Neurochemistry International 58 (2011) 281–294

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

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

ARTICLE INFO

Article history:
Received 7 October 2010
Received in revised form 25 November 2010
Accepted 30 November 2010
Available online 4 December 2010

Keywords:
Dopamine heterooligomer receptor
Phospholipase C
Calcium
Apoptosis
Dendrite
Schizophrenia

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.

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 psychomemitic effects of dopamine enhancing drugs such as amphetamine, phencyclidine and ketamine and strong D2 antagonisms 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

Abbreviations: DA, dopamine; IP3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; ER, endoplasmic reticulum; DAT, dopamine transporter; MAO, monoamine oxidase; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*9,*N*9-tetraacetic acid acetoxymethyl ester; 2-APB, 2-aminoethoxydiphenylborate; BK, bongkrekic acid; VE, Vitamin E; mPTP, mitochondrial permeabilization transition pore; DCF, 2,7-dichlorofluorescin diacete; PI, propidium iodide; ROS, reactive oxygen species; SOD, superoxide dismutase; CREB, cAMP response element binding protein; ERK, extracellular-regulated kinase; MAPKs, mitogen activated protein kinases; MAC, mitochondrial apoptotic channel; VDAC, voltage-dependent anion channel; ANT, adenine nucleotide translocase.

* Corresponding author. Tel.: +86 010 83911461; fax: +86 010 83911458.
E-mail address: lzhang@ccmu.edu.cn (L. Zhang).

[1] They are co-corresponding authors.

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doi:10.1016/j.neuint.2010.11.021

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 cortex 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 (Wayment 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 neuodegeneration and alleviating symptoms (Murphy et al., 1996; Jann, 2004; Perez-Neri et al., 2006; Hulshoff Pol and Kahn, 2008; Kim et al., 2008; Park et al., 2009).

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 Quinipirole (Jin et al., 2003). Multiple evidence from postmortem schizophrenia brains have demonstrated that disturbed calcium and mitochondrial function are present in the prefrontal cortical area (Lidow, 2003; Novak et al., 2006; Rezin 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 (Sczekan and Strumwasser, 1996). Given the disturbances of calcium homeostasis and mitochondrial dysfuntion 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-dopamine 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 retractions 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). Polyclonal 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 iodide were purchased from Molecular Probe (Eugene, OR). Monoclonal mouse anti-phospho-ERK1/2 (Thr202/Tyr204), polyclonal rabbit anti-ERK1/2, mouse-anti-phosph-AKT (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, SKF83959, SKF38393, DOI, quinpirole, Forskolin, SCH23390, Sulpiride, U73122, 2-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-GIO3/7 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 Hanks' 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 pipetts into single-cell suspensions. The dissociated cells were plated in the density of 10^5 cells/cm^2 in culture plates that had been coated with 0.01% polylysine (Sigma). Then the cells were maintained in the Neural Basal medium plus B27 and antibiotics in a 5% CO_2 and 95% O_2 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 experiment.

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 1% 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 iodide (PI) and analysis by FACS Vantage