Calcium-related signaling pathways contributed to dopamine-induced cortical neuron apoptosis

Ling Zhang *,1, Hui Yang 1, Huanying Zhao, Chunli Zhao

Department of Neurobiology, Beijing institute of Neuroscience, Capital Medical University, Beijing, China

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ABSTRACT

Accumulating pathological evidence showing layer-specific neuronal reduction, dendrite deficits and brain volume loss have implicated an apoptotic process in schizophrenia, but the exact mechanism remains elusive. Dopamine hyperactivity at D2 receptor sites was considered as an important mechanism in schizophrenia pathogenesis. Recently, a newly identified D1 and D2 receptor heterooligomer activated by the specific agonist SKF83959 has been shown to stimulate phospholipase C-related intracellular calcium release in the brain. In this study, we intend to test the hypothesis that overstimulation of this calcium-related signaling pathway by high concentration of dopamine and SKF83959 is capable of inducing cortical neuronal apoptosis through calcium disturbance. Our experimental results demonstrated that 10–100 μM dopamine and 10–50 μM SKF83959 treatments for 72 h were able to induce cortical neuronal apoptosis via the D1 and D2 receptor heterooligomer mediated calcium overload and mitochondria dysfunction-dependent pathways. Meanwhile, we found that although 24 h dopamine and SKF83959 treatments have not produced major apoptosis, they induced significant neuronal dendrite retraction as well as reduction of neurotrophic molecules such as phosphorylated AKT, ERK and Bcl-2 through PLC-sensitive pathways. Taken together, prolonged stimulation of dopamine and SKF83959 in cortical neurons can reduce dendrite extension at early stage and induce neuronal apoptosis later on through PLC–calcium related pathways, which might provide important apoptotic mechanisms for schizophrenia pathogenesis.

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1. Introduction

Schizophrenia is known as one of the most deteriorating psychiatric disorders so far, which can intensively affects a wide range of higher human cognitive functions such as attention, motivation, execution and emotion. In spite of decades of research efforts, its exact pathological mechanisms remain elusive (Ross et al., 2006). Neuropathological data on postmortem brains have suggested that layer-specific synaptic and dendrite deficits and neuronal reduction occurred in several brain regions including prefrontal cortex, hippocampus, and thalamus in schizophrenia patients (Benes et al., 1986; Selemon et al., 1995; Perez-Neri et al., 2006). Functional neuroimaging studies have also shown progressive gray matter loss and enlargement of brain ventricle early in the disorder (Lawrie and Abukmeil, 1998; Zipursky et al., 1998; Hulshoff Pol and Kahn, 2008; Cahn et al., 2009; Crespo-Facorro et al., 2009). Assays of postmortem tissues have found a lower Bcl-2/Bax ratio and an absolute reduction in Bcl-2 levels in temporal cortex in schizophrenia (Jarskog et al., 2005). Based on these findings, an apoptotic mechanism in the pathophysiology of schizophrenia appears increasingly plausible. However, how the apoptotic mechanisms are actually invoked during pathogenesis remains unclear.

Prefrontal cortex controls multiple cognitive functions of human brains and dysregulation of dopamine system in this area appears to underlie several cognitive impairments in schizophrenia (Goldman-Rakic and Selemon, 1997; Lidow, 2003). Dopamine hyperactivity hypothesis of schizophrenia is mainly based on the psychomimetic effects of dopamine enhancing drugs such as amphetamine, phencyclidine and ketamine and strong D2 antagonists shared by most effective antipsychotic drugs (Carlsson et al., 2000; Laruelle et al., 2003). Neuroimaging studies using radio tracers provided further evidence that more dopamine...
neurotransmitter release and D2 receptor binding high states were detected in schizophrenia brain and disease models (Benes, 2000; Seeman and Kapur, 2000; Takahashi et al., 2006; Seeman et al., 2007; Lehrer et al., 2010). Several animal disease models further testified the hyperdopaminergic state in the prefrontal cortical area and striatum (Javitt and Zukin, 1991; Lindefors et al., 1997; Balla et al., 2001, 2003; Sershen et al., 2008). Since the over-released dopamine at prefrontal cortical by disinhibited dopaminergic terminal in schizophrenia patients can be removed by dopamine transporter (DAT) reuptake and monoamine oxidase (MAO) metabolism, the high rise of dopamine at cortical synapses should be both tonic and phasic events (Waymert et al., 2001). Although the involvement of dopamine in neuronal toxicity has been reported by both in vivo and in vitro studies, the exact mechanisms via oxidation or dopamine receptor actions remain controversial, depending on drug concentration, time of exposure, different brain regions and cell types (McLaughlin et al., 1998; Cheng et al., 1996; Jones et al., 2000; Jiang et al., 2008; Chen et al., 2009). Despite that dopamine oxidation might contribute partially to the neuronal apoptosis in schizophrenia (Wood et al., 2009), the stronger neuroprotective effects provided by the antipsychotic drugs suggested that dopamine receptor blockade especially at D2-related site might also be a major component in the prevention of neurodegeneration and alleviating symptoms (Murphy et al., 1956; Jann, 2004; Perez-Neri et al., 2006; Hulshoff Pol and Kahn, 2008; Kim et al., 2008; Park et al., 2008).

The adenylcyclase-CAMP pathway has long been considered as the major signaling cascade following classical dopamine D1 and D2 receptor activation. Recently, D1 and D2 receptor hetero-oligomer has been shown to specifically activate phospholipase C (PLC) signaling and intracellular calcium store release in the brain (George and O'Dowd, 2007; Ming et al., 2006; Rashid et al., 2007). SKF83959 acts as the specific agonist of this D1 and D2 receptor complex, which showed distinct pharmacological profile from classical D1 and D2 receptor agonist such as SK38393 and Quinpirole (Jin et al., 2003). Multiple evidence from postmortem schizophrenia brains have demonstrated that disturbed calcium and mitochondrial dysfunction are present in the prefrontal cortical area (Lidow, 2003; Novak et al., 2006; Reniz et al., 2009; Martins-de-Souza et al., 2009). It was also reported that atypical antipsychotics were capable of inhibiting dopamine and IP3 sensitive calcium release in cortical neurons, suggesting that control of calcium release through D2 related sites is associated with alleviation of schizophrenia symptoms (Szczekan and Strumwasser, 1996). Given the disturbances of calcium homeostasis and mitochondrial dysfunction were the common causes for neurodegeneration in several pathological conditions such as stroke, ischemia–reperfusion, Alzheimer's disease and Huntington's disease (Mattson et al., 1992; Tang et al., 2005), it is possible that overstimulation of this unique calcium-related D1–D2 dopaminergic receptor signaling cascade can also induce neuronal death, which might contribute to the apoptotic process in schizophrenia. In addition, alterations in several important neuronal survival and dendrite growth promoting molecules such as AKT, ERK and Bcl-2 and massive neurite retraction have been reported in the brains of schizophrenia patients and animal models (Arguello and Gogos, 2008; Jarskog et al., 2005; Enomoto et al., 2005; Hansen et al., 2004; Pereira et al., 2009), therefore stimulation of this dopamine receptor pathway probably might also make cortical neurons lose dendritic structures and increase apoptotic vulnerabilities through downregulation of these relevant molecules in disease progression.

In the present study, we used primary culture of cortical neurons as a model to investigate whether prolonged dopamine and SKF83959 treatments could induce neuronal apoptosis through D1D2 receptor mediated calcium disturbances and mitochondrial dysfunction. Furthermore, we evaluated whether short-term dopamine and SKF83959 treatments in cortical neurons can affect AKT, ERK signaling components and Bcl-2 family proteins without induction of apoptosis, thus cause shortening of neuronal dendrites and increasing vulnerabilities.

2. Materials and methods

2.1. Reagents

Neurobasal medium and B27 were purchased from Invitrogen (Carlsbad, CA). Polycional rabbit anti-glutamate and GABA antibodies came from Chemicon (Temecula, CA); monoclonal mouse β-actin antibody was purchased from Proteintech group (Chicago, IL). Alex-488 conjugated goat anti-mouse IgG, Alex-488 conjugated donkey anti-rabbit IgG and Alex-594 conjugated donkey anti-goat IgG, DAPI and propidium iode were purchased from Molecular Probe (Eugene, OR). Monoclonal mouse anti-phospho-ERK1/2 (Thr202/Tyr204), polyclonal rabbit anti-ERK1/2, mouse-anti-phosph-ASK (serine 473) and rabbit-anti-AKT antibody were purchased from Cell Signaling, Inc. (Beverly, MA); Mouse monoclonal antibody against MAP-2, Bax and rabbit polyclonal antibody against Bcl-2 were from Sigma (St. Louis, MO). Dopamine, SKF93959, SKF83939, DOI, quinpirole, Forskolin, SCH23390, Sulpiride, U73122, Z-APB, BAPTA-AM, ruthenium red, bongkrekic acid, Vitamin E, pan-caspase inhibitor Z-VAD-FMK were all from Sigma (St. Louis, MO). Innocyte cytochrome C releasing kit was from Calbiochem (Gibbstown, NJ); Caspase-GLO3/7 assay kit was from Promega (Madison, WI). Other chemicals were purchased from the Chinese Chemical Co.

2.2. Primary culture of cortical neurons

Timed pregnant Sprague–Dawley rats were purchased from the Experimental Animal Center at Beijing University Medical School (Beijing, China) and housed under standard laboratory conditions. Experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of Capital Medical University. Rat fetuses were removed on embryonic day 18 from maternal rat killed by intraperitoneal injection of 6% chloral hydrate. The fetal cortex was dissected in Hank's balanced salt solution (HBSS), followed by digestion in 0.25% trypsin at 37 °C for 15 min with occasional shaking. After inactivation of trypsin by the serum containing medium, the cortical tissue blocks were collected by centrifugation and further triturated in Neural Basal medium plus B27 supplement (Invitrogen, Carlsbad, CA) with fire-polished pipettes into single-cell suspensions. The dissociated cells were plated in the density of 10⁶ cells/cm² in culture plates with approximately 5 cells/cm² in culture plates. The cell viability was measured with 0.01% propidium iodine (Sigma). The cell viability was measured with 0.01% propidium iodine (Sigma). The cell viability was measured with 0.01% propidium iodine (Sigma). Then the cells were maintained in the Neural Basal medium plus B27 and antibiotics in a 5% CO2 and 95% O2 incubator at 37 °C, and half of the medium was changed every three days. Cortical neurons were allowed to grow for 10-14 days before processing for further experiments.

2.3. Immunocytochemistry

Rat cortical neurons were grown on 0.01% polylysine-coated coverslip for 10-14 days and then processed with immunocytochemical studies. First, cells were washed with PBS for three times, fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 10 min, followed by blocking with 3% bovine serum albumin for 30 min. Then the cells were incubated in primary antibodies: rabbit polyclonal anti-glutamate (1:1000) and anti-GABA (1:1000), or mouse anti-dopamine D1 receptor (1:1000) and rabbit anti-dopamine-D2 receptor (1:1000), mouse anti-map2 (1:1000), Bax (1:1000) and VDAC (1:100) for 1 h at room temperature. After washing with PBS for three times, the cells were visualized with Alexa-fluor 488 or Alexa-fluor 594 conjugated goat anti-mouse IgG or donkey anti-mouse or rabbit secondary antibodies. Cell nuclei were counterstained with DAPI. Fluorescent images were obtained and analyzed under Leica confocal microscope (Leica, Germany). Measurement of neuronal dendritic features including total dendritic branch length, dendrite tips, soma area was through Leica qwin analysis software (Leica, Germany) of 50 randomly chosen neurons stained with MAP-2 antibodies. The individual dendrite morphology was traced by the electronic marker and the dendrite length of each dendrite was automatically quantified, then the total dendrite branch length was the summation of each dendrite length from the neuron. Dendrite tips are manually counted by the number of dendrite branches that each neuron has. Soma area of each neuron was determined by manually defining the boundary of the cell bodies and the soma area was given automatically by the software. The mean ± SE of total dendritic length, dendrite tips, soma area of neurons in each drug treatment group were averaged from 50 randomly chosen neurons in the slides of each drug group from three independent experiments.

2.4. Measurement of cell apoptotic death

Methods used to establish apoptotic cell death included staining with fluorescent DNA-binding dyes propidium iode (PI) and analysis by FACSVantage

2.5. Measurement of cell viability

50 randomly chosen neurons in the slides of each drug group from three independent
Flow Cytometer (Becton Dickinson). After drug treatment, cortical neurons were detracted by trypsin and collected by centrifugation at 300 g. Cells were then washed with PBS twice and fixed in 70% cold ethanol at 4 °C for 30 min. PI staining of cellular DNA was achieved by incubation of fixed cells with PI (5 μg/ml) (Molecular Probes) in the presence of 1 mg/ml RNase (Sigma) at 37 °C for 30 min. Then at least 10³ cells from each sample were measured by flow cytometer. The percent of cells with sub-G1 peak DNA fragments among total cell populations was regarded as rate of apoptosis.

2.5. Measurements of mitochondrial membrane potential

The JC-1 (Molecular Probes) was used as a measure of mitochondrial transmembrane potential. Briefly, after drug treatment cells were incubated for 30 min at the presence of 10 μM of the dye and then washed in HBSS. Cellular fluorescence was imaged using a FACS Vantage Flow Cytometer (Becton Dickinson) with excitation at 488 nm and emission at 510 nm. The ratio of cells with green/orange fluorescence was regarded as the representative of mitochondrial membrane potential.

2.6. Measurements of mitochondrial cytochrome C release and caspase 3/7 activation

The primary cortical neurons were maintained with increasing concentration of dopamine and SKF83959 for 72 h. The cells with cytochrome C released into the cytosol were examined by InnoCyt Cytochrome C releasing kit (Calbiochem). In addition, the activities of these cultures treated with dopaminergic and different inhibitors were evaluated by Caspase-Glo3/7 assay kit (Promega).

2.7. Measurements of cytotoxic ROS production

The cell-permeable fluorescent probe DCF (Sigma) was used to measure reactive oxygen species (ROS) within cells by detection of enzymatically formed hydrogen peroxide. Following drug treatment, cells were loaded with 10 μM DCF for 30 min at 37 °C in a 5% CO₂ incubator and then were washed with Hank’s solution. Cellular fluorescence was quantified with excitation at 504 nm and emission at 529 nm by spectrophotometer (Perkin Elmer).

2.8. Immunoprecipitation and Western blot

Adult rat cortex and day 14 cortical cultures were collected, washed with PBS and homogenized by sonication in cell lysis buffer (containing 20 mM Tris, pH 7.5, 50 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 50 mM sodium fluoride, 40 mM β-glycerophosphate, 0.5 mM sodium orthovanadate, 1 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride, 20 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A/ (Sigma, St. Louis, MO). Cell lysate was incubated on ice for 30 min and then clarified by centrifugation at 12,000 rpm at 4 °C for 20 min. For immunoprecipitation assays, the supernatant was incubated with 5 μg primary dopamine D1 receptor antibody (Santa Cruz) or D2 receptor antibody (Chemicon) at 4 °C on a rocker for 4 h, followed by immunoprecipitation with protein G agarose beads (Sigma) at 4 °C overnight. The eluted protein was then mixed 1:5 with loading buffer, resolved by polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes for immunoblot analysis. Membranes were probed using the same D2 (dilution: 1:1000) or D1 receptor antibodies (dilution 1:2000). For Western blot analysis, the protein concentrations of the supernatants were first determined by a BCA Protein Assay kit (Pierce, Upland, IN). Before gel loading, Western blot analysis, the protein concentrations of the supernatants were first determined by a BCA Protein Assay kit (Pierce, Upland, IN). Before gel loading, resolved protein samples were mixed 1:5 with a sample buffer containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.04% bromophenol blue, and 0.125 M Tris–HCl, (pH 6.8) and boiled for 5 min. The resultant mixtures were loaded onto 10% polyacrylamide gels and run at 150 mV for 1 h. Proteins resolved by electrophoresis were transferred onto Hybond ECL nitrocellulose membranes (Amersham, Piscataway, NJ) at 100 mA for 1 h. After blocking in 5% dry milk and 0.1% Tween 20 in PBS (pH 7.6), the membranes were processed for immunoabelling for 1 h at room temperature with one of the following primary antibodies: mouse monoclonal diabetes D1 receptor (dilution 1:2000; Santa Cruz), rabbit polyclonal antibody D2 receptor (dilution 1:1000; Chemicon), rabbit monoclonal antibody Bax (dilution 1:2000; Sigma, St. Louis, MO); rabbit polyclonal antibody Bcl-2 (dilution 1:1000; Cell Signaling Technology), rabbit anti-P42/44 ERK1/2 (dilution 1:1000; Cell Signaling Technology), mouse anti-phosphorylated P42/44 ERK1/2 (dilution 1:100; Upstate, Waltham, MA), mouse anti-AKT, rabbit anti-phosphorylated Akt (dilution 1:1000; Cell Signaling Technology) or rabbit anti-actin (1:50; ProteinTech). Incubation with secondary peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibodies (dilution 1:2000; Sigma, St. Louis, MO) was conducted for 1 h at room temperature. The labeled bands were visualized on X-Omat AR Films (Kodak, Rochester, NY) using a SuperSignal Chemiluminescence kit (Pierce, Upland, IN). We used the software called Image for Doctor One (Biorad) to quantify the Western blot bands. Three replicates of Western blots were performed for each experiment. For each experiment, the band density of phosphorylated and total Akt and ERK, Bax, Bax and actin was scanned and analyzed by the software. The ratio of phosphorylated Akt and ERK was calculated by the percentage of phosphorylated versus total protein band density. The ratio of Bcl2 and Bax was calculated by the percentage of Bcl2 versus Bax band density.

2.9. Statistical analysis

All experiments were performed three times and the values were presented as means ± SE. For statistical analysis of data from multiple groups, one-way ANOVA followed by a Dunnett’s post hoc test was performed. The temporal effects of drug exposure were examined using a two-way ANOVA, with the presence of drugs and duration of exposure as variables. This was followed by a Tukey’s post hoc test. The p threshold value mentioned after each experiment such as “p < 0.01,” “p < 0.05” are all considered with significant difference.

3. Results

3.1. Characterization of primary cortical neuronal cultures in vitro

To first characterize the major neurotransmitter phenotypes of primary cultured neurons derived from fetal rat cortex, cultures were fixed and immunostained with both glutamate and GABA antibodies. The immunocytochemical results suggested that 95% of the cultured cells were glutamatergic neurons, whereas less than 5% of them were GABAergic interneurons (Fig. 1A–D). Therefore, our culture model is more likely representative of glutamatergic pyramidal projection neurons in cortex.

3.2. Characterization of dopamine D1 and D2 receptor subtype expression and their associations in rat cortex and cortical cultures

In addition, we attempted to determine the co-localization of dopamine D1 and D2 receptors to cortical neuronal structures by simultaneous immunostaining with both D1 and D2 receptor antibodies. The results showed that both receptor subtypes were widely distributed and colocalized to most of the cortical neurons (Fig. 2A–D) and their subcellular structures including soma, axon, dendrite (Fig. 2E), implying their possible constitutive interactions. Furthermore, our immunoblot and co-immunoprecipitation analyses proved that D1 and D2 dopamine receptors are abundantly expressed and endogenously associated in rat cortex and cortical primary cultures (Fig. 2F).

3.3. Cortical neuron apoptosis induced by dopamine and SKF83959 was through PLC–calcium-dependent pathway

To assess whether activation of dopamine receptors is capable of triggering neuronal apoptosis and calcium-related signaling pathway is involved in these cytotoxic events, cortical neurons were treated with an array of dopaminergic drugs such as dopamine (100 μM), D1 receptor specific agonist SKF38393 (50 μM), D2 receptor specific agonist quinpirole (50 μM), cAMP stimulator forskolin (30 μM), and D1D2 heteroregulator agonist agonist SKF83959 (50 μM) for 48 and 72 h. Cell apoptotic assays showed that only dopamine and SKF83959 have significantly induced around 40–50% neuronal apoptosis, compared with less than 10% cell apoptosis in control cultures. In contrast, drugs of cAMP signaling modulators such as SKF38393, forskolin, quinpirole have not enhanced cell apoptosis under similar conditions (Fig. 3A). Then we specifically characterized the time-dependent apoptotic effects of dopamine and SKF83959. Both drugs have not induced significant neuronal apoptosis within 24 h, however, the apoptotic rate increased to 30–40% at 48 h, reaching over 50% at 72 h (Fig. 3B). We also delineated the dose-dependent apoptotic effects by dopamine and SKF83959 at 72 h (Fig. 3C and D). Based on these results, PLC–calcium-related signaling pathways were probably specifically involved in dopamine and SKF83959-induced toxicity of cortical neurons. To confirm these putatively involved signaling cascades, we pre-incubated cortical neurons with selective D1 receptor antagonist SCH23390, D2 receptor antagonist Sulpiride, PLC inhibitor U73122, IP3 sensitive calcium channel blocker 2-APB, intracellular calcium chelator BAPTA/AM for 2 h before treatment with 50 μM dopamine and 30 μM SKF83959 for
another 72 h, we found in both drug groups the apoptotic events were remarkably prevented by these series of inhibitors (Fig. 3 E and F).

3.4. Mitochondrial membrane potential was downregulated dose-dependently by dopamine and SKF83959 in cortical neurons

To determine whether impaired mitochondrial function contributes to dopamine and SKF83959 induced neurotoxicity, cortical neurons were stimulated with increasing concentration of dopamine and SKF83959 for 72 h and mitochondrial membrane potential was assayed with JC-1 indicator. The results demonstrated that dopamine and SKF83959 dose-dependently depolarized mitochondrial membrane potential, which can be largely prevented by pretreatment with D1 receptor antagonist SCH23390, D2 receptor antagonist Sulpiride, specific PLC inhibitor U73122, IP3 receptor blocker 2A-PB, intracellular calcium chelator BAPTA/AM. Meanwhile, preincubation with mitochondrial calcium uniporter blocker ruthenium red (RUT), mPTP inhibitor bongkrekic acid (BK) and antioxidant Vitamin E (VE) could partially prevent the mitochondrial membrane depolarization (Fig. 4A–D). These findings altogether proved that in cortical neurons under dopamine and SKF83959 treatments, calcium arising mainly from intracellular ER stores contributed to the elevation of mitochondrial calcium level, then leads to opening of MPTP, and induced mitochondrial membrane depolarization. Furthermore, ROS might also contribute partially to the mitochondrial membrane depolarization.

3.5. Cytochrome C release was induced dose-dependently by dopamine and SKF83959 in cortical neurons

Cytochrome C plays very important roles in mediating mitochondria-dependent cell apoptosis, which is released from intermembrane space after mitochondria membrane depolarization and opening of MPTP. It then forms complexes with Apaf-1 and ATP in the cytosol and catalyzes the activation of caspases 9 and caspases 3, 7 cascades, leading to DNA fragmentation and cell apoptosis (Hengartner, 2000). We employed a cytochrome C releasing kit from Calbiochem to evaluate whether prolonged dopamine and SKF83959 treatments in the cortical cultures induced cytochrome C release into cytosol during the apoptotic process. We found 72 h of dopamine and SKF83959 caused dose-dependent Cytochrome C release into the cytosol. The results showed that 72 h treatment of 50 μM of dopamine and 30 μM SKF83959 induced nearly 30–50% of cells releasing most cytochrome C from their mitochondria (Fig. 5A and B).

3.6. Caspase 3/7 activation was induced dose-dependently by dopamine and SKF83959 in cortical neurons

We further examined whether prolonged dopamine and SKF83959 treatments have induced caspases 3, 7 activation after cytochrome C release. Our data have shown that dopamine and SKF83959 dose-dependently induced caspases 3, 7 activation, which could be alleviated by pretreatment with Sulpiride, 2A-PB, VE and BK, suggesting calcium overload, ROS and MPTP opening all contributed to caspase3, 7 activation (Fig. 6A and B).

3.7. ROS generation by dopamine and SKF83959 was through mitochondria-dependent and independent pathways

To test the hypothesis that reactive oxygen species (ROS) are generated both extracellularly and after mitochondrial dysfunction, which are also contributing factors of dopamine-induced toxicity (Camello-Almaraz et al., 2006; Feissner et al., 2009). Cortical neurons were exposed to a series of concentrations of dopamine and SKF83959 for 48 h, and then the cytosolic ROS level was examined by fluorescent indicator DCF. We found that dopamine and SKF83959 dose-dependently induced ROS production (Fig. 7A and B), and pretreatment with Sulpiride, IP3 receptor blocker 2-APB, antioxidant VE and MPTP inhibitor BK partially prevented the generation of ROS. Therefore ROS probably is partially derived from mitochondrial calcium overload, mitochondrial membrane depolarization and disruption of electron trans-
mission chains. We further tested whether the neuronal apoptosis induced by dopamine and SKF83959 could be alleviated by pretreatment with mitochondrial uniporter blocker RUT, MPTP blocker BK, antioxidant VE and pan-caspase inhibitor Z-VAD-FM, the results showed that these series of inhibitors could partially inhibit neuronal apoptosis induced by dopamine and SKF83959 (Fig. 7C and D), indicating that calcium uptake into mitochondria, MPTP opening, ROS and caspases activation all contributed to this neuronal apoptotic process.

3.8. Inhibitory effects on dendrite process extension of cortical neurons by dopamine and SKF83959

Dendrites are the primary sites in which neurons receive, process and integrate inputs from their multiple presynaptic partners. The functions of dendrites are critically dependent on the branching patterns of the dendritic tree, which is controlled by both intrinsic genetic programme and neurotrophic signals (Miller and Kaplan, 2003). In this study we tested the hypothesis that short-time comparatively high dose dopamine and SKF83959 treatments induced dendrite retraction in cortical neurons without apoptosis induction. Cortical neurons were incubated for 24 h with 50 \( \mu \)M dopamine and 30 \( \mu \)M SKF83959 in the presence or absence of U73122 and VE, then neurite morphology of neurons from different drug groups were revealed by MAP-2 immunological analysis. The control cultures showed extended and complex dendrite processes, whereas the cortical neurons with dopamine and SKF83959 treatments displayed shortened and much less complex dendrites. The pretreatment with PLC inhibitor U73122 could alleviate dopamine and SKF83959 mediated dendrite inhibitory effects (Fig. 8A–F). Then the dendrite features were further quantified by parameters such as total dendrite branch length (TDBL), dendrite tips and soma area and the results showed that dopamine and SKF83959 treatments displayed shortened and much less complex dendrites. The pretreatment with PLC inhibitor U73122 and VE could alleviate dopamine and SKF83959 mediated dendrite inhibitory effects (Fig. 8A–F). Then the dendrite features were further quantified by parameters such as total dendrite branch length (TDBL), dendrite tips and soma area and the results showed that dopamine and SKF83959 significantly decreased TDBL (Fig. 8G) and dendritic tips (Fig. 8H) of cortical neurons compared with control, whereas the soma area (Fig. 8I) remains basically the same, suggesting no significant apoptotic morphology was detected. U73122 treatment largely alleviated the drug effects.
of SKF83959, while U73122 plus VE mostly blocked the dopamine effect, indicating the dual role of calcium and ROS in mediating the neurodegenerative effects by dopamine.

3.9. Inhibitory effects on AKT and ERK phosphorylation and Bcl-2/Bax ratio exerted by dopamine and SKF83959 treatments

Mitogen-activated protein kinases (MAPKs) and PI3K-AKT, two important mediators of growth factor signal transduction from the cell surface to the nucleus, have been implicated in a wide variety of cellular processes, such as proliferation, differentiation and apoptotic cell death (Kyosseva, 2004). Bcl-2 and Bax belong to the bcl-2 family proteins which are crucially involved in the control of mitochondria-dependent apoptotic process (Breckenridge and Xue, 2004). Bcl-2 protein is localized to the outer mitochondrial membrane and regulates cell apoptosis and survival by neutralizing pro-apoptotic proteins Bax or BH-3 only peptide such as bid and bad (Chipuk and Green, 2008), whereas Bax normally localizes to the cytosol and was translocated to the mitochondrial outer membrane for PTP opening and release of apoptotic factors (Scorrano and Korsmeyer, 2003; Lalier et al., 2007). We evaluated the 24 h drug effect of dopamine and SKF83959 on AKT and ERK phosphorylation, representatives of growth factor signaling and Bcl-2/Bax ratio which shows cellular apoptotic susceptibilities. The Western blot results showed that dopamine and SKF83959 dose-dependently reduced the percentage of phosphorylated AKT, ERK and Bcl-2/Bax ratio, which were partially blocked by pretreatment with PLC inhibitor U73122 (Fig. 9A–D). Thus, we postulate that prolonged dopamine and SKF83959 stimulation could block MAPKs and PI3K–AKT pathways and Bcl-2 upregulation through mechanisms involving PLC–calcium signaling in cortical neurons.

3.10. Translocation of Bax to nucleus and mitochondria by dopamine and SKF83959 treatments

Although we did not detect the alteration of Bax protein level, Bax might induce neuron apoptosis through translocation to the nucleus and mitochondria as demonstrated by previous studies (Gill et al., 2008; Gill and Perez-Polo, 2009). Our immunocytochemical studies proved that 24 h dopamine and SKF83959 treatments induced Bax translocation to the nucleus and mitochondria (Fig. 10) and contributed to apoptosis.

4. Discussions

Schizophrenia has been regarded as a neurological disorder of developmental or genetic origin due to its high genetic susceptibility background. However, the presence of large number of sporadic schizophrenia patients might suggest that abnormalities in neurotransmitter system function also play important roles in
Fig. 4. Mitochondrial membrane potential was downregulated by dopamine and SKF83959 in cortical neurons through calcium–ROS dependent pathways. Cortical neurons were treated with dopamine (A) and SKF83959 (C) for 72 h, and mitochondrial membrane potential was evaluated by JC-1 staining. Note the dose-dependent inhibitory effects on mitochondrial membrane potential by dopamine and SKF83959 in cortical cultures. (B and D) Cortical neurons were pretreated for 2 h with the following series of compounds: D1 receptor antagonist SCH23390 (10 μM), D2 receptor antagonist Sulpiride (20 μM), PLC inhibitor U73122 (20 μM), IP3 sensitive ER channel blocker 2A-PB (20 μM), intracellular calcium chelator BAPTA-AM (20 μM), mitochondrial calcium uniporter blocker ruthenium red (RUT) (25 μM), MPTP inhibitor BK (10 μM) antioxidant VE (10 μM), and then treated with 50 μM dopamine and 30 μM SKF83959 for 72 h. Note the mitochondrial membrane depolarization induced by dopamine and SKF83959 was mostly or partially blocked by the series of inhibitors shown above. Each data point in the column was mean ± SE from three experiments with 

* \( p < 0.05 \) compared with control groups.

Fig. 5. Cytochrome C release by dopamine and SKF83959 from cortical neurons was dose-dependent. Cortical neurons were treated with dopamine (A) and SKF83959 (B) for 72 h and the percentages of cytochrome c releasing cells were examined. Note the dose-dependent effects of both dopamine and SKF83959 on cytochrome C release in cortical neurons. Each data point was mean ± SE from three experiments with 

* \( p < 0.01 \), * \( p < 0.05 \) compared with control groups.

Fig. 6. Caspases 3, 7 activation by dopamine and SKF83959 in cortical neurons were through calcium, MPTP and ROS sensitive pathway. Cortical neurons were treated with dopamine (A) and SKF83959 (B) in the absence and presence of Sulpiride (20 μM), 2A-PB (20 μM), VE (10 μM) and BK (10 μM) for 72 h, then caspases 3, 7 activation of cortical neurons from each group were examined. Note the dose-dependent effects of dopamine and SKF83959 on ROS production, and the effects were partially blocked by Sulpiride, 2-APB, VE and BK. Each data point was mean ± SE from three experiments with 

* \( p < 0.01 \), * \( p < 0.05 \) compared with control groups.
increasing evidence has shown discrete reductions in cortical neurons and neuritic atrophy may occur with laminar and regional specificities, the underlying mechanisms remain unclear. In the present study, we achieved the novel finding that stimulation of primary cultured cortical neurons for 72 h with 10–100 μM dopamine and 10–50 μM SKF83959 could activate D1 and D2 receptor heterooligomers, resulting in specific PLC-related intracellular calcium overload and mitochondria-dependent cortical neuronal apoptosis, providing another possible pathway for dopaminergic toxicity in addition to its oxidative effects. Meanwhile, we found that 24 h similar drug treatment which did not induce major neuronal apoptosis significantly reduced AKT and ERK phosphorylation and Bcl-2 protein, caused Bax translocation to the nucleus and mitochondria, made neurons lose their neurite branches and become more vulnerable to toxic insults. Altogether, these findings might provide new insight for dopamine hyperactivity-based apoptotic mechanisms in schizophrenia.

The functional dopamine D1 and D2 binding sites shown by D1-specific [3H]SCH23390 binding and D2-specific [3H]raclopride binding were detected throughout the cortex by previous studies. (Lidow et al., 1991; Goldman-Rakic et al., 1992; Vincent et al., 1995). Our immunocytochemical and immunoprecipitation studies have further proved the colocalization of both D1 and D2 receptors to the same cortical neuronal structures and formation of functional complexes. SKF83959 specifically activates the heteromeric D1D2 complex and exhibits the greatest potency among tested D1-dopamine receptor agonists including SKF81297 and SKF38393 with regard to stimulation of IP3 production in brain, but elicits no cAMP production (Panchalingam and Undie, 2001; Jin et al., 2003). It has also been shown to induce Gq-mediated intracellular calcium release and promote CaMKII and Cdk5 enzyme activities in transfected cells and brain slices (Lee et al., 2004; Zhen et al., 2004; Hasbi et al., 2009). One study on primary hippocampal neurons further detailed that SKF83959 induced sustained PLCβ sensitive intracellular calcium elevation due to early phase of intracellular calcium release and late phase of voltage-gated and ligand gated calcium influx (Ming et al., 2006). Despite that disruption of cellular calcium homeostasis has been proposed to be a critical event in apoptosis (Kruman et al., 1999; Giacomello et al., 2007), whether dopamine hyperactivities are capable of inducing cortical neuronal toxicity through deregulation of intracellular calcium remain uncertain. On one hand, some evidence have suggested that dopamine toxicity is mediated through an extracellular oxidative stress mechanism because in their studies dopamine-induced death can be prevented by antioxidants and reductive substances instead of either D1 or D2 antagonist (Alagarsamy et al., 1997; Jones et al., 2000; Jiang et al., 2008). On the other hand, our
earlier study have revealed that 48 h treatment of 50 μM SKF83959 can dose-dependently induce apoptosis in proliferating cortical precursor cells through a PLCβ dependent pathway (Zhang et al., 2005). In the present study, we provided further evidence that dopamine and SKF83959 can induce cortical neuronal apoptosis through D1D2–PLC–IP3 dependent loss of intracellular calcium homeostasis. Interestingly, one research group recently also reported that dopamine could mediate calcium-dependent cell apoptosis in mouse young striatal neurons but not in neonatal striatal neurons, due to gradually increased expression of PLC during mouse striatal maturation (Iwatsubo et al., 2007). Although the cortical precursor cells used in our earlier study and neurons generated in our present studies were arising from embryos and developed for 14 days in vitro, a much earlier developmental origin compared with the striatal neonatal neurons they used, we still observe potent activities of PLC in our cultures. Therefore, the discrepancies regarding mechanisms of dopamine-induced neuronal death from different research groups might result from different experimental approaches, variabilities in developing stage, patterns of dopamine receptor and PLC expression, and downstream signaling events in cortical, striatal neurons and other neuronal cell types.

Fig. 8. Retraction of dendrite complexities by dopamine and SKF83959 in cortical neurons. (A–F) Typical images of MAP-2 immunolabeling of dendrite extensions in cortical cultures treated with dopamine (50 μM) and SKF83959 (30 μM) for 24 h in the absence and presence of PLC inhibitor U73122 (20 μM) and VE (20 μM). Note neurons show normal long and complex dendrite processes in control cultures (A), in contrast to cultures treated by SKF83959 (B) and dopamine (D) displaying shrunked and less complex dendrites. The pretreatment of U73122 in SKF83959-treated cultures prevented the dendrite loss (C), whereas pretreatment with both U73122 and VE in dopamine-treated cultures blocked the dendrite loss (D and E). The bar graphs show mean ± SE for total dendrite branch length (TDBL) (G), number of dendrite terminal tips (H), and soma area (I) for neurons from these cultures. Data were quantified from at least 50 cells from each culture of three experiments with “p < 0.01 compared with control group. Note the significant reduction in total dendrite branch length, dendrite terminal tips but not soma area in cultures treated with dopamine and SKF83959 compared with control cultures, and the blockage of such effects by pretreatment with U73122 and VE.
The mechanisms underlying cytosolic calcium rise and apoptosis are not fully understood, although much evidence is available showing downstream mitochondrial calcium accumulation might be a major event during apoptosis induction (Kruman and Mattson, 1999; Tang et al., 2005; Jeong and Seol, 2008). Mitochondria and ER are regionally closely distributed in 100-nm range in several cellular compartments. Mitochondria can serve as high capacity calcium sinks and regulate the local spatiotemporal cytosolic calcium signal through calcium uniporter and Na+/Ca2+ exchanger, thus modulate many calcium-dependent cellular processes (Szalai et al., 1999; Hajnoczy et al., 2000). Our pharmacological data indirectly demonstrated that in our culture system cytosolic calcium rise induced by dopamine and SKF83959 is mediated through opening of ER-IP3 sensitive channel and then calcium is uptake into mitochondria, which could be prevented by addition of IP3 channel blocker 2-APB, intracellular calcium chelator BAPTA-AM and mitochondrial calcium uniporter blocker, ruthenium red. Previous functional studies have shown that mitochondrial calcium uptake is very slow when the cytoplasm has submicromolar Ca2+, whereas large amounts of Ca2+ can be taken up and stored by the mitochondria at higher [Ca2+] levels (Kd approx. 10 μM). Therefore, we could speculate that prolonged dopamine and SKF83959 treatments of cortical neurons have accumulated over micromolar range of cytosolic calcium and led to the calcium overload of mitochondria. This is consistent with Rizzuto and co-workers in their studies showing that mitochondria undergo large increases of [Ca2+] following stimulation by IP3-linked agonists in a wide variety of cell types (Rizzuto et al., 1993, 1998). It has been shown that the physiological range of mitochondria Ca2+ sustains the activation state of the mitochondrial dehydrogenases and the tricarboxylic acid cycle, and modulate ATP production. Conversely, excessive mitochondria...
Ca\textsuperscript{2+} uptake can open mitochondrial permeability transition pore (MPTP) depending on calcium range. For example, in brain mitochondria, low micromolar Ca\textsuperscript{2+} thresholds (1–4 μM) determine PTP-independent and reversible cytochrome c release, and the high micromolar one (≈200 μM) leads to the opening of MPTP (Schild et al., 2001; Giacomello et al., 2007). The underlying mechanisms might be that calcium can modulate ANT and VDAC conformation through direct interaction or indirect binding with cardiolipin and induce MPTP (Brustovetsky and Klingenberg, 1996; Halestrap and Brenner, 2003; Hansson et al., 2004; Báthori et al., 2006). We found that ruthenium red, bongkrekic acid can partially alleviate dopamine and SKF83959-induced mitochondria membrane depolarization and neuronal apoptosis in cortical cultures, suggesting that mitochondrial calcium uptake and MPTP-dependent membrane depolarization are actually involved in the apoptotic process during 72 h overstimulation by the dopaminergic drugs.

MPTP appears to be a major step in mitochondria-dependent cell death that leads directly to marked mitochondrial membrane depolarization and release of apoptotic factors such as cytochrome c, activation of caspases cascade and induce nuclear chromatin condensation and DNA fragmentation, then apoptosis (Ricci et al., 2003; Grimm and Brdiczka, 2007; Rasola and Bernardi, 2007; Tsujimoto and Shimizu, 2007). Our experimental results have shown that mitochondrial calcium uptake after prolonged stimulation of dopamine and SKF83959 has dose-dependently induced mitochondria membrane depolarization, PTP-dependent cytochrome c release and subsequent caspases 3, 7 activation. Moreover, the caspases 3, 7 activation can be prevented by pretreatment of Sulpiride, 2-APB, BK and VE, suggesting D1D2 activation, IP3 sensitive calcium rise, ROS production, MPTP opening, cytochrome C release and caspases 3, 7 activation are serial events in this apoptotic process. These findings are consistent with other reports that both calcium and MPTP serve as important mediators of mitochondria-dependent cell death pathway (Richter, 1993; Kruman et al., 1998; Kruman and Mattson, 1999; Hajnóczky et al., 2006).

Dopamine has long been considered as potential, endogenous neurotoxic agents, since ROS could be generated by the MAO-catalyzed oxidative deamination of dopamine and also by autoxidation (Miyazaki and Asanuma, 2008). In our experiment, we found D1 and D2 receptor antagonists could only partially block dopamine-induced but not totally block SKF83959-induced down-regulation of mitochondrial membrane potential and cell death, suggesting that extracellular dopamine oxidation is partially involved in this dopamine-induced neuronal death. Meanwhile, intracellular calcium rise by D1D2 heterooligomer receptor activation can also modulate ROS homeostasis by regulating ROS generation and annihilation mechanisms in both the cytoplasm and the mitochondria. In addition to regulation of multiple extramitochondrial ROS generating enzymes such as cell-surface NADPH oxidases and nitric oxide synthase, Ca\textsuperscript{2+} can directly activate antioxidant enzymes, such as catalase and GSH reductase to increase the level of SOD. Therefore the net Ca\textsuperscript{2+} effects on ROS generation and annihilation appear to be tissue-specific and context-sensitive (Yan et al., 2006). In mitochondria, after cytochrome c release by calcium overload, the activation of caspases can feed back on the permeabilized mitochondria to damage its function and generate ROS through effects of caspases 3 on complex I and II in the electron transport chain (Cadenas and Sies, 1998; Ricci et al., 2003), another possible pathway leading to ROS is mediated through inhibition of complex I and IV by nitric oxide and peroxynitrite (Brookes et al., 2004). Our results demonstrated that the ROS generation by prolonged dopamine and SKF83959 stimulation in the cortical neurons were remarkably elevated and preapplication of Sulpiride, 2-APB, BK and Vitamin E.
can reduce ROS generation and neuronal death, which argues that ROS can also be generated downstream of mitochondrial calcium overload and dysfunction. Thus, both mitochondria-dependent and independent pathways have participated in ROS production during dopamine-induced cortical neuronal death. This is consistent with the clinical finding that the dietary supplementation of either antioxidants or EPUFAs, particularly omega-3 has already been found to improve some clinical outcomes of schizophrenia patients (Mahadik et al., 2006).

According to the results discussed above, dopamine and SKF83959 can induce cortical neuronal death through calcium and ROS-dependent mitochondria pathways. Nevertheless, cortical neuronal death and degeneration might also be promoted by inhibition of neurotrophic pathways enhancing neuronal survival and differentiation (Hetman and Xia, 2000). MAPK cascade of raf–MEK–ERK and PI3K–AKT pathways are the two most important neuronal growth factor signaling cascades contributing to the promotion of neuron survival (Garrington and Johnson, 1999; Kandel and Hay, 1999; Kyosseva, 2004; Frebel and Wiese, 2006). For example, ERK activity promoted survival through regulation of transcriptional factors such as CREB, RSK and modulation of functions of Bcl-2 family proteins such as bad, bim, bag-1 and Bcl-2 (Hetman and Gozd, 2004), whereas AKT phosphorylation directly modulates bad, caspase 9, SAPK, NF-KB and Mdm2 to control survival (Song et al., 2005). Therefore, our detection of downregulation of AKT and ERK activity under dopamine and SKF83959 treatments should directly affect neuronal survival. Furthermore, ERK phosphorylation has also been found to be highly correlated with neuronal differentiation and extension of great complex dendritic structure during neuronal maturation. In matured neurons, ERK phosphorylation was tremendously enhanced and these neurons are more susceptible to dephosphorylation compared with younger neurons (Waetzig and Herdegen, 2005; Ha and Redmond, 2008; Zhou et al., 2009).

Other studies have shown that the PI3K–AKT–mTOR pathway activation alone controlled dendrite size, a coordinated activation together with the ras–MAPK signaling pathway was required for increasing dendritic complexity in neuronal cultures (Jaworski et al., 2005; Kumar et al., 2005). Our experiments showed that 24 h dopamine and SKF83959 treatments which have not induced significant neuronal apoptosis have remarkably reduced the dendrite extension and complexity in cortical cultures, meanwhile similar drug treatment had induced dose-dependent decrease in the ERK and AKT phosphorylation through PLCβ-sensitive pathways. Based on stereological studies on schizophrenia postmortem brains, reduced gray matter volume and enlargement of brain ventricle resulted from degeneration of neuronal dendrite branches and subsequent cell loss (Selemion et al., 1995; Rajkowska et al., 1998), so the inhibitory effects of dopamine and SKF83959 on cortical ERK and AKT phosphorylation and dendrite growth at early stage and induction of neuronal apoptosis later on probably provided a convincing explanation for dopamine-hyperactivity based neurodegeneration and subsequent cortical volume loss in schizophrenia.

Apart from its neutralizing role at mitochondria to promote cell survival, Bcl-2 also promotes axonal regeneration and neurite extension in the retinal ganglion cells and the midbrain dopaminergic neurons (Myers et al., 1995; Chen et al., 1997; Eom et al., 2003). One study reported that Bcl-2 expression at ER supports neurite growth by enhancing intracellular Ca2+ signaling and activating CREB and ERK. Several other studies have demonstrated that Bcl-2 upregulation is downstream of AKT and ERK1/2 phosphorylation, which underlies BDNF and retinoic acid induced neuronal differentiation and neuroprotection (Almeida et al., 2005; Wang et al., 2006; Lim et al., 2008). In our study, dopamine and SKF83959 reduced the Bcl-2 protein level and showed no obvious change of bax, displaying matching dose-response inhibitory effects on ERK and AKT phosphorylation via PLC-mediated pathways. These results are in line with several studies of postmortem tissues from schizophrenia patients showing that there was around absolute 25% reduction in Bcl-2 levels and reduced Bcl-2/Bax ratio in temporal cortex in schizophrenia and 2-fold increase after antipsychotic drug treatment (Jarskog et al., 2000, 2005). In schizophrenia patients, reduction of BDNF expression was found in the prefrontal area and atypical antipsychotics treatment can enhance BDNF production (Duran and Thome, 2004; Angelucci et al., 2005, 2007). Therefore, in our culture system dopaminergic drug might function through PLC–calcium sensitive pathway to decrease BDNF mediated ERK and AKT activities and subsequently reduce Bcl-2 level to inhibit neurite outgrowth, anti-apoptotic activities and increase neuronal death. However, the exact mechanisms underlying how dopamine and SKF83959 affect BDNF neurotrophic activities and Bcl-2 level are currently under our investigation.

Meanwhile, although there is no significant change in Bax protein level, we did detect Bax translocation to the nucleus and mitochondria in cortical neurons after dopamine and SKF83959 treatments. The Bax activation can be downstream of calpain activation due to the elevated cytosolic calcium level and subsequent cleavage of BH3-only peptide such as bid and bad (Juin et al., 2005). The translocated Bax at mitochondria can form oligomer-pores as mitochondrial apoptotic channel (MAC) on the outer membrane, promote cytochrome c release and early apoptosis without affecting membrane potential and ATP production (Kinnally and Antonsson, 2007). This might explain why ruthenium red and bongkrekic acid only partially block dopamine and SKF83959 induced neuronal apoptosis. Cytochrome C released from the MAC channel might contribute to some of the apoptotic events. The role of Bax at nucleus can be Ser15 phosphorylation-mediated activation of p53 (Gill et al., 2008). P53 can promote apoptosis by enhancing expression of p53-up-regulated modulator of apoptosis (PUMA) or blocking NF-kappaB-mediated survival signaling. It can also translocate to mitochondria to release cytochrome C (Hong et al., 2010). Altogether Bax activation and translocation in cortical neurons after dopamine and SKF83959 treatments might contribute to apoptosis through multiple mechanisms.

5. Conclusions

Our present study provided new findings on a PLC–calcium related D1–D2 oligomer-receptor signaling cascade and their relevant neural degenerative effects in the cortical neuron after prolonged treatment. These new findings might expand our understanding of the dopaminergic hypothesis of schizophrenia, which is crucial for developing rational therapeutic approaches for prevention of neuronal death and for improving clinical outcome in schizophrenia therapy.

Conflict of interest

We declare that there is no conflict of interest about any data published in this article.

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