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CDK5 knockdown in astrocytes provide neuroprotection as a trophic source *via* Rac1

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ABSTRACT

Astrocytes perform metabolic and structural support functions in the brain and contribute to the integrity of the blood–brain barrier. Astrocytes influence neuronal survival and prevent gliotoxicity by capturing glutamate (Glu), reactive oxygen species, and nutrients. During these processes, astrocytic morphological changes are supported by actin cytoskeleton remodeling and require the involvement of Rho GTPases, such as Rac1. The protein cyclin-dependent kinase 5 (CDK5) may have a dual effect on astrocytes because it has been shown to be involved in migration, senescence, and the dysfunction of glutamate recapture; however, its role in astrocytes remains unclear. Treating a possible deregulation of CDK5 with RNAi is a strategy that has been proposed as a therapy for neurodegenerative diseases. Models of glutamate gliotoxicity in the C6 astroglioma cell line, primary cultures of astrocytes, and co-cultures with neurons were used to analyze the effects of CDK5 RNAi in astrocytes and the role of Rac1 in neuronal viability. In C6 cells and primary astrocytes, CDK5 RNAi prevented the cell death generated by glutamate-induced gliotoxicity, and this finding was corroborated by pharmacological inhibition with roscovitine. This effect was associated with the appearance of lamellipodia, protrusions, increased cell area, stellation, Rac1 activation, BDNF release, and astrocytic protection in neurons that were exposed to glutamate excitotoxicity. Interestingly, Rac1 inhibition in astrocytes blocked BDNF upregulation and the astrocyte-mediated neuroprotection. Actin cytoskeleton remodeling and stellation may be a functional phenotype for BDNF release that promotes neuroprotection. In summary, our findings suggest that CDK5 − knockdown in astrocytes acts as a trophic source for neuronal protection in a Rac1-dependent manner.

1. Introduction

Astrocytes are specialized glial cells of the central nervous system (CNS) that perform various functions to regulate brain homeostasis (Chen and Swanson, 2003; Wang and Bordey, 2008). These functions include providing metabolic and structural support to neurons and synapses, regulating excess potassium, sodium, and neurotransmitters in the synaptic cleft (Sofroniew and Vinters, 2010) and making contact with the vasculature *via* their end-feet to generate a protective layer that contributes to the integrity of the blood–brain barrier (BBB) (Koehler et al., 2009; Posada-Duque et al., 2014).

Astrocytes respond to brain damage through a process called astrogliosis. This response is characterized by morphological and metabolic changes that allow them to recapture neurotransmitters such as glutamate, sodium ions, ROS, and other potentially toxic substances (Chen and Swanson, 2003; Rothstein et al., 1996; Swanson et al., 2004). Simultaneously, astrocytes release compounds that may favor neuroprotection, such as BDNF, NO, VEGF, and TNF-α (Buskila et al., 2005; Chung and Benveniste, 1990; Koyama et al., 2012; Oka et al., 2004). However, when the reactive response persists, it can become a pathological condition in which the astrocytes cease to respond to glutamate-induced gliotoxicity and generate a glial scar, thus impeding the connections between neurons in the affected area (Barreto et al., 2011; Chen and Swanson, 2003). Like neurons, astrocytes are sensitive to gliotoxicity; thus, high concentrations of extracellular glutamate trigger changes in calcium homeostasis that lead to mitochondrial damage, apoptosis, and cell death (Chen et al., 2000; Guimaraes et al., 2010; Matute et al., 2002, 2007).

Cyclin-dependent kinase 5 (CDK5) is a protein that has been implicated in neurogenesis, dendrite formation, and neurotransmission under normal conditions and is involved in the regulation of various cytoskeletal proteins (Lai and Ip, 2009; Su and Tsai, 2011). However, overactivity of CDK5 has been widely reported to be related to excitotoxic

Abbreviations: CDK5, cyclin-dependent kinase; GLU, glutamate; AD, Alzheimer's disease; DIV, day *in vitro*; ROS, reactive oxygen species; BDNF, brain-derived neurotrophic factor; ROCK, Rho kinase; LDH, lactate dehydrogenase; G-LISA, GTP enzyme-linked immunosorbent assay; Raci, Rac inhibitor; EAAT, excitatory amino-acid transporter; 3×Tg-AD, triple transgenic Alzheimer mice; AAV, adeno-associated virus; shRNA-miR, short hairpin RNA in a microRNA backbone; PAK, p21-activated kinase; SCR, scrambled; GFAP, glial fibrillary acidic protein; MAP-2, microtubule-associated protein-2; SAMP, senescence-accelerated mouse prone.

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processes related to the development of Alzheimer's disease (AD) and neurodegenerative disorders such as ischemia, Parkinson's disease, and Huntington's disease (Su and Tsai, 2011). Under pathological conditions, the microtubule-associated protein tau (a substrate of CDK5) is hyperphosphorylated by CDK5 and other kinases, which induces the formation of toxic intracellular aggregates in both neurons and astrocytes. These aggregates are known as neurofibrillary tangles (NFTs), and are considered a pathological marker in AD and other tauopathies (Castro-Alvarez et al., 2014a, 2014b; García-Matas et al., 2008).

CDK5 inhibition has been studied as a possible therapeutic target for the treatment of AD and other dementias (Alvarez et al., 1999; Weishaupt et al., 2003). Studies from our group have demonstrated that CDK5 silencing with RNA interference reduces pathological markers, such as NFTs, and improves cognitive function in triple-transgenic AD mice (Castro-Alvarez et al., 2014a; Piedrahita et al., 2010). However, the possible roles of the CDK5 protein and p35, the specific activator of CDK5 in astrocytes, are associated with regulation of the microtubule cytoskeleton and formation of the glial scar after scratch wounds (He et al., 2007).

Astrocytic reactivity following brain injury has been associated with the capacity to migrate to the lesion site. This process requires the activation of Rho GTPase proteins, which finely regulate the actin cytoskeleton (Kong et al., 2013; Narayanan et al., 2013; Villarreal et al., 2014). The rearrangement of the actin cytoskeleton and the generation of a wavy extension of the cell membrane called a lamellipodium during astrocytic stellation are directed by Rac1 (Bustelo et al., 2007; Ellenbroek et al., 2012; Racchetti et al., 2012). Recent studies in astrocytes have demonstrated that this protein may participate in the regulation of senescence, survival, and cell-cycle control pathways (Alexander et al., 2004; S.-Y. Hwang et al., 2006).

Rac1 and CDK5 are involved in the regulation of brain-derived neurotrophic factor (BDNF) production, specifically in neurons, as a mechanism underlying plasticity and learning and memory (Haditsch et al., 2013; Lai et al., 2012). Additionally, it is well known that astrocytes mediate BDNF-induced neuroprotection against brain injury (Giralt et al., 2010; Gutierrez-Vargas et al., 2014; Husson et al., 2005; Ruiz et al., 2012). However, the roles of CDK5 and Rac1 in the production of this trophic factor in astrocytes remain unknown, and this issue is the focus of the present study.

2. Materials and methods

2.1. C6 cell line cultures

The C6 (ATCC, CCL-107) rat glioma cell line was used as the astrocyte model. C6 cells express the S100 protein and EAAT (Agulhon et al., 2012; Baber and Haghighat, 2010). The C6 cells were maintained in DMEM (Sigma-Aldrich) supplemented with 2.5% fetal bovine serum (FBS, GIBCO) and penicillin–streptomycin mixture (GIBCO) and were incubated at 37 °C with 5% CO_2 and subcultured using a 0.25% trypsin/EDTA mixture (GIBCO) in 6- or 24-well plates at densities of 2.5×10^5 and 3.5×10^4, respectively.

2.2. Primary cortical astrocyte cultures

The cortices of neonate Wistar rats (PN1–2) were dissected, trypsinized, dissociated, and cultured in 75-cm^2 flasks at 37 °C and 5% CO_2 (De Marinis et al., 2013). The culture medium was changed after one day of culture and then twice per week. The astrocyte-enriched cultures were obtained beginning at 8 days *in vitro* (DIV), and the flasks were shaken at 37 °C at 350 rpm in a sequence of 6 h, 18 h, and 24 h in which the culture medium was washed at each time interval. This procedure was performed to minimize the presence of oligodendrocytes and microglia. The cell confluence was observed at DIV10 and was approximately 4×10^6 astrocytes that were characterized by a flat, polygonal morphology. Subsequently, the astrocytes were subcultured using 0.25% trypsin/EDTA (GIBCO) in 6-, 12-, or 24-well plates at densities of 1.5×10^5, 7.5×10^4, and 3.5×10^4, respectively.

2.3. Primary neuronal cultures

Cortical cultures from Wistar rat embryos (E18–19) were dissected, trypsinized, dissociated and cultured on poly-L-lysine (Sigma-Aldrich) pre-coated coverslips in neurobasal medium (GIBCO) containing B-27 supplement (GIBCO), L-glutamine (Sigma) and a penicillin–streptomycin antibiotic mixture (GIBCO) at 37 °C in a 5% CO_2-humidified atmosphere for a maximum of 19 days *in vitro* (DIV19) (Kaech and Banker, 2006). The DIV7 neurons were transferred to astrocyte primary cultures for co-culture assays. Isolated primary neurons were plated at a density of 5.0×10^4 cells in 12 multi-well plates for the LDH cytotoxicity and BDNF assays and for immunofluorescence.

2.4. Glutamate-induced gliotoxicity assay and treatments in C6 cells

Initially, cells were treated with 0.125, 0.5, 1, 2.5, 5, or 10 mM glutamate (Sigma-Aldrich) for 20 min or 24 h to determine the concentrations that exhibited gliotoxic effects. Next, cells were treated with glutamate and 10, 25, 50, or 100 μM of roscovitine (Calbiochem) to determine the concentration that exhibited a protective effect. Roscovitine was prepared in dimethyl sulfoxide (DMSO, 0.01%) and diluted in culture medium. The cells that were transfected with the Scr or CDK5 shRNA-miR version (Piedrahita et al., 2010) were then treated with glutamate buffer (vehicle) as controls or with 5 mM glutamate for 24 h at 1 day post-transfection (n = 4, per duplicate). The cells that were transfected with different versions of Rac1 activators were then co-treated with 5 mM glutamate and 25 μM Roscovitine for 24 h at 1 day post-transfection (n = 4, per duplicate).

2.5. Treatments and inhibitors of the primary astrocytes

DIV25 astrocytes were treated with 0.125 mM glutamate (Sigma) for 20 min. After this treatment, the proteins were collected for 30 min for use in the Rho GTPase activation assays. At 24 h post-glutamate, the cells and media were collected for protein isolation or LDH assay or fixed for immunofluorescence analyses. For the time course experiment, the DIV25 astrocytes were treated with 0.125 mM glutamate for 20 min and after 1, 6, 12, 18, or 24 h, the cells were collected (n = 4, per duplicate).

2.6. Plasmid DNA and C6 transfections

The experimental plasmid, pAAV-U6miRCDK5.2-hrGFP, contained the interferent version of CDK5, CDK5 shRNA-miR (shRNA-miR: short hairpin RNA in a microRNA backbone). The control plasmid, pAAV-U6miRCDK5.Scr-hrGFP, contained the Scrambled version, Scr shRNA-miR. These constructs had been validated previously (Piedrahita et al., 2010). The cells were transfected at a concentration of 1 μg/μL of DNA using DMEM supplemented with 0.5% FBS and Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). The transfection medium was left for 5 h and then replaced with DMEM + 0.5% FBS. For the Rac1 activation assays, the following three plasmids were used: pcDNA3-eGFP-Rac1-WT (wild-type), pcDNA3-eGFP-Rac1-T17N (dominant negative, DN), and pcDNA3-eGFP-Rac1-Q16L (constitutively active, CA). These transfections were conducted in the same manner as described previously. At 24 h after transfection, 5 mM glutamate treatments were performed with and without 25 μM roscovitine for 24 h. Two days after transfection, the cells were fixed and permeabilized for immunofluorescence analysis. The Scr and CDK5 shRNA-miR transfection efficacies were 50%, and that for the Rac versions was 30% (n = 4, per duplicate).