Dopamine facilitates dendritic spine formation by cultured striatal medium spiny neurons through both D1 and D2 dopamine receptors

Caroline Fasano a, Marie-Josée Bourque a, Gabriel Lapointe b, Damiana Leo a, Dominic Thibault a, Michael Haber d, Christian Kortleven a, Luc DesGroseillers b,c, Keith K. Murai d, Louis-Éric Trudeau a,c,*

a Department of Pharmacology, Faculty of Medicine, Université de Montréal, Montréal, Québec, Canada
b Department of Biochemistry, Faculty of Medicine, Université de Montréal, Montréal, Québec, Canada
c Neuroscience Research Group, Faculty of Medicine, Université de Montréal, Montréal, Québec, Canada
d Centre for Research in Neuroscience, Department of Neurology and Neurosurgery, The Research Institute of the McGill University Health Centre, Montreal General Hospital, Montréal, Québec, Canada

Abstract

Variations of dopamine (DA) levels induced by drugs of abuse or in the context of Parkinson’s disease modulate the number of dendritic spines in medium spiny neurons (MSNs) of the striatum, showing that DA plays a major role in the structural plasticity of MSNs. However, little is presently known regarding early spine development in MSNs occurring before the arrival of cortical inputs and in particular about the role of DA and D1 (D1R) and D2 (D2R) DA receptors. A cell culture model reconstituting early cellular interactions between MSNs, intrinsic cholinergic interneurons and DA neurons was used to study the role of DA in spine formation. After 5 or 10 days in vitro, the presence of DA neurons increased the number of immature spine-like protrusions. In MSN monocultures, chronic activation of D1R or D2R also increased the number of spines and spinophilin expression in MSNs, suggesting a direct role for these receptors. In DA-MSN cocultures, chronic blockade of D1R or D2R reduced the number of dendritic spines. Interestingly, the combined activation or blockade of both D1R and D2R failed to elicit more extensive spine formation, suggesting that both receptors act through a mechanism that is not additive. Finally, we found increased ionotropic glutamate receptor responsiveness and miniature excitatory postsynaptic current (EPSC) frequency in DA-MSN co-cultures, in parallel with a higher number of spines containing PSD-95, suggesting that the newly formed spines present functional post-synaptic machinery preparing the MSNs to receive additional glutamatergic contacts. These results represent a first step in the understanding of how dopamine neurons promote the structural plasticity of MSNs during the development of basal ganglia circuits.

1. Introduction

Synapses are strategic, highly specialized sites for neuronal communication. Excitatory synaptic contacts are often located on specialized postsynaptic domains called dendritic spines. The number and the shape — thin, stubby and mushroom — of these dendritic spines are critical determinants of the level of input integration by neurons and of the function of neuronal circuits in the brain (McKinney, 2010). Persistent morphological changes of dendritic spines are commonly associated with synaptic plasticity and memory formation (Segal, 2010). For example, the number of dendritic spines is regulated under physiological conditions, such as motor learning in vivo (Xu et al., 2009; Yang et al., 2009). Alterations in spine shape and number are also found under pathological conditions, such as Alzheimer’s disease (Knobloch and Mansuy, 2008; Baloyannis, 2009), Parkinson’s disease (Solis et al., 2007) and schizophrenia (Glantz et al., 2000; Flores et al., 2005). In the striatum, the main population of intrinsic neurons, called medium spiny neurons (MSNs), bears a large number of spines on their dendrites. Glutamate released by cortico-striatal afferents, but also by cholinergic interneurons (Gras et al., 2002, 2008; Gras et al., 2008; El Mestikawy et al., 2011; Higley et al., 2011) and dopamine (DA) neurons (Sulzer et al., 1998; Dal Bo et al., 2004; Mendez et al., 2008; Stuber et al., 2010; Tecuapetla et al., 2010), activates postsynaptic ionotropic receptors located on the head of these MSN spine protrusions, whereas DA released by midbrain DA neurons activates
postsynaptic metabotropic receptors located on the neck of dendritic spines (Bouyer et al., 1984; Freund et al., 1984; Smith et al., 1994). This tripartite configuration, commonly known as the striatal synaptic triad (Dani and Zhou, 2004), is thought to allow for DA-mediated gating of cortico-striatal glutamatergic synaptic transmission (Calabresi et al., 1992; Levine et al., 1996; Cepeda et al., 2001; Kerr and Wickens, 2001; Paille et al., 2010). There is an increasing body of evidence showing that in addition to its acute role in regulating synaptic transmission, DA is also critical for the morphological integrity of dendritic spines in MSNs. Indeed, elevation of DA levels by drugs of abuse, such as cocaine and amphetamine, increases the number of dendritic spines in MSNs (Robinson and Kolb, 1997, 1999; Li et al., 2003; Lee et al., 2006; Singer et al., 2009), whereas a decrease in DA levels or loss of DA neurons in Parkinson’s disease models reduces the number of dendritic spines (Ingham et al., 1993; Solis et al., 2007; Garcia et al., 2010). However, little is presently known concerning the specific mechanisms linking DA receptor activation to spine formation and/or maintenance in MSNs. In particular, it is presently unclear if DA regulates spines by acting directly on DA receptors on MSNs, or whether it acts indirectly by regulating cortico-striatal glutamate release (Garcia et al., 2010). Ontogeny of the striatum, arrival of the dopaminergic innervation (Sprecht et al., 1981; Voon et al., 1988) and appearance of DA receptors (Goffin et al., 2010) all occur prior to birth in the rodent, whereas maturation of dendritic spines (Jakowec et al., 2001; Zhuravin et al., 2007) as well as striatal innervation by cortical afferents (Christensen et al., 1999; Inaji et al., 2011) are postnatal phenomena occurring during the second week after birth. Although the prior development of the DA system places it in a favourable condition to contribute to spine development, whether it does indeed play a role in early spine development in addition to regulating spine maintenance in the mature striatal synaptic triad is undetermined.

Here we used a primary culture model to investigate the role of DA in early MSN dendritic spine formation. We tested the hypothesis that co-culturing MSNs with DA neurons stimulates the formation of dendritic spines by MSNs.

2. Methods

2.1. Primary neuronal monocultures and co-cultures

All experiments were performed in accordance with the Université de Montréal animal ethics committee guidelines. All efforts were made to reduce the number of animals used and minimize animal suffering. Postnatal day 0 to postnatal day 2 pups from the transgenic mouse line TH-EGFP/21-31 carrying the enhanced green fluorescent protein (eGFP) gene under the control of the tyrosine hydroxylase promoter (Sawamoto et al., 2001; Matsuhashi et al., 2002) were used. DA neurons in these transgenic mice express eGFP and present the same electrophysiological properties as wild type DA neurons (Jomphe et al., 2005). For the MSN monocultures (called thereafter MSN cultures), we adapted the protocol recently described by Fasano et al. for midbrain DA neurons (Fasano et al., 2008b). Briefly, after the brain was harvested, a 1 mm-thick coronal slice containing the substantia nigra and the ventral tegmental area (Fasano et al., 2008b). All cultures were incubated at 37 °C in a 5% CO2 atmosphere in a Neurobasal-A1299 medium (Gibco, Logan, UT, USA) supplemented with penicillin/streptomycin, Gluta-MAX-1 (Gibco) and 10% foetal calf serum (HyClone Laboratories, Logan UT, USA). In the MSN-DA cultures the number of DA neurons present on the coverslips was not statistically different from culture to culture.

2.2. Viral infection

Dendritic spine counting was made possible by infection of neurons with a Semliki Forest virus (SFV) carrying the gene encoding the farnesylated red fluorescent protein mCherry one day prior to imaging (4 DIV or 10 DIV according to the experimental design). SFV were synthesized according to a recently described protocol (Haber et al., 2006). The coverslips were transferred to 12-well plates containing 500 µL of culture medium pre-heated at 37 °C. Vial suspension was added to each coverslip and cultures were incubated until used.

2.3. Semi-quantitative multiplex RT-PCR

Total RNA was isolated from neuronal cultures using TRIzol (Invitrogen, Burlington, ON). Two micrograms of RNA were reverse transcribed using random hexamers as primers (Applied Biosystem, Stockholm, Sweden). 20 U of RNase out (Invitrogen, Burlington, ON) and 20 U of moloney-murine leukaemia virus reverse transcriptase (M-MLV, Invitrogen). 20% of the reverse transcribed cDNA was amplified in a 15 µL reaction mixture containing 1.5 mM MgCl2, 0.5 mM dNTPs mix, 10 pmol of each primer (spinhophilin-fw 3’- CCGCT/CACAGTTCTATG; spinhophilin-rev 3’- ATACAGGCTCACCAGTGTTG; b-actin-fw 3’- CTCTTTTTCACCAGCTCTTCT; b-actin-rev 3’- AGTACTTCTCTTACCTGCTT; AlphaDNA, Montreal, QC, Canada) and 5 units Taq-DNA polymerase (Qiagen, Mississauga, Ontario) in PCR buffer (20 mM Tris–HCl, 50 mM KCI, pH 8.3). After a first denaturing step at 94 °C for 8 min, PCR amplification was performed using a Biometra thermocycler. After 30 cycles as follows: 95 °C for 30 s; 60 °C for 30 s; 72 °C for 40 s. This was followed by a final extension step (72 °C for 5 min). The number of cycles was within the exponential phase of the amplification reaction. BLAST searches against the databases determined the specificity of PCR primers. Mock controls always ran in PCR reactions and never gave amplification products. The amplified products were separated by electrophoresis in 1.5% agarose gel. The gels were imaged with a Kodak DCi2 camera and the PCR products were quantified by using ImageJ software (Molecular Dynamics, Sunnyvale, CA). Spinhophilin mRNA expression was normalized to actin mRNA. Raw values of the ratio of the spinhophilin mRNA to actin mRNA were used for the statistical analysis.

2.4. Pharmacological treatments and drugs

After 5 days in vitro (DIV), young developing neurons were treated chronically, one treatment per day for 5 consecutive days, with DA receptor agonists or antagonists or appropriate vehicles. Analyses were performed after 11 DIV. Unless otherwise stated, drugs were purchased from Sigma-Aldrich (Saint-Louis, MO, USA): SKF38339 (SFK, 4 µM), quinproline (Quin, 1 µM), SCH23390 (SCH, 1 µM), sulpiride (Sulp, 1 µM), CNOX (20 µM), -Glutamic acid monosodium salt monohydrate (Glut, 100 µM), Tetrodotoxin (TTX, 1 µM) was purchased from Alomone Labs (Jerusalem, Israel). All drug concentrations used for the present study are in the lower range of the ones commonly reported in the literature (Onn et al., 2003; Centonze et al., 2006; Fasano et al., 2008a; Zhang et al., 2009; Stuber et al., 2010) and do not affect neuron survival.

2.5. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 and nonspecific binding was blocked with 10% normal goat serum or 5% donkey serum. Cells were incubated overnight with primary antibodies: rabbit polyclonal anti-α-Agarose antibody (1:2500, Sigma-Aldrich, USA), mouse monoclonal anti-tyrosine hydroxylase (TH) antibody (1:5000, Sigma–Aldrich, USA), rabbit polyclonal anti-TH antibody (1:1000, Millipore), mouse monoclonal anti-type 2 vesicular glutamate transporter (VGLUT2) antibody (1:2000, Millipore), mouse 12CA5 monoclonal anti-α-HA antibody (1:3000), goat anti-choline acetyl-transferase (CHAT) (1:200, Millipore), guinea-pig anti-type 3 vesicular glutamate transporter (VGLUT3) (1:5000, generously gifted by Prof. S. El Meftikawy, McGill University) or mouse monoclonal anti-PDF-95 (1:100, NeuroMab). The cells were then incubated for 1 h with goat anti-mouse Alexa-fluor 488 or 647 conjugated secondary antibodies (1:200, Molecular Probes Inc., USA) or goat anti-rabbit Alexa-fluor 488 or 546 conjugated secondary antibodies (1:200, Molecular Probes Inc., USA) or donkey anti-goat Alexa-fluor 488 conjugated secondary antibodies (1:1000, Molecular Probes Inc., USA) or donkey anti-rabbit Alexa-fluor 488 conjugated secondary antibodies (1:1000, Molecular Probes Inc., USA). Dendritic spine counting was made possible by infection of neurons with a Semliki Forest virus (SFV) carrying the gene encoding the farnesylated red fluorescent protein mCherry one day prior to imaging (4 DIV or 10 DIV according to the experimental design). SFV were synthesized according to a recently described protocol (Haber et al., 2006). The coverslips were transferred to 12-well plates containing 500 µL of culture medium pre-heated at 37 °C. Vial suspension was added to each coverslip and cultures were incubated until used.

2.6. Imaging

Epi-fluorescence microscopy was used to perform Sholl analysis of dendritic complexity and to quantify the number of dendritic spines established on the first 50 µm of primary dendrites by living MSNs. Coverslips were transferred to a recording chamber placed onto the stage of a Nikon Eclipse TE-200 inverted fluorescence microscope and superfused at room temperature with saline solution consisting of (in mM): NaCl 140, KCl 5, MgCl2 2, CaCl2 1, sucrose 6, glucose 10, HEPES
10 (305–310 mOsm and pH adjusted to 7.35). Images were acquired using a Hamamatsu Orca-III digital-cooled CCD camera (Hamamatsu, Bridgewater, NJ, USA) and the Image-Pro Plus 6.2 software suite (Media Cybernetics, Bethesda, MD, USA). Fluorescence was excited using a Sutter DG4 xenon lamp (Sutter Instruments, Novato, CA, USA) and collected after passing through a 460/500-nm filter to detect eGFP-DA neurons and through a 510/550-nm filter to select m-Cherry infected neurons. Z-stack images of TH/VGLUT2, ChAT/VGLUT3 and eGFP/mCherry/PSD-95 immunostained material were acquired on a point-scanning confocal microscope (FV1000, Olympus) equipped with multi-argon and helium/neon lasers.

2.7. Dendritic spine counting

A z-series projection of 3–7 images with 1 μm step size interval was captured. Images were analysed using Image J software (NIH; http://rsbweb.nih.gov/ij/); the first 50 μm of all dendrites was measured using the straighten plugin, and then the total number of dendritic spines was counted blindly. Protrusions from primary dendrites shorter than 5 μm were considered as spines (Supplementary Fig. 1). Counting the number of dendritic spines on the first 50 μm of dendrites and not on distal dendrites where the spine density is higher was motivated by a concern of reproducibility of the results. Indeed, cultured MSNs display crossings of their respective dendritic arborisation, making it difficult to consistently analyse comparable distal dendrites segments. The number of spines expressing PSD-95 was counted blindly from z-series projections of 3–5 images with 0.5 μm step size interval, according to the same criteria used for spine identification.

2.8. Sholl analysis

The complexity of dendritic arborisation was evaluated by performing a Sholl analysis. Images were analysed using Image J software (NIH); a threshold was applied to trace the outline of MSNs, the images were then binarized and a home-made plugin was used to calculate the number of dendritic branches that intersected concentric circles spaced by incremental 20 μm radii starting from the soma centre.

2.9. Electrophysiology

Spontaneous miniature EPSCs (mEPSCs) and membrane currents evoked in MSNs in response to glutamate receptor activation were recorded at room temperature by using the patch-clamp technique in whole-cell configuration. 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 (see imaging section) containing 1 μM TTX to block action potentials. MSNs were clamped at a holding potential of −60 mV and currents were recorded with a Warner PC-505 patch-clamp amplifier (Warner Instruments Corp., Hamden, CT, USA) using PClamp 10 software (Molecular Devices, Sunnyvale, CA, USA). Borosilicate glass patch pipettes (5–7 MΩ resistance, World Precision Instruments Inc., Sarasota, FL, USA) were filled with a potassium methylsulfate intrapipette solution consisting of (mM): K鼾SO4 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 285–295 mOsm. Spontaneous mEPSCs were detected and analysed using Mini Analysis (v.5.6; Synaptosoft, Inc., Leonia, NJ, USA) and Clampfit (v.10, Molecular Devices) softwares. mEPSC frequency was measured over a period of 3 min. The threshold for mEPSC detection was set at twice the mean amplitude of the background noise. Glutamate-mediated responses were evoked with 1-glutamic acid (100 μM) delivered every 20 s for 5 ms by local pressure application, using a pneumatic picopump (World Precision Instruments, Sarasota, FL, USA). The pipette was positioned within a few microns from a MSN dendrite, at a distance of about 50 μm from the soma. The inward current elicited by glutamate was analysed using Clampfit 10 software (Molecular Devices) and normalized to cell capacitance (pA/pF). Cell capacitance was estimated using the square pulse method, as implemented in the PClamp 10 Membrane Test module. The average cell capacitance was 30 ± 2 and 29 ± 3 pF (p = 0.75) in MSN and DA-MSN culture groups, respectively. Pipette capacitance and series resistance (typically between 10 and 20 MΩhm) were both adjusted prior to cell capacitance measurements, at the start of each recording.

2.10. Statistical analysis

Each series of experiments were obtained from at least 3 distinct cultures, each one including 2 to 4 coverslips per group. All values are expressed as mean ± SEM. Statistical analysis between groups were performed using a t-test or an analysis of variance (ANOVA one- or two-way) followed by a Tukey post-hoc test, as appropriate. Values were taken to be statistically different if p < 0.05.

3. Results

3.1. Characterisation of a dopamine neuron and medium spiny neuron co-culture system

All experiments were performed by evaluating the development of spine-like protrusions in cultured mouse MSNs. Striatal cultures containing only neurons intrinsic to the striatum, and thus a majority of GABA neurons (MSN cultures) (Fig. 1 A) were compared to mixed cultures including MSNs growing together with added DA neurons purified from TH-GFP transgenic mice (DA-MSN cultures) (Fig. 1B). Although glutamatergic inputs from cortical afferents are known to increase dendritic spine density in cultured MSNs (Segal et al., 2003), cortical glutamate neurons were not added to the cultures in the present study as we aimed to specifically examine the contribution of dopamine neurons. Although immature spines without synapses are likely to be present in the cultures examined, a modest level of glutamatergic inputs to MSNs was nonetheless present in both MSN cultures and DA-MSN cultures, probably

![Fig. 1. MSN and MSN-DA cultures. Monoculture of MSNs (A) and co-culture of MSNs with purified DA neurons (B). From left to right, the panels show GABA immunostaining, identifying MSNs (red), TH immunoblabelling, identifying DA neurons (green), the merge of GABA and TH immunoinstaining and a phase contrast image showing the neurons growing on a striatal astrocyte monolayer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
contributing to the detected basal spinogenesis. In MSN cultures, glutamatergic inputs were provided by the small contingent of cholinergic interneurons, otherwise recently described as co-releasing glutamate due to expression of the type 3 vesicular glutamate transporter (VGLUT3) (Gras et al., 2002, 2008; El Mestikawy et al., 2011; Higley et al., 2011). The presence of mixed cholinergic-glutamatergic interneurons in our cultures was confirmed by double-immunocytochemistry for ChAT and VGLUT3. Numerous VGLUT3-positive terminals were present in both MSN cultures (Fig. 2A) and in DA-MSN cultures (Fig. 2B). In DA-MSN cultures, glutamatergic axon terminals can also be established by DA neurons, a subset of which is known to express type 2 vesicular glutamate transporter (VGLUT2) (Dal Bo et al., 2004; Mendez et al., 2008). The presence of glutamatergic terminals was confirmed in our DA-MSN cultures by TH-VGLUT2 double immunocytochemistry (Fig. 2B). No VGLUT2 positive terminals were detected in MSN cultures, as expected (Fig. 2A).

3.2. Co-culture with dopamine neurons increases dendritic spine number in medium spiny neurons

In both MSN cultures and DA-MSN cultures, filopodia/thin, stubby and mushroom-like dendritic spines could be observed (Supplementary Fig. 2). However, a majority of dendritic spines were of the thin or filopodial shape (Fig. 3). After 5 DIV, MSNs cultured without DA neurons exhibited $3.35 \pm 0.24$ dendritic spine-

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**Fig. 2.** Presence of glutamatergic axon terminals in MSN and MSN-DA cultures. (A) MSN culture (A) and MSN-DA culture (B). The upper series of images, from left to right, shows ChAT immunostaining identifying a striatal cholinergic interneurons, VGLUT3 immunostaining revealing the presence of glutamatergic terminals and a merge image illustrating the mixed phenotype of cholinergic interneurons. The lower series of images, from left to right, shows TH immunostaining identifying mesencephalic DA neurons sorted by FACS, VGLUT2 immunostaining revealing the presence of glutamatergic terminals and a merge image. The inserts show a magnified view of VGLUT-containing axon terminals.
like protrusions on the first 50 μm of their primary dendrites (n = 89 neurons). When co-cultured with DA neurons at low density (443 ± 92 DA neurons per coverslip) or high density (779 ± 125 DA neurons per coverslip), the number of spine-like protrusions increased to 4.43 ± 0.30 (n = 90 neurons) or 5.60 ± 0.29 (n = 102 neurons), respectively (Fig. 3A). This represented a statistically significant increase of 32 ± 6% (p < 0.01) and 67 ± 9% (p < 0.001), respectively. The difference in the number of dendritic spines formed by MSNs in the presence of low and high DA neuron density was statistically significant (p < 0.05). When neurons were allowed to grow for 10 DIV, MSNs established 4.78 ± 0.27 dendritic spine-like protrusions (n = 129 neurons), whereas in DA-MSN cultures with a low density (289 ± 46 DA neurons per coverslip) or high density (504 ± 74 DA neurons per coverslip) of DA neurons, MSNs exhibited 9.00 ± 0.41 (n = 121 neurons) or 11.58 ± 0.40 (n = 111 neurons) spine-like protrusions, respectively (Fig. 3B,C). This represented a statistically significant increase of 88 ± 9% (p < 0.001) and 142 ± 8% (p < 0.001), respectively. The difference in the number of dendritic spines formed by MSNs between low and high DA neuron density was statistically significant (p < 0.001). Taken together these results demonstrate that co-culture with DA neurons potently increases the number of dendritic spine-like protrusions in MSNs. This effect, induced at early stages of postnatal MSN culture development, was clearly dependent on the number of DA neurons present in the co-cultures.

3.3. The presence of dopamine neurons increases dendritic branching in MSNs

Our initial experiments indicate that the presence of DA neurons enhances spine formation in cultured MSNs. However it is also possible that DA neurons have a broader neurotrophic effect and globally enhance the morphological development of MSN dendrites. If this is the case, a prediction is that in DA-MSN cultures, an increase in dendritic branching should occur to an extent that is proportional to the increase in spine number. To examine this possibility, a Sholl analysis was performed to study dendritic branching of MSNs at 10 DIV (Fig. 4A). We compared the number of dendritic crossings with the Sholl concentric circles between MSN cultures (n = 101 neurons) or DA-MSN cultures (n = 76 neurons) in low density co-cultures and n = 132 in high density co-cultures, Fig. 4B). No difference was observed in the number of crossings between MSNs and MSNs cultured with a low density of DA neurons. However a small increase in the number of crossings was detected in MSNs co-cultured with DA neurons at high density. In these DA-MSN cultures, a significant increase in the number of crossings between 6.5 ± 3.8% to 16.0 ± 4.6% (p < 0.05 to p < 0.01) was found at 40 μm, 60 μm, 80 μm and 100 μm from the soma center in comparison to MSN cultures. When the analysis was restricted to the first 50 μm (circles at 20, 40 and 60 μm from the soma center), which correspond to the location of the previous spine density calculations, MSNs in monoculture intersected 9.67 ± 0.13 times the Sholl circles in total (Fig. 4C). In comparison, MSNs co-cultured with a low density of DA neurons crossed the circles 9.86 ± 0.18 times, a value not significantly different than for MSN cultures (p > 0.05), whereas MSNs in co-culture with a high density of DA neurons displayed 10.39 ± 0.19 crossings, which represented a small but statistically significant increase in dendrite branching compared to MSN cultures (p < 0.05). Hence, innervation by DA neurons only modestly enhances global dendritic development. These results strongly argue that the presence of DA neurons acts specifically to enhance early spine formation in MSNs.

3.4. Dopamine-induced dendritic spine formation involves both D1 and D2 dopamine receptors

The innervation of MSNs by DA neuron axon terminals could have facilitated spine induction through activation of D1 or D2
receptors (D1R, D2R). Alternately, spine growth could have resulted exclusively from the presence of additional glutamatergic inputs provided by VGLUT2-expressing DA neurons, without any obligatory implication of DA receptors.

To evaluate if D1R and D2R activation is involved, we first determined if chronic treatment of MSN cultures with DA receptor agonists increases spine number. Under control conditions, MSNs established $4.43 \pm 0.29$ dendritic spine-like protrusions on the first $50 \mu m$ of primary dendrites ($n = 62$ neurons, Fig. 5 A and B). In comparison, when MSNs were treated chronically with the D1R agonist SKF38393 (SKF, $4 \mu M$, $n = 54$ neurons), they established $6.62 \pm 0.46$ dendritic spine-like protrusions, representing a statistically significant increase of $49 \pm 10\%$ ($p < 0.01$). Similarly, when MSNs were exposed chronically to the D2R agonist quinpirole (QUINP, $1 \mu M$, $n = 50$ neurons), they established $6.83 \pm 0.28$ dendritic spine-like protrusions, representing a statistically significant increase of $54 \pm 6\%$ ($p < 0.01$). A combination of SKF38393 ($4 \mu M$) and quinpirole ($1 \mu M$) increased spine number to $7.10 \pm 0.53$, which represented a statistically significant increase of $60 \pm 12\%$ ($p < 0.01$, $n = 25$ neurons) compared to the control group. However, there was no additive effect of the two receptor agonists, thus suggesting that these receptors share a common downstream signalling pathway to stimulate dendritic spine formation.

Arguing in favour of a regulation of spine plasticity by DA receptor activation, we found that chronic treatment of monocultured MSNs with DA receptor agonists also increased expression...
of spinophilin, a protein enriched in dendritic spines (Allen et al., 1997). Compared to the untreated group, chronic treatment with SKF38393 (4 μM) or quinpirole (1 μM) increased the ratio of spinophilin to actin mRNA by 119 ± 3% (p < 0.05) and 67 ± 13% (p < 0.05), respectively (n = 19 coverslips for each groups, Fig. 5C and D), as determined by semi-quantitative RT-PCR. When MSNs were treated with a combination of SKF38393 (4 μM) and quinpirole (1 μM), this ratio was increased by 111 ± 52% (p < 0.05, n = 7 coverslips), which was not significantly higher than in response to the individual agonists alone.

Although the positive effect of DA receptor agonists on spine formation argues in favour of the implication of D1R and D2R in mediating the effects of DA neurons on spinogenesis, we performed additional experiments with DA receptor antagonists to validate this hypothesis. Experiments were performed by inhibiting D1Rs and/or D2Rs in DA-MSN cultures containing a high density of DA neurons (Fig. 6). Under control conditions, MSNs in these cocultures exhibited 9.22 ± 0.34 dendritic spine-like protrusions on the first 50 μm of primary dendrites (n = 47 neurons). In comparison, after chronic inhibition of D1Rs with SCH23390 (1 μM, n = 41 neurons), the number of dendritic spine-like protrusions was decreased to 6.46 ± 0.43, thus representing an inhibition of 30 ± 5% (p < 0.01). After chronic treatment with the D2R antagonist sulpiride (1 μM, n = 44 neurons), MSNs exhibited 7.16 ± 0.30 dendritic spine-like protrusions, corresponding to a reduction of 22 ± 3% (p < 0.01). Finally, after combined blockade of D1Rs and D2Rs, MSNs displayed 6.47 ± 0.45 dendritic spine-like protrusions, representing a decrease of 30 ± 5% (p < 0.01, n = 45 neurons) and a lack of additive effect. These results thus confirm a role for D1R and D2R in regulating early spinogenesis in MSNs and argue for a lack of additive effect between the two receptors.

3.5. Co-culture with dopamine neurons increases sensitivity to glutamate and the number of functional glutamate synapses onto MSNs

Although a DA-mediated increase in spine number should facilitate the establishment of functional glutamatergic synapses in MSNs, this should ideally be associated with a parallel upregulation of glutamate receptor number or function. To test this possibility, we compared glutamate-mediated membrane currents in MSN and MSN-DA cultures after 10 DIV. In these experiments, whole-cell recordings were obtained under voltage-clamp from MSNs, distinguished from DA neurons by their lack of eGFP fluorescence. Recordings were performed in the presence of TTX (1 μM) to block all activity-dependent synaptic activity. Local pressure ejection of glutamate (100 μM) along MSN dendrites evoked CNQX-sensitive AMPA receptor-mediated inward currents (Fig. 7 A). In monocultured MSNs, glutamate-induced currents displayed an average amplitude of 1.74 ± 0.27 pA/pF (n = 12 neurons) (Fig. 7B, C). In comparison, in co-cultured MSNs, the amplitude of glutamate-induced currents was 4.43 ± 1.19 pA/pF (n = 13 neurons), thus representing an increase of 155 ± 68% (p < 0.05, Fig. 7B, C). The responsiveness of MSNs to glutamate was thus enhanced in the presence of DA neurons. Also arguing that some of the newly formed spines induced in response to co-culture with DA neurons present functional post-synaptic machinery, these recordings also revealed that the frequency of glutamate-mediated mEPSCs was

Fig. 5. Dopamine-induced dendritic spine formation involves both D1 and D2 dopamine receptors. (A) Neurons in MSN cultures were chronically treated with vehicle (CTRL), SKF (4 μM) or QUINP (1 μM) or both SKF (4 μM) and QUINP (1 μM). Both SKF and QUINP increased the number of dendritic spines established on the first 50 μm of MSN primary dendrites. When applied together, SKF and QUINP increased spine density to the same extend than each drug separately, indicating a lack of additive effect of D1R and D2R activation. (B) Examples of 50 μm of primary dendrites. When applied together, SKF and QUINP increased spine density to the same extend than each drug separately, indicating a lack of additive effect of D1R and D2R activation. (C) Histogram summarizing RT-PCR densitometry. Data are presented as the ratio of spinophilin mRNA content to actin mRNA content, per coverslip of MSN cultures at 10 DIV. MSNs were chronically treated with vehicle (CTRL), SKF (4 μM), QUINP (1 μM), or both SKF (4 μM) and QUINP (1 μM). Separately, both SKF and QUINP increased the ratio spinophilin to actin. However, when SKF and QUINP were applied together, they failed to elicit an additive effect. (D) Example of a RT-PCR gel showing amplicons of spinophilin and actin under the different pharmacological conditions. *: p < 0.05, **: p < 0.01.
significantly higher in co-cultured MSNs (0.92 ± 0.24 Hz; n = 5) than in monocultured MSNs (0.21 ± 0.06 Hz; n = 5) (Fig. 7D). This represents an increase of 335 ± 113% (p < 0.05). Finally, compatible with these functional observations, we found that dendritic spines containing the post-synaptic scaffolding protein PSD-95 were more abundant in MSN-DA cultures than in MSN cultures, parallelling the global increase in spine number (Fig. 7F). While the proportion of spines with PSD-95 was not significantly increased (43 ± 4% in MSN cultures and 50 ± 3% in MSN-DA cultures, p = 0.08), there was a significant net increase of 157 ± 19% (p < 0.001) in the number of spines containing PSD-95 in the presence of DA neurons (1.9 ± 0.2 per 50 μm in MSN cultures (n = 30 neurons) and 4.9 ± 0.4 per 50 μm in MSN-DA cultures (n = 32 neurons)) (Fig. 7G). Taken together, these results suggest that a subset of the newly formed spines induced in response to co-culture with DA neurons present functional post-synaptic machinery.

4. Discussion

4.1. Structural role of dopamine

Multiple lines of previously published evidence argue for a regulatory role of DA in spine maintenance and stability in MSNs (Glantz and Lewis, 2000; Flores et al., 2005; Solis et al., 2007; Villalba and Smith, 2010). However, considering the multiple and complex cellular interactions occurring in vivo, it has been difficult to provide support for a direct instructive role of DA on MSN spines, independently from presynaptic effects of DA. In the present study, we thus took advantage of a unique in vitro model of primary neuron co-culture to investigate the direct role of DA on MSN dendritic spine formation. By using DA neurons purified from TH-eGFP mice (Sawamoto et al., 2001; Matsushita et al., 2002), it was possible to evaluate the impact of adding DA neurons on MSN spine formation without the complication of adding other non-dopaminergic mesencephalic neurons which could otherwise have had an impact on spine formation through regulation of MSN activity (Segal et al., 2003; Fishbein and Segal, 2007).

Compared with other brain structures such as the hippocampus, the mechanisms underlying the formation and maintenance of dendritic spines in the striatum are much less understood. However, it has been established that there is a loss of dendritic spines in MSNs following DA denervation in Parkinson’s disease (Villalba and Smith, 2010), suggesting a role for DA in spine maintenance. In addition, growth factors such as BDNF also appear to play a key role in spine development and plasticity (Saylor et al., 2006; Jia et al., 2010; Rauskolb et al., 2010). Whether DA acts directly on MSNs to regulate spines is the subject of debate. Based on the knowledge that DA gates glutamatergic transmission onto MSNs by acting through D2R to inhibit glutamate release (Bamford et al., 2004a, 2004b), recent in vivo and in vitro studies have shown that spine loss following DA denervation can be prevented by cortical denervation, supporting the hypothesis that DA regulates spine maintenance through its presynaptic effect on cortico-striatal synapses (Neely et al., 2007; Garcia et al., 2010).

In the present study, we focussed our attention on developing MSNs and demonstrate for the first time that co-culture with DA neurons or direct pharmacological activation of D1R or D2R promotes the formation of dendritic spines. These spines were mostly of the long and thin subtype or filopodial-like, suggesting that they were immature, an observation that is in line with the relatively young age of the cultures and the absence of cortical glutamatergic neurons. These cultures nonetheless contained VGLUT2- and VGLUT3-positive glutamatergic axon terminals, originating respectively from DA neurons and cholinergic inter-neurons, explaining in part the presence of a minimal number of synapses and spines, even in MSN cultures. It has been previously demonstrated that co-cultures of MSNs together with cortical neurons for four weeks can lead to the establishment of immature spines of the mushroom subtype (Segal et al., 2003), a class of spines otherwise known to represent sites of mature, stable synapses (Kasai et al., 2003). A first conclusion from our work is therefore that although DA or the presence of dopaminergic terminals is not required for MSNs to establish an initial repertoire of spines, DA neurons can nonetheless directly enhance the establishment of immature spines in developing MSNs. We hypothesize that following the spine enhancing effect of DA neurons, cortical glutamate inputs may be subsequently required for spine maturation and stabilization. Additional experiments will be required to test this hypothesis.

Considering the immaturity of the spines formed by MSNs in our model, a potential concern could be that a subset of the dendritic protrusions we observed was in fact developing dendritic branches and not immature spines. Four observations argue against this possibility. First, we observed that while the presence of DA neurons induced a small increase in the complexity of MSN branching, this effect was very small in comparison to the effect of DA neurons or DA receptor agonists on spine formation. A second argument is that in parallel to their ability to enhance spine...
number, the DA receptor agonists SKF38393 and quinpirole both increased the levels of spinophilin mRNA, a scaffolding protein enriched in dendritic spines (Allen et al., 1997). Third, mEPSC frequency was increased when MSNs were cultured in the presence of DA neurons, providing physiological evidence for an increase in the number of functional synapses. Finally, we observed an increase in PSD-95-containing spines on MSN primary dendrites when co-cultured with DA neurons, suggesting that at least a subset of the protrusions that were counted were indeed functional dendritic spines.

The main objective of the present study was to examine the role of DA and DA receptors in spine formation. We therefore did not specifically examine the role of glutamate neurons and glutamate receptors, which clearly also play a critical role. The potential role of...
GABA and acetylcholine were also not examined. However, we showed that VGLUT2- and VGLUT3-positive glutamatergic terminals were present in the cultures; such axon terminals are likely to have contributed in part to the formation and stabilization of spines in the absence of cortical neurons. Although our finding that co-culture with DA neurons increased spine number argues for a role of DA in spine induction, it is important to consider that a subset of DA neurons also expresses VGLUT2 and establishes glutamatergic axon terminals (Rayport, 2001; Dal Bo et al., 2004; Dal Bo et al., 2008; Mendez et al., 2008; Birgner et al., 2010) (see reviews by Trudeau and Gutierrez (2007), Descaries et al. (2008) and El Mestikawy et al. (2011)). Glutamate synapses established by DA neurons may therefore have contributed to the effect of DA neurons on spine number. Additional experiments will be required to directly examine this possibility. However, our finding that D1 and D2 agonists are able to directly enhance spine formation in the absence of DA neurons strongly suggests that DA receptors directly act on MSNs to regulate the formation of nascent spines. Also in support of a role for DA receptors, we found that DA receptor antagonist treatment reduced the enhancement in spine number induced by co-culture with DA neurons. Considering that DA receptor antagonists did not reduce spine number to the level found in MSN cultures, it is likely that the residual enhancement in spine number induced by co-culture with DA neurons is due to the establishment of glutamatergic synapses emanating from DA neurons. This might also explain why the pharmacological treatment of MSNs with DA agonists did not enhance spine number as effectively as that induced by co-culture with DA neurons.

In light of the present findings showing a role for DA in promoting dendritic spine formation by MSNs, the recent demonstration that tonic activity of D1R and D2R inhibits the number of functional GABAergic collateral synapses established by embryonic MSNs (Goffin et al., 2010) suggests that DA may play a multi-faceted role in the synaptic refinement of striatal circuits during early development. The demonstration that DA, through D2R, also acts presynaptically on DA neuron terminals to inhibit their growth (Fasano et al., 2008a, 2010) further demonstrates that DA acts both pre- and postsynaptically to establish and fine-tune synaptic structure in the striatum.

4.2. Non-additive effects of D1 and D2 DA receptors

The now classical model of basal ganglia circuitry proposes that striatal MSNs can be classified in two distinct populations, the direct and indirect pathway (Bolam et al., 2000; Kravitz et al., 2010). D1R and D2R are thought to be expressed in distinct populations of MSNs, a conclusion supported by observations made with transgenic mice expressing fluorescent reporter proteins in MSNs (Wang et al., 2006; Day et al., 2008; Gertler et al., 2008). Although a global segregation of D1R- and D2R-containing MSNs seems clear, a certain level of co-expression of D1R and D2R in individual MSNs also seems to occur, as revealed by single cell RT-PCR (Surmeier et al., 1992, 1996), immunocytochemistry (Falk et al., 2006; Goffin et al., 2010), immunohistochemistry (Goffin et al., 2010), or co-immunoprecipitation (Lee et al., 2004; Pei et al., 2010). A combination of in vivo and in vitro studies has shown that D1R and D2R can heterodimerize and form a signalling complex able to activate phospholipase C and mobilize intracellular calcium (Lee et al., 2004; Hasbi et al., 2009). Furthermore, this cascade has been shown to trigger BDNF production, leading to MSN growth (Hasbi et al., 2009), a finding that is compatible with our observation of an enhancement of spine formation by DA.

In the present study, we demonstrate that activating or blocking D1R or D2R affected spine formation to approximately the same extent. In addition, we found that activating or blocking both receptors simultaneously did not lead to an additive effect. Two possible interpretations can be considered. First, it could be that a majority of MSNs under our conditions expressed both D1R and D2R and that their ability to enhance spine formation is due to the activation of a final common pathway, perhaps implicating receptor heteromers and stimulation of BDNF production. A second possibility is that a majority of MSNs expressed only D1R or D2R, but that through reciprocal synaptic interactions between MSNs (Tepper and Plenz, 2006), chronic D1 or D2 agonist treatments had an impact on both cell populations.

4.3. Functional role of dopamine

During the post-natal maturation of synapses, the number of dendritic filopodia decreases, while the number of stable spines increases, suggesting that filopodia represent the precursors of mature dendritic spines (Yuste and Bonhoeffer, 2004; Yoshihara et al., 2009). The work described in the present study suggests that DA and DA receptors facilitate spine formation in MSNs by promoting the establishment of dendritic filopodia long and thin spines. Whether these immature spines are functional was not extensively examined. However, we observed that in parallel with the increase in spine formation, co-culture of MSNs with DA neurons led to an increased response of the MSNs to local glutamate perfusion, an increased spontaneous mEPSC frequency as well as an increased number of spines containing PSD-95, a scaffolding protein accumulating at the postsynaptic site of excitatory synapses. These findings collectively argue that the availability and delivery of ionotropic glutamate receptors to spines is enhanced by DA neuron innervation, compatible with the hypothesis that these nascent spines may present functional post-synaptic machinery, preparing them to receive potential glutamatergic contacts from cortex. The specific contribution of glutamatergic terminals established by cholinergic and DA neurons in nascent spine formation remains to be examined. However, our work argues in favour of a positive role for such terminals, thus highlighting a potential new role for glutamate cotransmission in the developing brain.

Based on the complete set of results presented in this study, we propose the hypothesis that during brain development, innervation of the striatum by DA neurons induces the formation of immature but functional dendritic spines in MSNs. These glutamate-sensitive spines would then be ready to detect the arrival of cortical and subcortical glutamate terminals, an event that would lead to further formation of spines and their subsequent maturation. At later stages, DA would otherwise play an additional, critical role, in the maintenance of mature spines by regulating MSN excitability (Hernandez-Lopez et al., 2000; Surmeier et al., 2007; Azad et al., 2009) and intrasynaptic Ca2+ concentration (Hernandez-Lopez et al., 2000; Yang, 2000; Day et al., 2006; Higley and Sabatini, 2010). The model proposed here is compatible with a number of observations including that (1) nigro-striatal and meso-striatal dopaminergic connections are already established at birth, (2) in the striatum, dopaminergic fibres expressing TH are present as early as E14 (Specht et al., 1981; Voorn et al., 1988), (3) D1R and D2R are already present at E17 (Goffin et al., 2010) and (4) maturation of dendritic spines in the striatum of rat and mice (Jakowec et al., 2001; Zhuravin et al., 2007) as well as striatal innervation by cortical afferents (Christensen et al., 1999; Inaji et al., 2011) are postnatal phenomena occurring during the second week after birth.

4.4. Pathological implications and conclusion

The relationship between DA and dendritic spine density is well established in the adult: increases of DA levels in response to drugs of abuse, such as cocaine and amphetamine lead to an elevation of the
number of dendritic spines in MSNs (Robinson and Kolb, 1997, 1999; Li et al., 2003; Lee et al., 2006; Singer et al., 2009). In addition, a decrease in DA levels or loss of DA neurons in Parkinson’s disease models reduces the number of spines (Ingham et al., 1993; Solis et al., 2007; Garcia et al., 2010). However, little is known presently regarding the regulatory role of DA in spine genesis at early stages of development. The present report represents a first step in this direction by revealing that DA neurons can act on developing MSNs to promote the development of new spines and increase responsiveness to glutamate through AMPA receptors. A better understanding of the role of the DA system in regulating MSN development is of importance, especially considering that in a number of pathological conditions, such as schizophrenia and attention deficit with hyperactivity disorder (ADHD), alterations in brain DA levels during development are hypothesized to occur (for reviews see (van der Kooy and Glennon, 2007; Tripp and Wikens, 2008; Heinz and Schlagenhauf, 2010)), which could therefore lead to a perturbation of spine formation and maintenance in the striatum.

Conflicts of interest

The authors declare presenting no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2012.11.030.

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