Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2012 | 120 | 765–778 doi: 10.1111/j.1471-4159.2011.07618.x

ORIGINAL ARTICLE

Pre-synaptic dopamine D_3 receptor mediates cocaine-induced structural plasticity in mesencephalic dopaminergic neurons via ERK and Akt pathways

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Abstract

Exposure to psychostimulants results in neuroadaptive changes of the mesencephalic dopaminergic system including morphological reorganization of dopaminergic neurons. Increased dendrite arborization and soma area were previously observed in primary cultures of mesencephalic dopaminergic neurons after 3-day exposure to dopamine agonists via activation of D_3 autoreceptors (D_3R). In this work, we showed that cocaine significantly increased dendritic arborization and soma area of dopaminergic neurons from E12.5 mouse embryos by activating phosphorylation of extracellular signal-regulated kinase (ERK) and thymoma viral proto-oncogene (Akt). These effects were dependent on functional D_3R expression because cocaine did not produce morphological changes or ERK/Akt phosphorylation neither in primary cultures of D_3R mutant mice nor following pharmacologic blockade with D_3R antagonists SB-277011-A and S-33084. Cocaine effects on morphology and ERK/Akt phosphorylation were inhibited by pre-incubation with the phosphatidylinositol 3-kinase inhibitor LY294002. These observations were corroborated *in vivo* by morphometrical assessment of mesencephalic dopaminergic neurons of P1 newborns exposed to cocaine from E12.5 to E16.5. Cocaine increased the soma area of wild-type but not of D_3R mutant mice, supporting the translational value of primary culture. These findings indicate a direct involvement of D3R and ERK/Akt pathways as critical mediators of cocaine-induced structural plasticity, suggesting their involvement in psychostimulant addiction.

Keywords: cocaine, dopamine receptor, mesencephalon, p-Akt, p-ERK, structural plasticity.

J. Neurochem. (2012) **120**, 765–778.

Received November 9, 2011; revised manuscript received December 5, 2011; accepted December 5, 2011.

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Abbreviations used: 7-OH-DPAT, 7-hydroxy-*N*,*N*-di-propyl-2-aminotetralin; Akt, thymoma viral proto-oncogene; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; D_3KO, D_3R null mutant mice; DA, dopamine; DAT, DA transporter; E, embryonic day; ERK, extracellular signal-regulated kinase; IR, immunoreactivity; PBS, phosphate-buffered saline; p-Akt, phosphorylated thymoma viral proto-oncogene; p-ERK, phosphorylated extracellular signal-regulated kinase; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; SN, substantia nigra; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

The evidence that cocaine addiction is associated with neuroadaptive changes of mesolimbic dopaminergic pathway is well established (Nestler *et al.* 1993; Koob *et al.* 1998; Thomas *et al.* 2008). While several studies addressed gene expression and neurochemical changes, fewer works describe changes of cell morphology, phenomena also defined as structural plasticity. These events are of potential interest because representative of long-lasting reorganization of synaptic input and signal processing underling the chronic features of addiction (Nestler *et al.* 1993; Robinson and Kolb 2004; Russo *et al.* 2010; Stuber *et al.* 2010). In rodents, exposure to cocaine or amphetamine produces structural changes of dendrite morphology of the dopaminoceptive medium spiny neurons of the nucleus accumbens and in pyramidal neurons of the prefrontal cortex (Robinson and Kolb 1999, 2004; Lee *et al.* 2005). In ventral mesencephalon, significant outgrowth of dendrites of dopaminergic neurons was observed after repeated exposure to amphetamine (Mueller *et al.* 2006), while a single dose of cocaine increased the number of synaptic spines in dopaminergic neurons of the mesencephalic ventral tegmental area (VTA) (Sarti *et al.* 2007). Prenatal exposure to cocaine can also result in long-term changes in the mesencephalic dopaminergic system of the adult offspring (Fang and Ronnekleiv 1999; Glatt *et al.* 2000).

The molecular mechanisms of structural plasticity to cocaine and other psychostimulants are only partially understood. A parsimonious hypothesis asserts that the extracellular increase of dopamine (DA) produced by these drugs is the critical factor leading to morphological changes. These changes may lead to excessive activation of DA D_1, D_2 and D_3 receptors (D_1R, D_2R and D_3R) in the nucleus accumbens or of D_2R and/or D_3R auto-receptors expressed in the mesencephalic dopaminergic neurons (Missale *et al.* 1998). A relevant role for D_3R was suggested because its expression and function is enhanced by cocaine exposure (Caine and Koob 1993; Le Foll *et al.* 2002; Neisewander *et al.* 2004; Heidbreder *et al.* 2005). Recent findings implicate D_3R in structural neuroplasticity and regeneration of mesencephalic dopaminergic neurons both *in vivo* (Joyce and Millan 2007) and *in vitro* (Collo *et al.* 2008). In the latter study, we observed increases of dendrite arborization and soma size of mesencephalic dopaminergic neurons in mouse primary cultures following treatment with the preferential D_3R agonists and amphetamine. These effects were dependent on phosphorylation of the extracellular signal-regulated kinase (ERK) pathway (Collo *et al.* 2008). Interestingly, ERK and the thymoma viral proto-oncogene (Akt) pathways play a critical role for dendrite outgrowth in hippocampal neurons (Jaworski *et al.* 2005; Kumar *et al.* 2005) and for soma size in dopaminergic neurons of the substantia nigra (SN) (Domanskyi *et al.* 2011; Kim *et al.* 2011).

In the present work, we investigated the effects of cocaine on structural plasticity of mesencephalic dopaminergic neurons. The roles of D_3R and of ERK/Akt pathways were explored in primary cultures from wild-type or D_3R knock-out mice (D_3KO) or using pharmacologic antagonists. The *in vivo* relevance of these observations was obtained by assessing structural plasticity in SN and VTA of newborn mice exposed to cocaine *in utero* at the same embryonic stage used for the *in vitro* cultures.

Materials and methods

Chemicals

Cocaine was purchased from Sigma (St Louis, MO, USA); 7-hydroxy-*N*,*N*-di-propyl-2-aminotetralin (7-OH-DPAT), sulpiride, SCH23990 and LY294002 from Tocris Bioscience (Bristol, UK); brain derived neurotrophic factor (BDNF) from Alomone Labs Ltd. (Jerusalem, Israel); SB-277011-A was a gift from C. Heidbreder, GlaxoSmithKline (Verona, Italy); S-33084 was provided by Institut Servier (Paris, France). Cocaine, 7-OH-DPAT, SCH23990, S-33084, and BDNF were dissolved in water; SB-277011-A and LY294002 were dissolved in dimethylsulfoxide (> 1 : 3000 final dilution), while sulpiride was dissolved in ethanol (> 1 : 2000 final dilution), all as recommended by producer. For each control treatment the vehicle was prepared with the respective dilution of dimethylsulfoxide or ethanol.

Animals

CD1 mice were obtained from Charles-River Laboratories (Italy). Mice genetically deprived of dopamine D_3R (D_3KO) and their singenic wild-type were obtained from the Jackson Laboratory (B6.129S4-Drd3Tm1Dac/J) (Accili *et al.* 1996). Homozygous mice were generated on a pure genetic background and compared with singenic wild-type mice. The genotype of D_3KO and singenic mice was assessed as described in Accili *et al.* (1996). Breeding of all mice was performed to achieve timed pregnancy with the accuracy of ± 0.5 days. The embryonic day (E) was determined by considering the day of insemination (determined by vaginal plug) as day E 0.5. All mice were cared and killed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in line with Italian law.

Primary mesencephalic cultures

Primary mesencephalic cultures were prepared as described in Collo *et al.* (2008). Briefly, ventral mesencephalon tissues were dissected from E12.5 mouse embryos and collected in ice-cold Hank's buffered salt solution (EuroClone, Milan, Italy). Tissues were transferred in Accumax (Sigma Aldrich), mechanically dissociated at 22°C and resuspended in Neurobasal medium (Gibco-Invitrogen, Carlsbad, CA, USA) containing 2 mM glutamine (EuroClone) and B27 supplement (Gibco-Invitrogen). Cells were counted and seeded on poly-D-lysine/laminin (Sigma-Aldrich) -coated coverslides (5×10^4/mL), or 12-well plates (5×10^5/mL), and cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. No fetal bovine serum was added to the cultures. Fifty percent of the medium was changed every 2 days until treatment. Under these conditions, the cultures contained > 99.5% neurons and < 0.5% astrocytes, as assessed by microtubule-associated protein 2 and glial fibrillary acidic protein immunostaining, respectively (data not shown). Pharmacological treatments were conducted *in vitro* after 5 days from seeding, time required for primary culture stabilization.

In vivo prenatal cocaine treatment

Pregnant D_3KO and singenic wild-type mice were individually housed in a climate controlled room on a 12/12-h light/dark cycle with *ad libitum* access to food and water. Treatments were performed during the light period (9:00 AM to 6:00 PM). All mice were weighed daily starting at day 10. At day 12.5, pregnant mice weighting 25–30 g were administered intraperitoneally (i.p.) with two daily dose of 20 mg/kg cocaine or saline as described by Crozatier *et al.* (2003). In this article, this regimen resulted in enhanced stereotypes to cocaine challenges in the offspring as young adults, indicating neuroadaptation. Treatments were repeated for 5 days, between E12.5 to E17. After each injection, animals were placed for 30 min in a clean cage and inspected for locomotor activity. After delivery, P1 newborn mice were killed and the brains were removed and processed for immunohistochemistry.

Immunocytochemistry and immunofluorescence in primary cultures

Immunocytochemistry and immunofluorescence were performed as described in Collo *et al.* (2008). Briefly, mesencephalic neurons were fixed in phosphate-buffered saline (PBS) containing 3% paraformaldehyde (Sigma-Aldrich)/3% sucrose (Sigma Aldrich), blocked in PBS-containing 0.1% Triton (Promega, Madison, WI, USA), 5% normal goat serum (Jackson ImmunoResearch, West Grove, PA, USA), 1% bovine serum albumin (BSA; Sigma Aldrich) and incubated overnight at 4°C with a rabbit polyclonal anti-tyrosine hydroxylase (TH) antibody (1 : 500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by a biotinylated goat anti-rabbit antibody (Jackson ImmunoResearch) (30 min at 22°C) and a final incubation with the ABComplex/horseradish peroxidase detection system (Vector Laboratories, Burlingame, CA, USA). Color development was achieved by addition of 3,3′diaminobenzidine (Sigma Aldrich) for 45 s. In immunofluorescence, the dopaminergic neurons were identified using either anti-TH or anti-DAT antibodies, whose co-distribution was previously tested. To study the expression of phosphorylated ERK (p-ERK) or Akt (p-Akt) in dopaminergic neurons, mesencephalic cultures were incubated with either a rabbit polyclonal antibody anti-TH (1 : 800; Santa Cruz Biotechnology) or a rat monoclonal antibody anti-DA transporter (1 : 400; DAT) (Santa Cruz Biotechnology), together with a mouse monoclonal antibody to p-ERK (1 : 400; Cell Signaling Technology, Beverly, MA, USA) or a rabbit polyclonal antibody to p-Akt (1 : 200; Cell Signaling Technology), respectively. DyLight™ 488- and Cy3-conjugated secondary antibodies (Jackson Immuno Research) were used. Coverslides were mounted with Vectashield Mounting Medium (Vector Laboratories). As negative control, cultured neurons were incubated with the secondary antibodies only. Immunofluorescence was visualized with an Olympus IX51 microscope (Hamburg, Germany). Each experiment was repeated three times and all dopaminergic neurons present in two coverslides for each treatment condition were assessed.

Immunohistochemistry for brain sections

Newborn mice were killed at P1 and brain removed, fixed overnight in PBS 4% paraformaldehyde, cryoprotected in 20% sucrose and rapidly frozen by immersion in isopentane on dry ice. For each brain, a complete set of coronal sections was cut through the SN and VTA at 30 μm. Sections were mounted on slides, blocked and permeabilized with PBS, 5% BSA (Sigma-Aldrich), 0.1% Triton (Sigma-Aldrich) for 30 min at 22°C. They were incubated with the rabbit anti-TH antibody (1 : 500; Santa Cruz) diluted in PBS, 1% BSA, overnight at 4°C. Then they were washed and incubated with a biotinylated goat anti-rabbit antibody (Jackson ImmunoResearch) diluted in PBS, 1% BSA for 30 min at 22°C, followed by avidin-biotinylated horseradish peroxidase complex (ABC; Vector Laboratories) and development with DAB.

Computer-assisted morphological analysis

Digital images from primary neuronal cultures or mesencephalic brain tissues were acquired with the Olympus IX51 microscope connected to an Olympus digital camera and a PC. Morphometric measurements were performed by a blinded examiner on digitalized images using Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA). For *in vitro* primary neuronal culture, the morphological indicators of structural plasticity were: (i) the maximal dendrite length, (ii) the number of primary dendrites and (iii) the soma area (Schmidt *et al.* 1996). Maximal dendrite length was defined as the distance from the soma (hillock base) to the tip of the longest dendrite for each neuron; dendrites shorter than 20 μm were excluded from the analysis. Primary dendrites were defined as those directly stemming from the soma. Soma area was assessed by measuring the surface (μm^2) included by the external perimeter drawn on the cell membrane of neurons identified by TH-IR staining. Two coverslides per treatment groups were examined so to obtain measurements from at least 30 neurons. For *ex vivo* studies computer assisted morphometry was performed using procedures modified from Russo *et al.* (2007). The effects of cocaine on newborn mice were assessed by measuring in each brain the soma area of 90–120 dopaminergic neurons at various rostrocaudal levels of SN and VTA, respectively. The same rostrocaudal levels were considered in each treatment group so to keep anatomical variance partially controlled. In a typical experiment 8 newborn mice from three different mothers per group were included. The soma area of dopaminergic neurons on histological preparations was measured as described for TH-IR primary neurons in cultures.

Western blotting

Western blotting was performed at different time points (1–10 min) following challenge with cocaine (10 μM) and/or after pre-treatments with either D_3R antagonist SB-277011-A (50 nM) or phosphatidylinositol-3-kinase (PI3K) antagonist LY294002 (10 μM). For studies in D_3KO and singenic wild-type mice, challenges were produced with cocaine (10 μM) and 7-OH-DPAT (50 nM). Mesencephalic cultures were washed with ice-cold PBS and lysed in a buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1% Igepal, 1 mM polymethanesulphonyl fluoride, and complete protease inhibitors (Roche Diagnostics, Mannhein, Germany) for 10 min in ice. Neurons were homogenized with a probe- type sonicator on ice, and centrifugated at 13 000 *g* for 10 min at 4°C. The protein concentration was measured with a DC-protein assay (Bio-Rad, Hercules, CA, USA). Twenty micrograms of total proteins were resolved by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis and blotted onto a PVDF membrane (Immobilon-P; Millipore, Billerica, MA, USA). Membranes were

blocked in TBS/T (25 mM Tris base, 137 mM NaCl, 2.7 mM KCl and 0.1% Tween-20, pH 7.6), 5% dry milk, and incubated overnight with the primary antibody diluted in 5% milk in TBS/T. The primary antibodies used were: anti-p-ERK (1 : 1000) and anti-ERK (1 : 1000) mouse monoclonal antibodies (both from Santa Cruz Biotechnology), anti-pAkt (1 : 2000) and anti-Akt (1 : 1000) rabbit polyclonal antibodies (both from Cell Signaling Technology) and anti-TH (1 : 1000) rabbit polyclonal antibody (Millipore). In a typical experiment, the same membrane was processed in the following order: incubation with anti-pERK antibody, stripping, incubation with anti-ERK antibody, incubation with anti-p-Akt antibody, stripping, incubation with anti-Akt antibody, and incubation with anti-TH antibody. At each step, blots were washed and incubated for 90 min with appropriate horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit or goat anti-mouse; Santa Cruz Biotechnology), and developed using ECL (Amersham Biosciences, Milan, Italy). Specific bands were analyzed by densitometric scanning of exposed film using Gel-pro analyzer software (Media Cybernetics). In each experiment, the specific signal of p-ERK or p-Akt proteins was normalised to the corresponding ERK or Akt signals, respectively, and then to the level of TH measured in the same preparation.

Assay of [^{3}H]DA uptake

[^{3}H]DA uptake was measured as described in Collo *et al.* (2008). To eliminate any residual cocaine and to prevent any effect caused by its direct binding to the DA transporter (DAT), mesencephalic cultures were washed and incubated with fresh medium 16 h before performing the [^{3}H]DA uptake test. At the moment of the test the mesencephalic neurons were washed twice with Krebs–Ringer buffer containing 16 mM sodium phosphate, 119 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.3 mM EDTA, and 5.6 mM glucose (pH 7.4), and incubated in Krebs–Ringer buffer containing 1 μM [^{3}H]DA (45.0 Ci/mmol; Amersham Biosciences) for 15 min at 37°C. At the end of the incubation, the cultures were washed three times with ice-cold Krebs–Ringer buffer, and the cells were collected in 1 M NaOH. The radioactivity was counted in a Beckman liquid scintillation counter.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) if not stated otherwise. Significant differences from control conditions were determined using either one- or two-way analysis of variance (ANOVA) followed by *a posteriori* Bonferroni's test for multiple comparisons provided by GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Cocaine-induced structural plasticity in primary cultures of mesencephalic dopaminergic neurons of the mouse

TH-IR dopaminergic neurons were visualized, as represented in Fig. 1a and b. The time-dependency of dendrite outgrowth of dopaminergic neurons, measured as maximal dendrite length and number of primary dendrites, and of soma size, measured as area, was assessed by studying the effects of various doses of cocaine (0.1, 1 and 10 μM) or vehicle at two different time points, 24 and 72 h. Data were analyzed using a two-way ANOVA. The maximal length of dendrites showed a significant treatment effects [$F(3,232) = 46.36$; $p < 0.0001$]

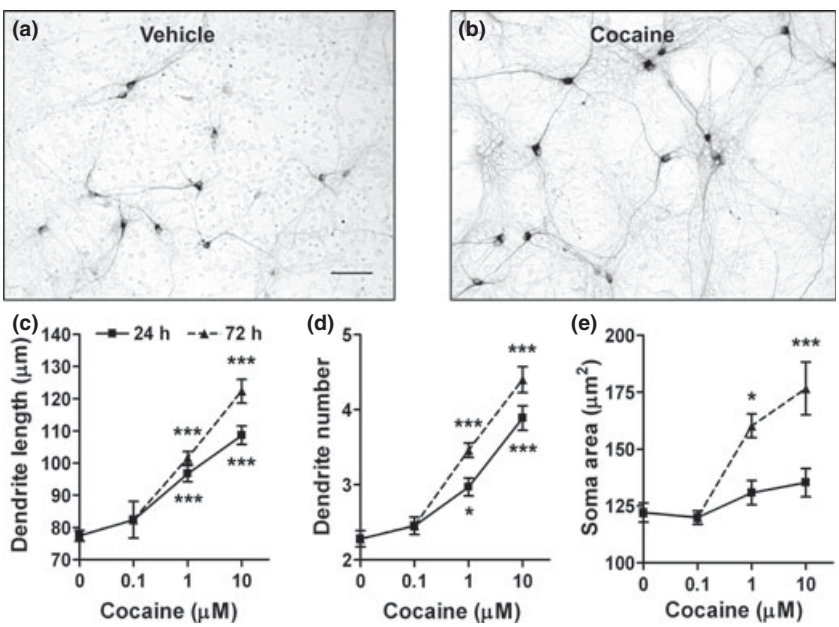

Fig. 1 Morphological effects of cocaine on mesencephalic dopaminergic neurons. (a, b) Representative micrographs of tyrosine hydroxylase immunoreactive (TH-IR) neurons following 72 h exposure to vehicle (a) or 10 μM cocaine (b). Scale bar: 60 μm. (c–e) Dose–response curve and time-dependence of morphological effects of cocaine on maximal dendrite length (c), number of primary dendrites (d), and soma area (e) measured 24 (continuous line) and 72 h (dotted line) after treatment (0 = vehicle). In all panels, values are represented as mean ± standard error of the mean (SEM) (*** $p < 0.001$; * $p < 0.05$ vs. vehicle, *post hoc* Bonferroni's test).

with significant difference from vehicle already at 1 μM and stronger effect at 10 μM (Bonferroni's test, $p < 0.001$ vs. vehicle), while time effect and interaction were not significant (Fig. 1c). The analysis of the number of primary dendrites showed a similar cocaine dose-related increase [$F(3,232) = 60.63$, $p < 0.0001$], with significant differences at 1 and 10 μM dose (Bonferroni's test, $p < 0.001$ vs. vehicle) and a stronger significant effect at 10 μM (Bonferroni's test, $p < 0.001$ 10 μM vs. 1 μM) (Fig. 1d). The main effect of time was also significant [$F(1,232) = 6.70$; $p < 0.02$] with the strongest signal at 72 h (Bonferroni's test, $p < 0.01$), while 'treatment × time' interaction was not significant. Differently, the soma area showed significant 'treatment × time' interaction [$F(3,224) = 6.26$; $p < 0.0005$], as well as treatment [$F(3,232) = 25.33$; $p < 0.0001$] and time effect [$F(1,232) = 17,02$; $p < 0.0001$]. *Post hoc* analysis indicated that the significant interaction was caused by a modest increase during the first 24 h and a concomitant strong growth at 72 h, mostly detected at the dose of 10 μM (Bonferroni's test, $p < 0.001$ vs. vehicle) (Fig. 1e). We concluded that the best test parameters for pharmacological studies were: a cocaine dose of 10 μM and an incubation time of 72 h.

Cocaine-induced structural plasticity in primary mesencephalic cultures: pharmacological studies with DA antagonists

The involvement of DA receptors in dendrite outgrowth of mesencephalic dopaminergic neurons exposed to cocaine (10 μM) for 72 h was initially tested using the D_2/D_3R antagonist sulpiride (5 μM) and the D_1R antagonist SCH23390 (1 μM) added to the culture 20 min before cocaine. On maximal dendrite length two-way ANOVA 'cocaine × antagonist' indicated significant cocaine effect [$F(1,174) = 155,10$; $p < 0.0001$], significant antagonist effect [$F(2,174) = 35,63$; $p < 0.0001$], and significant 'cocaine × antagonist' interaction [$F(2,168) = 35,83$; $p < 0.0001$] (Fig. 2a). *Post hoc* analysis indicated that sulpiride, but not SCH23390, significantly reduced maximal dendrite length to vehicle level (Bonferroni's test, $p < 0.001$). On number of primary dendrites, two-way ANOVA indicated significant cocaine effect [$F(1,174) = 111,10$; $p < 0.0001$], significant antagonist effect [$F(2,174) = 27,66$; $p < 0.0001$], and significant 'cocaine × antagonist' interaction [$F(2,168) = 26,64$; $p < 0.0001$] (Fig. 2b). *Post hoc* analysis indicated that pre-treatment with sulpiride, but not SCH23390, significantly attenuated the effects of cocaine ($p < 0.001$). SCH23390 lack of effects was expected, because no D_1R is expressed in the TH-IR neurons of rodents. Incubations with sulpiride or SCH23390 alone were ineffective.

To investigate the specific role of D_3R in structural plasticity similar experiments were performed with various doses of the D_3R antagonists SB-277011-A and S-33084. SB-277011-A produced a dose-dependent attenuation of cocaine induced maximal dendrite length [$F(5,201) = 18,55$; $p < 0.0001$] (Fig. 2c) and number of primary dendrites [$F(5,234) = 4,35$; $p < 0.001$] (Fig. 2d). For both axonal growth parameters, *post hoc* analysis showed significant effects of cocaine versus vehicle (Bonferroni's test, $p < 0.001$) and a blocking effect of SB-277011-A starting at doses of 50 nM (Bonferroni's test, $p < 0.05$ or lower, see Fig. 2c and d). The involvement of D_3R was confirmed using a more potent and selective D_3R antagonist, S-33084. Pre-treatments with S-33084 significantly antagonized the effects of cocaine on the maximal dendrite length [$F(5,498) = 16,94$; $p < 0.0001$] (Fig. 2e) and the number of primary dendrites [$F(5,381) = 12,94$; $p < 0.0001$] (Fig. 2f) at doses as low as 2 nM ($p < 0.001$). Exposure with 50 nM of SB-277011-A alone and 10 nM S-33084 alone did not show any effect.

To test if the presence of D_3R antagonists may interfere with dendrite outgrowth response independently from a D_3R selective blockade, mesencephalic cultures were exposed to SB-277011-A (50 nM) or vehicle followed by BDNF (10 ng/mL) or vehicle and incubated for 72 h. Two-way ANOVA showed that BDNF increased maximal dendrite length [$F(1,136) = 105.04$; $p < 0.0001$] that was not blocked by pre-treatment with SB-277011-A (Fig. 2g).

The [^{3}H]DA uptake paradigm (Fig. 2h) was used to assess the functional capacity of DAT in internalizing extracellular DA, a specific feature of dopaminergic neurons. Treatments with 10 μM cocaine produced a marked [^{3}H]DA uptake when tested after 72 h culture as shown by significant interaction [$F(1,8) = 79,68$; $p < 0.0001$]. Pre-treatment with SB-277011-A (50 nM) significantly blocked the effects of cocaine (Bonferroni's test, $p < 0.001$) to the level of vehicle treatment (Fig. 2h). Incubations with SB-277011-A alone did not affect the [^{3}H]DA uptake.

Cocaine-induced structural plasticity in primary mesencephalic cultures from D_3KO mice

Mesencephalic neurons obtained from D_3KO and singenic wild-type mice were processed in separated but parallel culture wells within the same experiment. Either cocaine (10 μM), the D_3R agonist 7-OH-DPAT (5 nM), or BDNF (10 ng/mL) were administered 72 h before morphological assessment to induce structural plasticity. Two-way ANOVA was used to assess the 'genotype × treatment' effect. Maximal dendrite length was affected by genotype [$F(1,229) = 3.84$; $p < 0.05$] and by treatment [$F(3,229) = 19.42$; $p < 0.0001$], the interaction being not significant. *Post hoc* assessment showed that cultures prepared from wild-type mice responded significantly to cocaine, 7-OH-DPAT and BDNF, while cultures prepared from D_3KO mice significantly responded only to BDNF (Bonferroni's test, $p < 0.001$ vs. control), but not to cocaine or 7-OH-DPAT (Fig. 3a). A similar profile was observed for the number of primary dendrites, showing a significant effect of genotype [$F(1,392 = 29.95$; $p < 0.0001$], treatment [$F(3,392) = 14.89$;

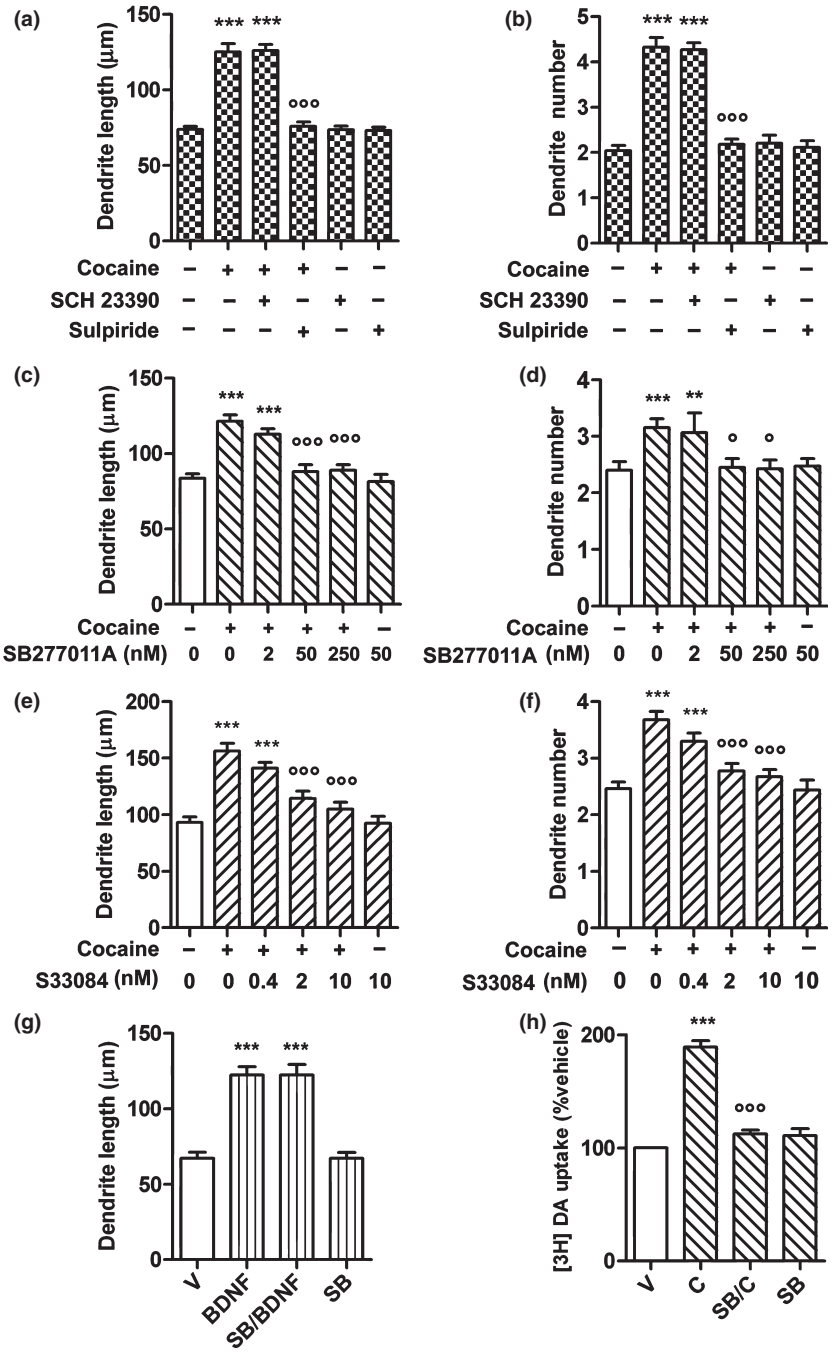

Fig. 2 Pharmacological studies with DA antagonists in primary mesencephalic dopaminergic neurons studied 72 h after cocaine exposure. (a, b) Effects of SCH23390 (1 μM), sulpiride (5 μM) or vehicle added 20 min before cocaine (10 μM) on maximal dendrite length and number of primary dendrites were shown. Treatments were indicated by the (+) symbols, vehicle by the (–) symbol on the *x*-axis. (c, d) Dose-dependent inhibition produced by the D_3R antagonist SB-277011-A (2, 50, 250 nM) added 20 min before cocaine (10 μM) on maximal dendrite length and number of primary dendrites. On the x-axis the treatments with cocaine or vehicle were indicated by the (+) or (–) symbols, respectively, while SB-277011-A doses with figures. (e, f) Dose-dependent inhibition produced by the D_3R antagonist S-33084 (0.4, 2, 10 nM) added 20 min before cocaine (10 μM) on maximal dendrite length and number of primary dendrites. On the *x*-axis, the treatments with cocaine or vehicle were indicated by the (+) or (–) symbols, respectively, while S-33084 doses with figures. (g) Lack of inhibition on maximal dendrite length produced by SB-277011-A (50 nM) added 20 min before BDNF (10 ng/mL) whose administration produced significant morphologic effects versus vehicle. (h) [^{3}H]Dopamine uptake enhancement produced by cocaine (10 μM) and blocking effect of the pre-treatment with SB-277011-A expressed as percentage of vehicle values. Experiments were replicated three times. All analyses reported in the figure were performed assessing cultures 72 h after treatments. Values are represented as mean ± standard error of the mean (SEM) (***$p < 0.001$ vs. vehicle; **$p < 0.01$ vs. vehicle; $^{ooo}p < 0.001$ vs. cocaine; $^{o}p < 0.05$ vs. cocaine; *post hoc* Bonferroni's test).

$p < 0.0001$], and also a significant interaction [$F(3,384) = 4.37$; $p < 0.005$] (Fig. 3b). *Post hoc* assessment showed that in cultures prepared from wild-type mice cocaine, BDNF and 7-OH-DPAT produced morphological changes, while cultures prepared from D_3KO mice significantly responded only to BDNF (Bonferroni's test, $p < 0.001$ vs. control), but not to cocaine or 7-OH-DPAT (Fig. 3b). Also soma area showed significant genotype [$F(1,231 = 9.94$; $p < 0.002$] and treatment effect [$F(3,231) = 9.09$; $p < 0.0001$], while interaction was not significant. *Post hoc* assessment showed that cultures prepared from wild-type mice responded significantly to cocaine, 7-OH-DPAT and BDNF, while cultures prepared from D_3KO mice significantly responded only to BDNF (Bonferroni's test, $p < 0.001$ vs. control), but not to cocaine or 7-OH-DPAT (Fig. 3c). This dissociation indicates that neurons carrying a D_3R mutation are still capable of morphological response to neurotrophic adaptive stimuli.

Cocaine-induced structural plasticity: ex vivo studies in wild-type and D_3KO mice

The translational relevance of the observed structural plasticity of dopaminergic primary neurons *in vitro* was investigated by exposing wild-type and D_3KO mouse embryos *in uterus* to a regimen of repeated daily treatment with cocaine and by analyzing their soma size in VTA and SN (Fig. 4a and b). Cocaine increased the soma area of TH-IR neurons in VTA of wild-type mice but did not in the D_3KO mice, as supported by highly significant genotype effects [$F(1,32 = 79.53$; $p < 0.0001$], treatment effects [$F(1,32 = 39.05$; $p < 0.0001$], and 'genotype × treatment' interaction [$F(1,28 = 49.18$; $p < 0.0001$] (Fig. 4c). Similar results were obtained assessing the soma area of dopaminergic neurons in SN showing a highly significant genotype effects [$F(1,32) = 86.69$; $p < 0.0001$], treatment effects [$F(1,32) = 73.76$; $p < 0.0001$] and 'genotype × treatment' interaction [$F(1,28 = 73.76$; $p < 0.0001$] (Fig. 4d).

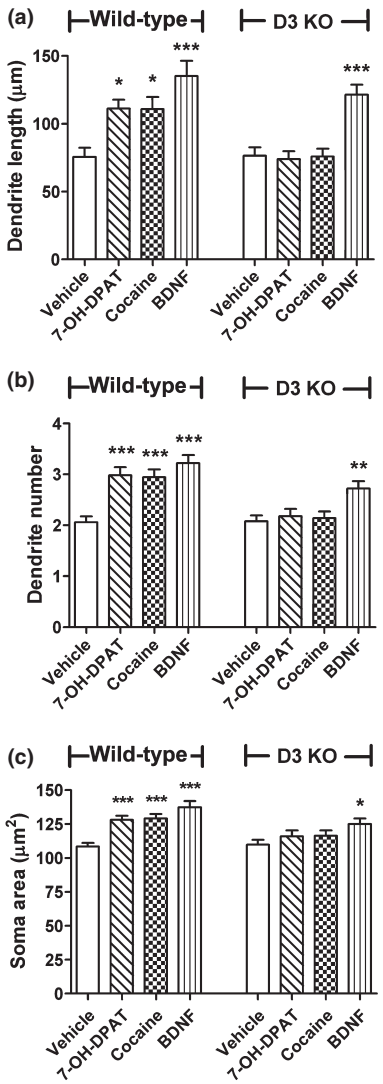

Fig. 3 Morphological effects of cocaine on TH-IR neurons from wild-type and D_3KO mice 72 h after treatment. Mesencephalic neurons are exposed to cocaine (10 μM), 7-OH-DPAT (5 nM) or BDNF (10 ng/mL) and the following endpoints are measured: maximal dendrite length (a), number of primary dendrites (b), soma area (c). (***$p < 0.001$; **$p < 0.01$; *$p < 0.1$ vs. vehicle; *post hoc* Bonferroni's test). In all panels, values are represented as mean ± standard error of the mean (SEM).

Intracellular ERK and Akt pathway activation by cocaine in primary dopaminergic neurons of mesencephalic cultures

The effect of cocaine on ERK phosphorylation was investigated by challenging mesencephalic neurons with 10 μM cocaine. Western blot analysis (one-way ANOVA) showed a rapid increase of p-ERK [$F(4,25) = 7.05$; $p < 0.001$] with a significant peak activity versus vehicle at 2 min (Bonferroni's test, $p < 0.001$) (Fig. 5a and b). Incubation with SB-277011-A (50 nM) 20 min before challenge significantly attenuated the p-ERK increase [$F(1,16) = 12.28$; $p < 0.001$] (Fig. 5c and d) providing evidence of D_3R involvement. No effect on p-ERK was produced by SB-277011-A when tested alone. In parallel experiments, cellular localization of p-ERK in

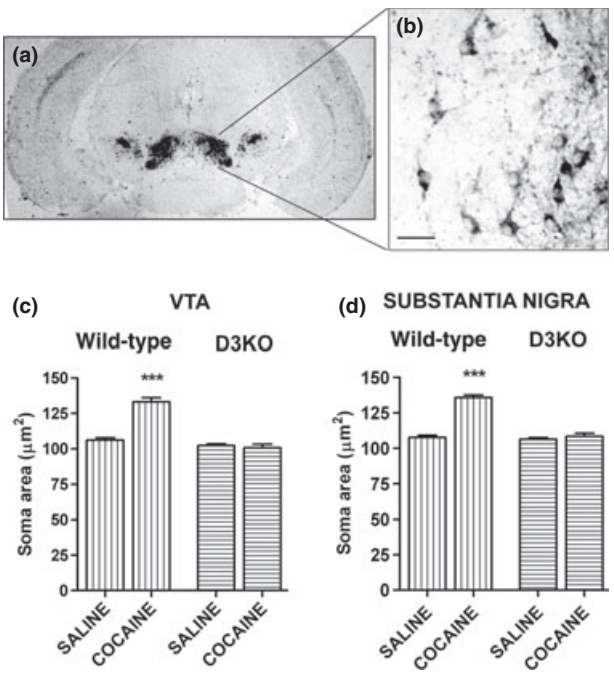

Fig. 4 Effects of prenatal cocaine exposure on structural plasticity of dopaminergic neurons of wild-type and D_3KO newborn mice. (a) Representative low-magnification photomicrograph of a coronal brain section of a P1 mouse exposed to daily treatment with cocaine (20 mg/kg × 2 ip) *in utero* from E12.5 to E17.5. Sections were immunostained with anti-TH antibody to identify dopaminergic neurons in ventral tegmental area (VTA) and substantia nigra (SN). (b) High magnification of TH-IR dopaminergic neurons from VTA whose soma area was investigated. (c, d) Cocaine effects on the soma area of TH-IR neurons in the VTA and SN, respectively, of wild-type and D_3KO mice. In all panels, values are represented as mean ± standard error of the mean (SEM) (***$p < 0.001$ vs. saline; *post hoc* Bonferroni's test). Scale bar: 60 μm.

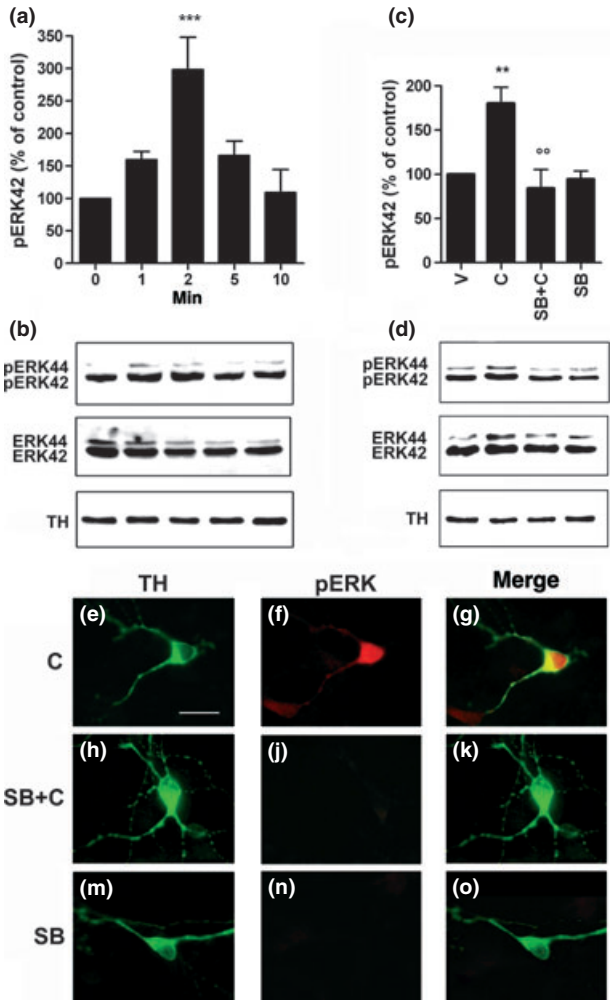

Fig. 5 Extracellular signal-regulated kinase (ERK) phosphorylation produced by cocaine challenge in mesencephalic TH-IR neurons and its reduction by pre-treatment with SB-277011-A. (a, b) Time course of ERK phosphorylation induced by cocaine (10 μM) at 0, 1, 2, 5, and 10 min. Cell lysates were processed for western blot and membranes stripped and re-probed as described in Methods. (a) Densitometric analysis of blots (n = 6) with specific levels of p-ERK normalised to the corresponding ERK and TH levels; (b) representative time–course blot. (c, d) Effects of SB-277011-A (50 nM) or vehicle added to the cultures 20 min before challenge with cocaine (10 μM) assessed after 2 min. (c) Densitometric analysis of blots (n = 6) normalised as described and (b) representative blot. (e–o) Immunofluorescence microphotographs of TH-IR (e, h, m), p-ERK-IR (f, j, n), and their colocalization (g, k, o) in mesencephalic dopaminergic neurons 2 min after treatment with cocaine (e–g), SB-277011-A followed by cocaine (h–k) or SB-277011-A alone (m–o). Scale bar: 20 μm. V, vehicle; C, cocaine; SB, SB-277011-A. Densitometric values were represented as percentage of vehicle values as mean ± SEM (***$p < 0.001$, **$p < 0.01$ vs. vehicle; $^{oo}p < 0.01$ vs. cocaine *post hoc* Bonferroni test).

dopaminergic neurons was investigated 2 min after cocaine exposure using double immunofluorescence. Cocaine activated p-ERK-IR in TH-IR neurons (Fig. 5e–g), while its effect was blocked by pre-treatment with SB-277011-A (Fig. 5h–k). In control experiments, incubation with SB-277011-A alone did not affect p-ERK expression (Fig. 5m–o).

The effect of cocaine on Akt phosphorylation was investigated by challenging mesencephalic neurons with 10 μM cocaine. Western blot analysis showed a rapid increase of Akt phosphorylation [$F(4,14) = 16.40$; $p < 0.001$] reaching the peak activity at 2 min exposure (Bonferroni's test. $p < 0.01$) (Fig. 6a and b). Incubation with SB-277011-A (50 nM) 20 min before challenge significantly attenuated the increase of Akt phosphorylation produced by cocaine [$F(1,12) = 22.78$; $p < 0.001$] (Fig. 6c and d).

In parallel experiments, cellular localization of p-Akt in dopaminergic neurons was investigated 2 min after cocaine exposure by double immunofluorescence. As TH and p-Akt antibodies were both generated in rabbit, a rat monoclonal antibody to DAT was used instead. These experiments showed that cocaine activated the expression of p-Akt-IR in DAT-IR neurons (Fig. 6e–g), while no p-Akt-IR was found in DAT-IR neurons after pre-treatment with SB-277011-A (Fig. 6h–k). In control experiments incubation with SB-277011-A alone did not affect p-Akt expression (Fig. 6m–o).

Lack of activation of ERK and Akt pathways induced by cocaine and D_3R agonist 7-OH-DPAT in primary mesencephalic cultures from D_3KO mice

Mesencephalic neurons obtained from embryos of D_3KO and singenic wild-type mice were processed in separated cultures within the same experiment. The effect of cocaine (10 μM) or 7-OH-DPAT (50 nM) on ERK phosphorylation was investigated using western blot analysis 2 min after treatment. Cocaine and 7-OH-DPAT significantly increased p-ERK in wild-type mice but not in D_3KO mice as indicated by the two-way ANOVA highly significant genotype effect [$F(1,18) = 165.8$, $p < 0.0001$], treatment effect [$F(2,18) = 35.38$, $p < 0.0001$], and 'treatment × genotype' interaction [$F(1,14) = 64.38$; $p < 0.0001$] (Fig. 7a). Similar response profiles were observed by studying p-Akt in mesencephalic neurons. Increase of p-Akt was produced by cocaine and 7-OH-DPAT in cultures from wild-type mice, while no effect was observed in mesencephalic neurons from D_3KO mice, as supported by two-way ANOVA significant genotype effect [$F(1,18) = 35.81$, $p < 0.0001$], treatment effect [$F(2,18) = 8.33$, $p < 0.005$], and 'treatment × genotype' interaction [$F(1,14) = 9.23$; $p < 0.0001$] (Fig. 7b).

Inhibition of PI3K in primary mesencephalic cultures blocks cocaine-dependent ERK and Akt pathway activation and prevents structural plasticity

The involvement of PI3K-ERK and -Akt pathways in dendrite outgrowth and soma size changes produced by cocaine was further studied using the PI3K inhibitor LY294002. Twenty minutes pre-treatment with LY294002 (10 μM) antagonized the significant increase of p-ERK levels [$F(3,16) = 9.71$;

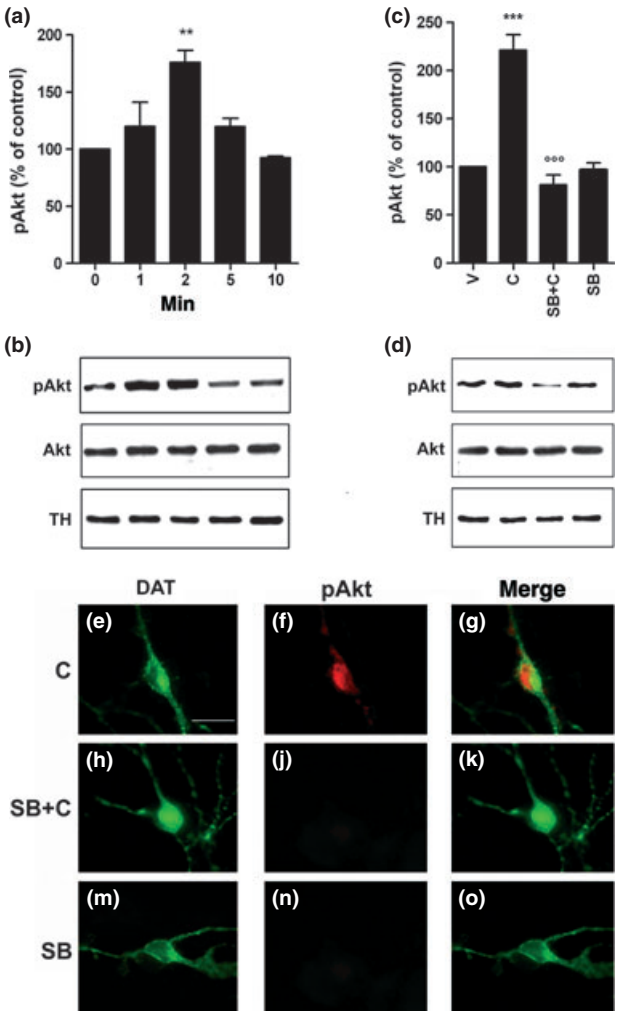

Fig. 6 Thymoma viral proto-oncogene (Akt) phosphorylation produced by cocaine in mesencephalic TH-IR neurons and its reduction by pre-treatment with SB-277011-A. (a, b) Time course of Akt phosphorylation induced by cocaine (10 μM) at 0, 1, 2, 5, and 10 min. Cell lysates were processed for western blot and membranes stripped and re-probed as described in Methods. (a) Densitometric analysis of blots ($n = 3$) with specific levels of p-Akt normalised to the corresponding Akt and TH levels; (b) representative time-course blot. (c, d) Effects of SB-277011-A (50 nM) or vehicle added to the cultures 20 min before challenge with cocaine (10 μM) assessed after 2 min. (c) Densitometric analysis of blots ($n = 3$) normalised as described and (b) representative blot. (e–o) Immunofluorescence microphotographs of DAT-IR (e, h, m), p-Akt-IR (f, j, n), and their colocalization (g, k, o) in mesencephalic dopaminergic neurons 2 min after treatment with cocaine (e–g), SB-277011-A followed by cocaine (h–k), or SB-277011-A alone (m–o). Scale bar: 20 μm. V, vehicle; C, cocaine; SB, SB-277011-A. Densitometric values were represented as percentage of vehicle values as mean ± SEM (***$p < 0.001$, **$p < 0.01$ vs. vehicle; °°°$p < 0.001$ vs. cocaine *post hoc* Bonferroni test).

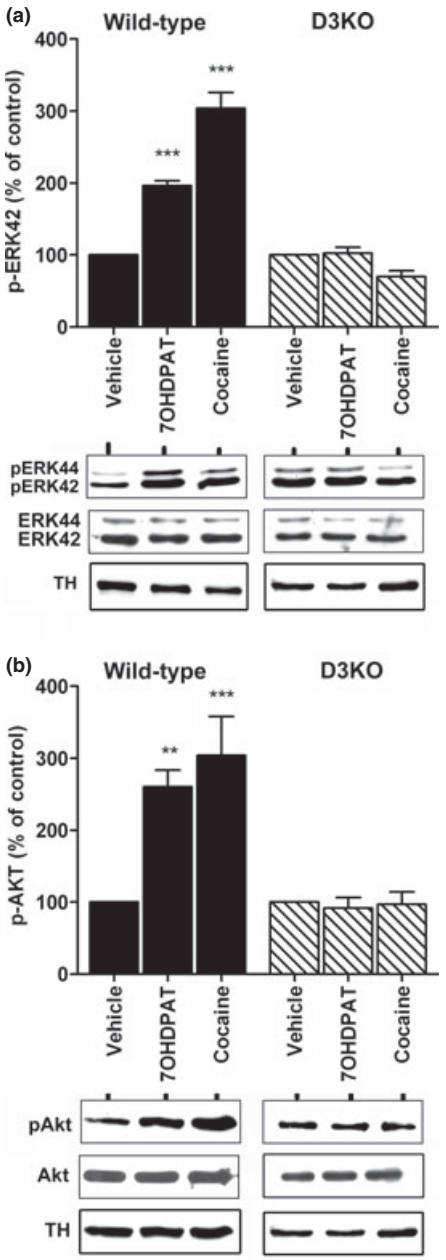

Fig. 7 Effect of cocaine and the D_3R agonist 7-OH-DPAT on ERK and Akt phosphorylation in mesencephalic cultures from wild-type and D_3KO mice. Cocaine was used at 10 μM and 7-OH-DPAT at 50 nM. Cell lysates were processed for western blot 2 min after challenge and membranes stripped and re-probed as described in Methods. (a) Densitometric analysis of blots ($n = 4$) with specific levels of p-ERK normalised to the corresponding ERK and TH levels; in the lower panel a representative blot is shown. (b) Densitometric analysis of blots ($n = 3$) with specific levels of p-Akt normalised to the corresponding Akt and TH levels; in the lower panel a representative blot is shown (***$p < 0.001$, **$p < 0.01$ vs. vehicle; *post hoc* Bonferroni test).

$p < 0.01$] (Fig. 8a) and p-Akt levels [$F(3,16) = 49.76$; $p < 0.001$] (Fig. 8b) measured by western blot in mesencephalic cultures 2 min after the challenge with cocaine (10 μM). Similar results were obtained in parallel experiments using double-staining immunofluorescence aimed to localize the expression of either p-ERK-IR or p-Akt-IR in

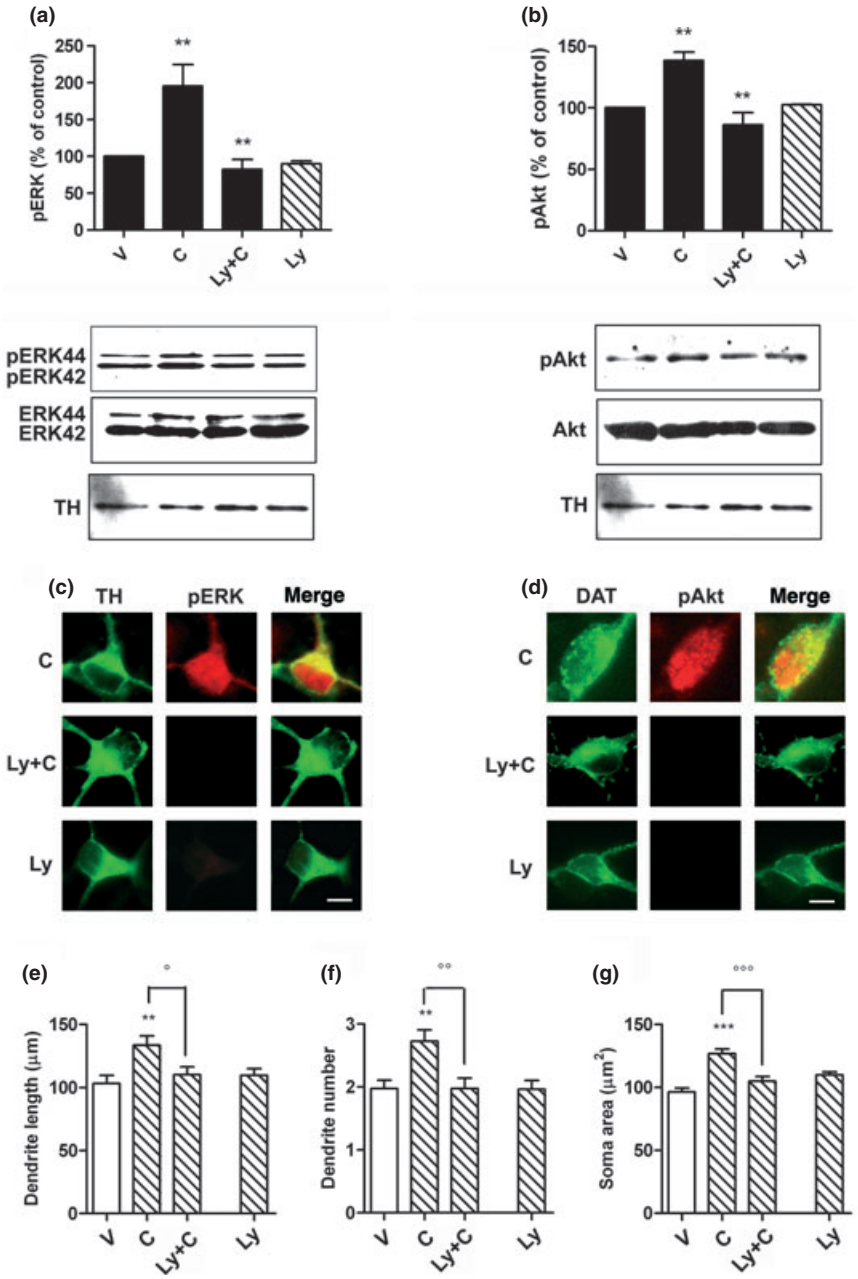

Fig. 8 Effect of phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002 on ERK and Akt phosphorylation and structural plasticity in mesencephalic dopaminergic neurons. (a–d) In the typical phosphorylation experiment, LY294002 (10 μM) or vehicle were added to the cultures 20 min before challenge with cocaine (10 μM) and assessed after 2 min. (a, b) Cell lysates were processed for western blot and membranes stripped and re-probed as described in Methods. (a) Densitometric analysis of blots ($n = 4$) with specific levels of p-ERK normalised to the corresponding ERK and TH levels; in the lower panel a representative blot is shown. (b) Densitometric analysis of blots ($n = 4$) with specific levels of p-Akt normalised to the corresponding Akt and TH levels; in the lower panel a representative blot is shown. Densitometric values were represented as percentage of vehicle values as mean ± SEM (c, d) Double immunofluorescence microphotographs of cellular localization of p-ERK and p-Akt in mesencephalic dopaminergic neurons. Dopaminergic neurons were identified (c) with TH-IR for p-ERK double staining and (d) with DAT-IR for p-Akt as described in the Methods. Scale bar: 15 μm. (e–g) Morphological effects of PI3K inhibition on cocaine-induced structural plasticity assessed as (e) maximal dendrite length, (f) primary dendrite number and (g) soma area. In a typical experiment, LY294002 (10 μM) or vehicle were added to the cultures 20 min before exposure to cocaine (10 μM) and measurements were performed after 72 h. V, vehicle; C, cocaine; Ly, LY294002 (***$p < 0.001$, **$p < 0.001$ vs. vehicle; $^{ooo}p < 0.001$, $^{oo}p < 0.01$, $^{o}p < 0.05$ vs. cocaine *post hoc* Bonferroni test).

dopaminergic neurons. Pre-treatment with LY294002 completely antagonized cocaine-induced p-ERK-IR (Fig. 8c) and p-Akt-IR (Fig. 8d) in dopaminergic neurons at 2 min after challenge. No effects on ERK or Akt phosphorylation were observed with LY294002 incubation alone (Fig. 8c and d).

The effects on cocaine-induced structural plasticity of the same dose of LY294002 used to block ERK and Akt phosphorylation were investigated at 72h after exposure to cocaine. LY294002 significantly attenuated cocaine effects on dendrite outgrowth and soma area (Fig. 8e–g). Accordingly, when maximal dendrite length was measured, two-way ANOVA indicated significant cocaine effect [$F(1,116) = 4,00$; $p < 0.05$] and interaction [$F(1,112) = 6,71$; $p < 0.02$], the *post hoc* test showing LY294002 attenuating the effect of cocaine (Bonferroni's test, $p < 0.05$) (Fig. 8e). When data from number of primary dendrites were investigated, two-way ANOVA indicated significant cocaine effect [$F(1,116) = 4,47$; $p < 0.05$] and interaction [$F(1,112) = 3,89$; $p < 0.05$], the *post hoc* test showing again the LY294002 blocking effect on the response to cocaine (Bonferroni's test, $p < 0.01$) (Fig. 8f). Similar result were observed for the soma areas, two-way ANOVA showing highly significant cocaine effect [$F(1,116) = 13.06$; $p < 0.0001$] and interaction [$F(1,112) = 30.55$; $p < 0.001$]. The *post hoc* test showed significant attenuation of cocaine effects by LY294002 (Bonferroni's test, $p < 0.001$) (Fig. 8g). For all measurements, no effect was observed when LY294002 was tested alone (Fig. 8e–g).

Discussion

The present study demonstrates that long-term exposure to cocaine increased soma area and dendrite arborisation of mesencephalic dopaminergic neurons via a mechanism dependent on dopamine D_3R activation. These morphological changes were detected both *in vitro,* using primary neuronal culture preparations from E12.5 mouse embryo mesencephalon, and *in vivo,* in the VTA and SN of P1 offspring prenatally exposed to cocaine during the same embryogenic period. The *in vitro* studies were conducted using a validated protocol of enriched primary neuronal cultures containing 5–7% TH-IR or DAT-IR neurons and less than 0.5% astrocytes (Collo *et al.* 2008). The maximal dendrite length and the number of primary dendrite were significantly increased 24 hours after cocaine exposure, while no significant effect was produced on the soma area, the proper increase occurring only at 72 h. The delayed effect in soma was probably related to differences in the geometry of soma surface-volume versus dendrites, and to the different molecular substrates associated with their growth: apposition of new membrane and development of Golgi outposts for the remodelling of primary dendrites (Horton *et al.* 2005), and changes of neurofilament proteins and of global axonal trafficking for the growth of the soma size (Nestler *et al.* 1993; Russo *et al.* 2007).

The morphological changes produced by 1–10 μM cocaine were paralleled by a significant increase of the [^{3}H]-DA uptake, indicating an augmented capacity of synaptic, dendritic and somatic DA transport. Interestingly, up-regulation of DAT was reported in the VTA and SN of rodents and monkeys exposed to cocaine *in utero* (Fang and Ronnekleiv 1999; Glatt *et al.* 2000). Increases of dendrite arborization, soma area and DA uptake in mesencephalic neurons were previously observed following exposure to 1–10 μM amphetamine (Mueller *et al.* 2006; Collo *et al.* 2008), another psychostimulant that increases extracellular DA levels (Rouge'-Pont *et al.* 1999).

In the present work, we investigated the relevance of specific subtypes of DA receptors that could be engaged by the increased extracellular DA levels produced by cocaine at first using pharmacologic antagonists and then knockout mice. An involvement of D_1R was excluded by showing that the selective antagonist SCH23390 was inactive, consistent with the known absence of expression of D_1R in mesencephalic dopaminergic neurons. Evidence for an involvement of D_2R and/or D_3R was obtained with sulpiride, which possesses similar affinity for both receptors: sulpiride suppressed the increases of dendrite arborization and soma area produced by cocaine. These effects were reproduced with the preferential D_3R antagonist SB-277011-A at concentration close to its affinity for D_3R (50 nM; Reavill *et al.* 2000) and confirmed using the more potent and selective D_3R antagonist S-33084 (Millan *et al.* 2000) at concentration as low as 2 nM. These results were in line with the blocking effect of low dose SB-277011-A on the morphological changes generated in mesencephalic dopaminergic neurons by amphetamine (Collo *et al.* 2008). Interestingly, in the present work we showed that pre-treatments with SB-277011-A did not block the increase of dendrite outgrowth and soma size produced by the neurothrophin BDNF that engages a different receptor but similar intracellular signalling pathways (Goggi *et al.* 2003).

The critical role of D_3R for structural plasticity induced by cocaine was confirmed by testing mesencephalic cultures prepared from D_3KO mice embryos. Dopaminergic neurons from D_3KO mice did not respond to cocaine and 7-OH-DPAT, a selective D_3R antagonist, while neurons from wild-type mice did. Interestingly, BDNF was effective increasing dendrite arborisation and soma size in both D_3KO and wild-type dopaminergic neurons, suggesting that the neurotrophin-operated signalling was indeed functional in D_3KO mice.

Independent support for a role of D_3R was obtained by the exploration of intracellular pathways activated by D_3R via PI3K in cell lines, which results in phosphorylation of ERK and Akt (Cussac *et al.* 1999; Beom *et al.* 2004; Mannoury la Cour *et al.* 2011) and other downstream genes known to participate to cytoskeletal regulations (Qian *et al.* 2004). More recently, using primary cultures of mesencephalic dopaminergic neurons, we showed that LY294002 blocked

D_3R agonist-dependent ERK phosphorylation within minutes, and D_3R agonist-dependent dendrite outgrowth after 72 h incubation (Collo *et al.* 2008). In the present work, we showed that exposure to cocaine produced a rapid phosphorylation of both ERK and Akt peaking after 2 min, increasing dendrite outgrowth and soma area after 72 h incubation. Cellular localization within dopaminergic neurons was supported by double-staining immunofluorescence obtained with antibodies recognizing dopaminergic markers (TH or DAT) and the phosphorylated kinases (p-ERK, p-Akt), respectively. These data are in line with evidence of psychostimulants increasing ERK phosphorylation *in vivo*, in particular in the VTA (Dietz *et al.* 2009), and producing different effects on PI3K phosphorylation depending to the brain regions (Zhang *et al.* 2006). To our knowledge, the present data are the first showing a critical role for PI3K/Akt activation in the structural plasticity effects of cocaine in dopaminergic neurons of the mesencephalon. The direct involvement of PI3K/Akt and ERK in dendrite outgrowth was previously shown in hippocampal neurons (Jaworski *et al.* 2005; Kumar *et al.* 2005), while its role of regulator of soma area was described in a variety of neurons (Scheidenhelm *et al.* 2005). In particular, genetic or pharmacologic manipulations aimed to reduce or increase activity of PI3K/Akt in dopaminergic neurons were associated with soma area reduction (Russo *et al.* 2007) and enhancement (Domanskyi *et al.* 2011; Kim *et al.* 2011), respectively. In the present work, we describe soma area increase in mesencephalic dopaminergic neurons of wild-type newborn mice after *in utero* exposure to cocaine. We also showed that this effect was absent in D_3KO newborn mice, corroborating the observations made in primary cultures and supporting the need of a viable D_3R to mediate morphological effects of cocaine. Structural plasticity depending on prenatal cocaine exposure was consistently described in prefrontal cortical neurons (Morrow *et al.* 2007; Lu *et al.* 2009); our data open to the possibility that changes in mesencephalic neurons could participate to the long-lasting behavioral liabilities to psychostimulants in adult life (Crozatier *et al.* 2003). However, more data are required to support this view. In this study, performed on newborns at P1, the prenatal effects of cocaine were observed in the dopaminergic neurons of both VTA and SN. This result was at variance with observations made in published studies on young adults (P-30-45 or older), when cocaine changes are restricted to VTA (Mueller *et al.* 2006; Russo *et al.* 2007; Sarti *et al.* 2007; Spiga *et al.* 2010), suggesting dependency on developmental stage.

Overall, the present investigation provided the first direct evidence that cocaine increases dendritic arborization and soma size in mesencephalic dopaminergic neurons, a phenomenon that could contribute to the maladaptive structural plasticity that is believe to constitute a pathogenetic drive for addiction (Nestler *et al.* 1993; Robinson and Kolb 2004; Sarti *et al.* 2007; Russo *et al.* 2010). Using *in vitro* and *in vivo* protocols, pharmacologic tools, knockout mice and intracellular signaling we provided evidence supporting a critical role of the DA D_3R in mediating cocaine-dependent structural plasticity in mesencephalic dopaminergic neurons. A robust body of pre-clinical data supports a role for D_3R in psychostimulant addiction, with D_3R antagonists preventing relapse to drug-taking in abstinent individuals (Caine and Koob 1993; Pilla *et al.* 1999; Sokoloff *et al.* 2001; Heidbreder *et al.* 2005). Therefore, it is possible that D_3R antagonists operate, among other mechanisms, via modifications of cocaine-induced structural plasticity in the mesencephalic dopaminergic neurons. Recent evidence obtained with Positron Emission Tomography indicates that the most abundant localization of D_3R in humans brain is in the ventral mesencephalon, where VTA and SN are located (Searle *et al.* 2010). These data further support the idea that targeting dopaminergic neurons with D_3R medicaments could provide novel therapeutics approach to psychostimulant addiction.

Acknowledgements

The authors thank Marzia Melotti for technical support. This study was supported by grant from ex 60%, University of Brescia to GC, and by grant from MIUR (PRIN 2008) to CM. EMP is full-time employee of GlaxoSmithKline. MJM. is full time employee of Institut de Recherches Servier. The other authors declare no conflict of interest in this work.

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