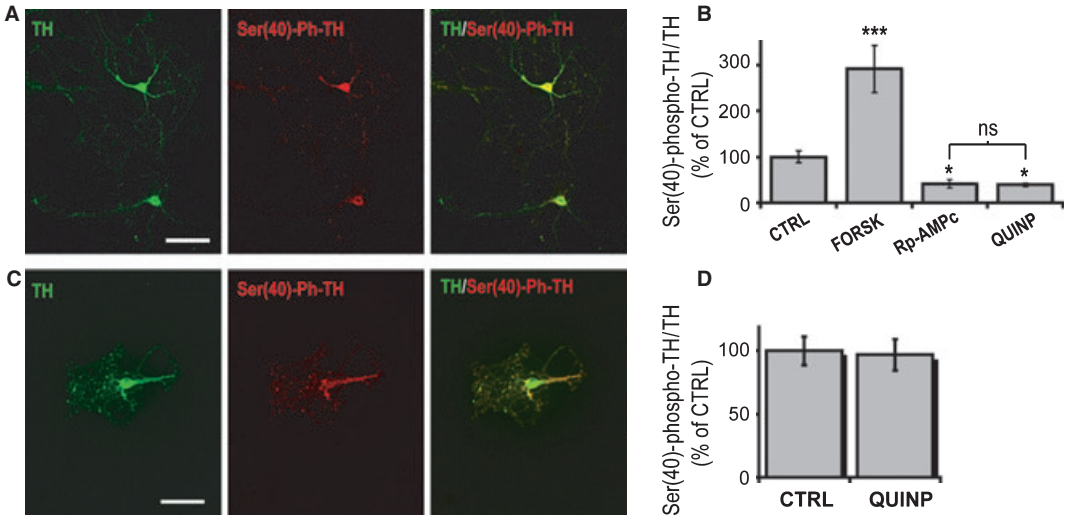

FIG. 3. The basal phosphorylation state of TH remains unchanged after chronic activation of the D2 autoreceptor. (A) TH (green) and Ser(40)-phospho-TH (red) immunostaining of neurons in standard mass culture. This merged image shows the specificity for DA neurons of the antibody directed against the Ser(40) residue of TH. (B) Histogram showing the relative phosphorylation state of TH at its Ser(40) residue after direct PKA activation with forskolin (FORSK) and direct PKA inhibition with Rp-AMPc or indirectly with quinpirole (QUINP). Forskolin (100 μM for 30 min) significantly increased the ratio of Ser(40)-phospho-TH to total TH ($n = 6$ coverslips) whereas Rp-AMPc (50 μM for 30 min) or quinpirole (1 μM for 15 min) significantly decreased the ratio ($n = 6$ and 8 coverslips, respectively). (C) TH and Ser(40)-phospho-TH immunostaining of an isolated DA neuron in microculture. (D) Histogram showing the relative phosphorylation state of TH at its Ser(40) residue after chronic treatment of isolated DA neurons in microcultures with quinpirole. Chronic activation of the D2R with 1 μM quinpirole was without effect. *$P < 0.05$ and ***$P < 0.001$ compared with the control group (CTRL); ns, not statistically different. Scale bar = 50 μm.

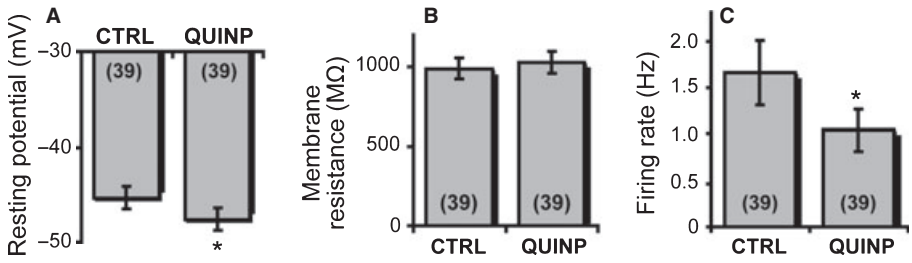

FIG. 4. Effect of chronic D2R activation on the resting potential and firing rate of DA neurons. (A) The resting membrane potential of DA neurons in standard cultures is decreased after chronic treatment with 1 μM quinpirole. (B) The membrane resistance of DA neurons was unaffected by the chronic treatment. (C) Chronic activation of D2Rs with quinpirole triggered a potent reduction of the spontaneous firing rate of DA neurons. *$P < 0.05$.

$P < 0.05$), respectively (Fig. 3A and B). These results thus confirm the suitability of this approach to detect the regulation of TH activity by PKA. In the microculture model, the Ser(40)-phospho-TH to total TH ratio measured in isolated DA neurons chronically treated with quinpirole was 97 ± 12% ($n = 45$) of the ratio measured in the control group ($n = 43$, $t = 0.192$, $P = 0.848$) (Fig. 3C and D). This result demonstrates that chronic D2R activation with quinpirole did not alter the basal level of TH activity. Combined with our previous demonstration that total TH expression is unchanged after chronic D2R activation, the present observation argues that the ability of neurons to synthesize DA is not modified by the chronic agonist treatment.

DA neuron spontaneous firing is reduced after chronic D2R activation

In addition to changes in the number of axon terminals, the quantity of DA released by DA neurons should be highly sensitive to the neurons' excitability and basal firing rate. Because the spontaneous firing rate of isolated DA neurons in microculture is low and unstable due to the formation of autapses, these recordings were performed in standard mass cultures. A total of 39 eGFP-expressing DA neurons were recorded. The resting membrane potential of DA neurons was −45.3 ± 1.2 mV in the control group and −47.6 ± 1.2 mV in DA neurons treated chronically with quinpirole (1 μM), representing a small but statistically significant hyperpolarization ($t = 2.029$, $P < 0.05$, Fig. 4A). The membrane resistance, calculated from a hyperpolarizing current injected through the recording pipette, was 987 ± 65 and 1025 ± 71 mΩ in control and quinpirole-treated groups, respectively. The difference was not statistically significant ($t = 0.580$, $P = 0.508$, Fig. 4B). Under control conditions, DA neurons fired spontaneously at a rate of 1.67 ± 0.35 Hz whereas their firing rate was significantly decreased to 1.04 ± 0.23 Hz after chronic D2R activation ($t = 2.189$, $P < 0.05$, Fig. 4C). Thus, their spontaneous firing rate was reduced by 42%. These results show that chronic activation of D2R with quinpirole affects the electrical membrane properties of DA neurons: their resting membrane potential was hyperpolarized and their firing rate was inhibited, but their membrane resistance remained unchanged.

This inhibition in the spontaneous firing rate of DA neurons could result from a number of mechanisms, including a reorganization in the neuronal network of the cultured DA neurons. It could also result more directly from a change in the intrinsic properties of these cells. To test this later possibility, we performed current-clamp recordings after chronic treatment with quinpirole and monitored recovery of firing after release from hyperpolarizing current injection initially maintaining neurons at −80 mV, an assay that is sensitive to changes in potassium A-type currents. Chronic treatment with quinpirole (1 μM) induced a statistically significant increase in both the latency to the first action potential and the first interspike interval after releasing neurons from current-clamp (Fig. 5A). The latency was 325 ± 65 ms for the control group ($n = 20$) and 769 ± 159 ms for the quinpirole-treated group ($n = 21$, $t = 2.528$, $P < 0.05$, Fig. 5B). The first interspike interval was 217 ± 35 and 604 ± 152 ms for the control and quinpirole-treated groups, respectively ($t = 2.429$, $P < 0.05$, Fig. 5B). These results are consistent with the hypothesis that chronic D2R activation can regulate the intrinsic electrical properties of DA neurons, perhaps through a enhancement of A-type potassium currents.

D2 receptors are desensitized after their chronic activation with quinpirole

In addition to intrinsic excitability, the spontaneous firing rate of DA neurons is also regulated by somatodendritic D2Rs that cause hyperpolarization through activation of G protein-activated inwardly rectifying K+ (GIRK) channels (Lacey *et al.*, 1987; Maslowski &

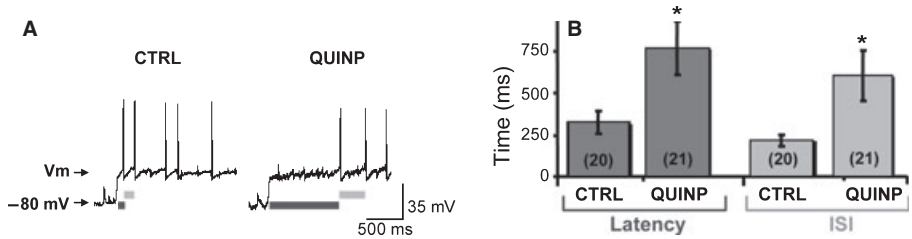

FIG. 5. Chronic D2R activation regulates the intrinsic properties of DA neurons. (A) Examples of current-clamp recordings in a control (left) and quinpirole-treated (right) DA neuron in standard mass culture. Neurons were maintained in current-clamp at −80 mV. Release of the clamp allowed DA neurons to return to their resting membrane potential and to resume spontaneous firing. Dark gray lines under the recordings indicate the latency prior to the first action potential. Light gray lines indicate the first interspike interval (ISI). (B) Histogram showing the quantification of the latency and first ISI in control and quinpirole-treated groups. After chronic treatment with quinpirole, both latency and ISI were increased. *$P < 0.05$.

Napier, 1991). Because it is known that D2Rs can become desensitized after prolonged agonist stimulation (Kim *et al.*, 2001; Bartlett *et al.*, 2005; Goggi *et al.*, 2007) or indirectly via the blockade of the DA transporter with methylphenidate (Thanos *et al.*, 2007; Hwang *et al.*, 2008), we next investigated whether the chronic treatment with quinpirole also induced a long-term desensitization of D2Rs in DA neurons. Using the patch-clamp technique in standard cultures, we first evaluated the inhibition of DA neuron firing rate induced by acute D2R activation with quinpirole. Also, in isolated DA neurons in microcultures, we evaluated the effect of brief D2R stimulation on the inhibition of TH phosphorylation state at its Ser(40) residue by immunofluorescence microscopy.

In standard cultures, when DA neurons presented a stable firing frequency, quinpirole was perfused in the recording chamber for 2 min, leading to a reduction in firing rate. Recovery of the basal firing frequency was observed after 5 min of washout. In control DA neurons, this brief activation of D2R by quinpirole decreased the firing rate by 49 ± 7% compared with the baseline ($t = 6.341$, $P < 0.001$, $n = 15$, Fig. 6A). In DA neurons chronically treated with quinpirole, the same perfusion with quinpirole still caused a significant decrease in firing rate, but only by 26 ± 9% compared with the baseline ($t = 3.663$, $P < 0.001$, $n = 15$). The acute effect of quinpirole on firing rate was thus reduced by 48% and was significantly less than in control cultures ($t = 2.205$, $P < 0.005$, Fig. 6A). During the washing period, the effect of quinpirole appeared to last longer in the quinpirole-treated group than in the control group, however, this was not a statistically significant difference ($t = 1.403$, $P = 0.177$). This result demonstrates that under our experimental conditions, chronic treatment with quinpirole decreased the ability of the D2R to regulate DA neuron firing rate.

To estimate desensitization in microcultures, cultures were exposed to quinpirole (1 μM) for 15 min directly in the culture medium and neurons were fixed for immunolabeling against Ser(40)-phospho-TH and total TH. In the control group, the ratio of Ser(40)-phospho-TH to total TH was decreased by 71 ± 29%, compared with vehicle, in response to this acute quinpirole application (Fig. 6B). In the chronically quinpirole-treated group, this ratio was only decreased by 36 ± 8% by the acute quinpirole treatment. Hence, the ability of quinpirole to decrease TH phosphorylation was inhibited by 50% ($t = 4.269$, $P < 0.01$) after chronic D2R activation. Together, these results show that in both standard and microcultures, the function of the D2R is inhibited after its chronic activation with quinpirole.

Discussion

In the present study we have demonstrated that chronic treatment with the D2R agonist quinpirole triggers multiple structural and functional

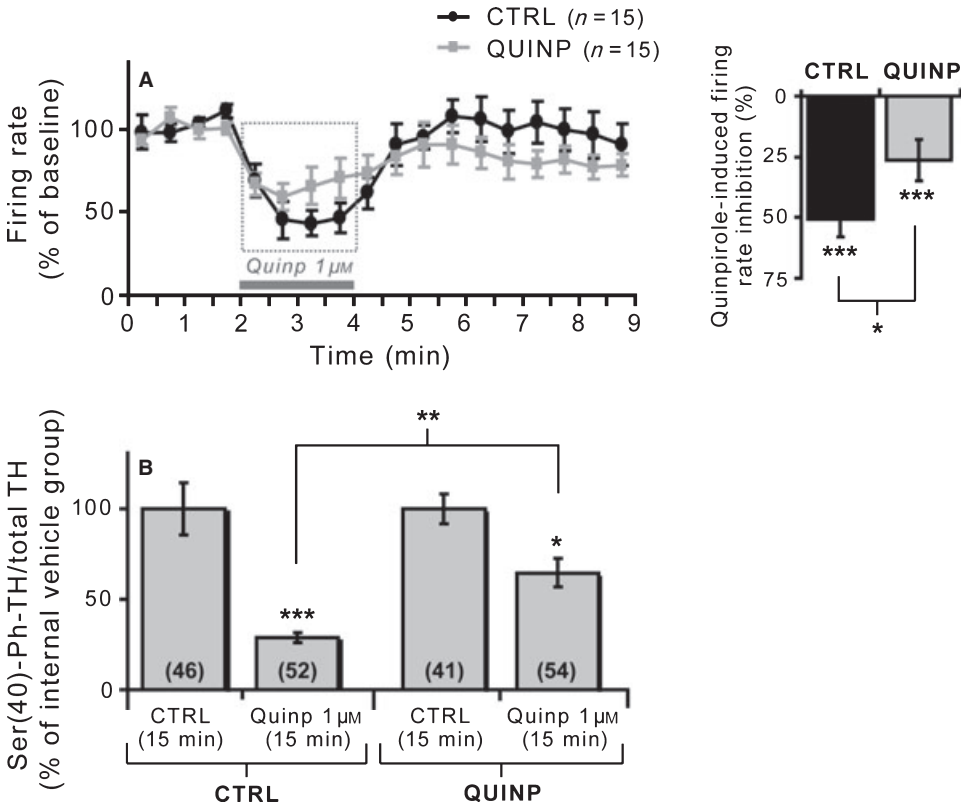

FIG. 6. Chronic treatment with the D2R agonist quinpirole inhibits the effects of acute D2R activation in DA neurons. (A) Patch-clamp whole-cell recordings of DA neurons in standard cultures. The graph (left section) shows the spontaneous firing rate as a function of time. Brief D2R activation by superfusion with quinpirole (1 μM) for 2 min produced a transient decrease in firing rate. Chronic treatment of neurons with 1 μM quinpirole decreased the ability of a brief quinpirole superfusion to inhibit firing rate. The histogram (right part) presents the mean inhibition of the firing rate during the 2 min of superfusion with 1 μM quinpirole in the control and quinpirole-treated groups. Chronic treatment with quinpirole significantly decreased the ability of acute quinpirole application to inhibit the firing rate of DA neurons. $*P < 0.05$ and $***P < 0.001$ compared with the baseline. (B) Quantification of the ratio of Ser(40)-Phospho-TH to total TH measured by immunofluoresence in isolated DA neurons in microcultures. D2R activation by adding quinpirole (1 μM) for 15 min in the culture medium before fixation decreased the phosphorylation state of TH at its Ser(40) residue. After chronic quinpirole treatment, short D2R activation with quinpirole for 15 min still inhibited the ratio of Ser(40)-Phospho-TH to total TH, although the magnitude of the effect was reduced compared with the control group ($**P < 0.05$). $*P < 0.05$ and $***P < 0.001$ compared with the internal vehicle group.

modifications in DA neurons: long-term D2R activation decreased the number of dopaminergic and glutamatergic synaptic terminals, reduced spontaneous firing rate, altered intrinsic membrane properties and induced partial desensitization of the D2R. However, there was no long-term change in the activity of TH.

Here we show that chronic D2R activation with quinpirole decreases the number of both dopaminergic and glutamatergic terminals established by DA neurons. We ensured that this was not due to a neurotoxic effect of the long-term treatment. Indeed, DA neuron survival in standard and in microcultures is not affected by the chronic treatment with quinpirole (see Fig. S1). A neurotoxic effect of chronic D2R activation was in any case unlikely, because the D2R is mostly known to exert neuroprotective effects (Sawada *et al.*, 1998; Kihara *et al.*, 2002; Marvanova & Nichols, 2007). Under our culture conditions, we did not observe any D2R agonist-mediated neuroprotection. This is consistent with the fact that such neuroprotection has only been described previously in an experimental context of stress-induced neurotoxicity (Sawada *et al.*, 1998; Kihara *et al.*, 2002; Marvanova & Nichols, 2007).

Several earlier studies have suggested that chronic D2R activation regulates DA neuron morphology and synapse formation. Indeed, chronic oral administration of a specific D2R agonist to rats and mice over several months induces a decrease in the size of the terminal arbour of substantia nigra pars compacta DA neurons (Parish *et al.*, 2001, 2002) whereas pharmacological blockade or genetic deletion of the D2R induced an increase in the terminal arbour volume (Parish *et al.*, 2002; Tripanichkul *et al.*, 2003). Whether these effects were direct or implicated postsynaptic receptors was, however, unclear. A direct, regulatory role of the D2R on synapse formation was recently demonstrated using an *in vitro* model of isolated DA neurons (Fasano *et al.*, 2008a). This study was performed by evaluating synapse number using a general synaptic vesicle marker, SV2, without considering the recently reported capacity of many DA neurons to establish VGLUT2-positive axon terminals that mediate glutamate release (Sulzer *et al.*, 1998; Dal Bo *et al.*, 2004; Mendez *et al.*, 2008; Yao & Bajjalieh, 2008; Birgner *et al.*, 2010), even in the presence of normal target neurons (Joyce & Rayport, 2000). Given that the glutamatergic phenotype of DA neurons is highly regulated by a number of physiological and pathological signals (Dal Bo *et al.*, 2008; Mendez *et al.*, 2008), it is possible that the previously reported reduction in axon terminal number induced by chronic D2R stimulation (Fasano *et al.*, 2008a) could have been due to a selective reduction in glutamatergic synapses established by DA neurons. In the present study, we found that chronic activation of the D2R in microcultures reduced the number of both types of terminals in the same relative proportion. This finding is in accordance with a previous electrophysiological study showing that the D2Rs regulate both DA and glutamate release by DA neurons in a similar fashion (Congar *et al.*, 2002). Thus, D2R-dependent inhibition of synapse formation in DA neurons is a general mechanism that presents no preference for the dopaminergic or glutamatergic phenotype of the terminals.

TH is the rate-limiting enzyme in the synthesis of DA. Its abundance and its activity directly regulate the quantity of DA synthesized and subsequently packaged in synaptic vesicles. Changes in the state of phosphorylation of TH critically regulate DA synthesis. When the Ser(40) residue of TH is specifically phosphorylated by PKA, TH activity is increased, which leads to an increased production of DA (Harada *et al.*, 1996; Jedynak *et al.*, 2002). We have previously demonstrated that chronic D2R activation decreased synapse formation through inhibition of PKA, but did not change the quantity of TH expressed in cultured DA neurons (Fasano *et al.*, 2008a). In the present study we extend these earlier findings by demonstrating that chronic D2R activation does not lead to a long-term reduction of TH phosphorylation at the Ser(40) residue. Hence, it is likely that basal DA synthesis is not affected by the chronic D2R agonist treatment.

DA neurons are typically spontaneously active *in vivo* (Mameli-Engvall *et al.*, 2006; Pillolla *et al.*, 2007) and *in vitro* (Werkman *et al.*, 2001; Appel *et al.*, 2006; Fasano *et al.*, 2008a). Tonic release of DA strongly relies on the basal firing rate of DA neurons. Here, we demonstrate that chronic activation of the D2R exerts a regulatory effect on the intrinsic electrophysiological properties of DA neurons, including a modest hyperpolarization of the resting membrane potential, a robust inhibition of spontaneous firing rate and a delay in recovery of firing upon release from hyperpolarization. Regulation of neuronal pacemaker activity by D2R activation is of major interest as antipsychotics and psychostimulants regulate DA neurotransmission by blocking this receptor (Walker *et al.*, 2006; Volz *et al.*, 2008; Roberts *et al.*, 2009). How long-term modulation of D2Rs regulates the electrical activity of DA neurons remains unclear. Indeed, it has been observed that chronic treatment with both D2R agonists or antagonists can trigger a decrease in the basal firing rate of DA neurons. *In vivo* delivery of a D2/D3 receptor agonist through an osmotic minipump in rats decreases the firing rate of DA neurons after 2 days of treatment, but recovery is observed after 14 days of treatment (Chernoloz *et al.*, 2009). In midbrain neuron cultures, chronic blockade of D2Rs with haloperidol, an antipsychotic, inhibits the firing rate of DA neurons (Hahn *et al.*, 2003, 2006) and decreases neuronal excitability via an upregulation of A-type potassium channels (Hahn *et al.*, 2006). In the present study, we observed a decrease in spontaneous firing (Fig. 4C) and a delay in recovery of firing upon release from hyperpolarization (Fig. 5) in neurons chronically treated with quinpirole. Given that the spontaneous firing rate of DA neurons strongly relies on A-type potassium currents (Putzier *et al.*, 2009), it may be hypothesized that as for haloperidol, chronic treatment with quinpirole upregulated A-type potassium channel expression, leading to the observed decrease in DA neuron pace-maker activity. Other channels, such as Ih, Im and calcium channels, also known to contribute to the excitability and pacemaker activity of DA neurons, could also be involved (Kang & Kitai, 1993; Seutin *et al.*, 2001; Liu *et al.*, 2003; Zhang *et al.*, 2005; Wanat *et al.*, 2008; Putzier *et al.*, 2009; Inyushin *et al.*, 2010). Whatever its mechanism, long-term changes in the firing rate of DA neurons could have extensive impact not only on DA release but also on the synaptic input integration by these neurons, as suggested by recent work using dynamic patch-clamp (Putzier *et al.*, 2009).

We found that chronic D2R activation triggered partial desensitization of D2R function in both standard cultures and microcultures. The agonist-dependent decrease of D2R sensitivity is in accordance with the literature (Kim *et al.*, 2001; Goggi *et al.*, 2007; Chernoloz *et al.*, 2009). A possible mechanism that could explain this reduced response after chronic activation is the interaction of D2Rs with G protein-coupled receptor-associated sorting protein (GASP), leading to degradation of the receptor (Bartlett *et al.*, 2005). Interestingly, the magnitude of inhibition of D2R function was in a similar range in standard and microcultures: 48 ± 16 and $50 \pm 11\%$, respectively (Fig. 6). This indicates that drug effects are direct and that DA neurons react very similarly to chronic D2R activation when they are isolated on microislands or when they are integrated in neuronal networks with postsynaptic targets. Although there may be differences between intercellular synapses and autapses, the microculture model offers a number of experimental advantages despite the fact that postsynaptic targets are absent. One such advantage is the ability to facilitate studies of synapse formation. This is particularly useful for neuronal populations, such as DA neurons, that express highly heterogeneous

properties. For example, prior studies performed with this microculture model have demonstrated that synaptogenesis by isolated DA neurons is sensitive to local environmental cues such as growth factors (Bourque & Trudeau, 2000) or other astrocyte-mediated signals (Forget *et al.*, 2006). This model has also been used to demonstrate that DA neurons express subsets of axon terminals that express VGLUT2 and can use glutamate as a co-transmitter (Dal Bo *et al.*, 2004).

In conclusion, the present study demonstrates that chronic activation of D2R induces profound and complex changes in DA neurons, including a decrease of both DA and glutamate axon terminal number and a reduction in cellular pacemaker activity. DA receptors in the brain are challenged chronically during pharmacotherapy of ADHD with psychostimulants or in the context of amphetamine or cocaine addiction. In future studies it would be intriguing to investigate whether psychostimulants or drugs of misuse induce a similar and overlapping pattern of structural and functional plasticity through repeated or prolonged D2R activation.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Primary culture of mesencephalic neurons and the effect of chronic treatment with quinpirole on DA neuron survival.

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Abbreviations

ADHD, attention deficit and hyperactivity disorder; D2R, D2 receptor; DA, dopamine; eGFP, enhanced green fluorescent protein; PKA, protein kinase A; SV2, synaptic vesicle protein 2; TH, tyrosine hydroxylase; VGLUT2, vesicular glutamate transporter 2; VMAT2, vesicular monoamine transporter 2.

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