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

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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 diseases (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 post-synaptic ionotropic receptors located on the head of these MSN spines, whereas DA released by midbrain DA neurons activates

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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 (Specht et al., 1981; Voorn 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 of 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; Matsushita 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 striatum was cut by hand with a scalpel blade and the dorsal striatum was isolated. After enzymatic digestion and mechanical dissociation, cells were pelleted, re-suspended at 240,000 living cells per mL and then plated on a monolayer of dorsal striatum astrocytes grown on collagen/poly-L-lysine pre-coated glass coverslips. To prepare co-cultures of MSNs and DA neurons (called thereafter MSN-DA cultures), 5000 or 10,000 eGFP DA neurons purified by fluorescence-activated cell sorting (FACS) were added to MSN cultures (called thereafter "low density" and "high density" MSN-DA cultures, respectively). Purified eGFP DA neurons were obtained by dissecting a piece of mesencephalic brain tissue containing the substantia nigra and the ventral tegmental area (Fasano et al., 2008b). After digestion and mechanical dissociation, the mesencephalic cell suspension consisted of glial cells and the two major neuronal populations, GABA and DA neurons. eGFP DA neurons were sorted using a BD FACSAria (BD BioSciences) flow cytometer/cell sorter according to a previously-described procedure (Mendez et al., 2008). All 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% 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). SFVs 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. Viral 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 hexanucleotides as primers (Applied Biosystem, Streetsville, Ontario), 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 $MgCl_2$, 0.5 mM dNTPs mix, 10 pmol of each primer (spinophilin-fw 3′ GGGCCTGCACTGATTTGTAT; spinophilin-rev 3′ ATACAAGGCCTCCAGGTGTG; b-actin fw 3′ CTCTTTTCCAGCCTTCCTTCTT; b-actin rev 3′ AGTAATCTCCTTCTGCATCCTGTC; AlphaDNA, Montreal, QC, Canada) and 5 units Taq-DNA polymerase (Qiagen, Mississauga, Ontario) in PCR buffer (20 mM Tris–HCl, 50 mM KCl, pH 8.3). After a first denaturing step at 95 °C for 8 min, PCR amplification was performed using a Biometra thermocycler T Gradient (Goettingen, Germany) and 30 cycles as follows: 95 °C for 30 s; 56 °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 DC290 system and the PCR amplicon were quantified by using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Spinophilin mRNA expression was normalized to actin mRNA. Raw values of the ratio of the spinophilin 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): SKF38393 (SKF, 4 μM), quinpirole (Quinp, 1 μM), SCH23390 (SCH, 1 μM), sulpiride (Sulp, 1 μM), CNQX (20 μM), L-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-GABA 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-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 Mestikawy, McGill University) or mouse monoclonal anti-PSD-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 (1:500, Molecular Probes) or donkey anti-guinea pig Cy3 conjugated secondary antibodies (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA, USA), according to each experimental design. Coverslips were then mounted with Vectashield (Vector laboratories, Burlingame, CA, USA).

2.6. Imaging

Epifluorescence 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, $MgCl_2$ 2, $CaCl_2$ 2, sucrose 6, glucose 10, HEPES