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Prenatal exposure to valproic acid increases the neural progenitor cell pool and induces macrocephaly in rat brain via a mechanism involving the GSK-3β/β-catenin pathway

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ABSTRACT

Autism is a spectrum of neurodevelopmental disorders characterized by social isolation and lack of interaction. Anatomically, autism patients often show macrocephaly and high neuronal density. To investigate the mechanism underlying the higher neuronal populations seen in ASD, we subcutaneously injected VPA (400 mg/kg) into pregnant Sprague-Dawley rats on E12, an animal model often used in ASD study. Alternatively, cultured rat neural progenitor cells were treated with VPA. Until E18, VPA induced NPC proliferation and delayed neurogenesis in fetal brain, but the subsequent differentiation of NPCs to neurons increased brain neuronal density afterward. Similar findings were observed with NPCs treated with VPA *in vitro*. At a molecular level, VPA enhanced Wnt1 expression and activated the GSK-3β/β-catenin pathway. Furthermore, inhibition of this pathway attenuated the effects of VPA. The findings of this study suggest that an altered developmental process underlies the macrocephaly and abnormal brain structure observed in the autistic brain.

1. Introduction

Autism is a severe neurobiological and developmental disorder that typically develops in the first 3 years of life. It is characterized by impairments in social interactions and communication, and by restricted and repetitive behaviors and interests.

Autism is not a single disease, but rather a complex phenotype of either multiple 'autistic disorders' or 'autism spectrum disorder'

Abbreviations: GSK-3β, glycogen synthase kinase 3 beta; β-catenin, cadherin-associated protein-β; NPCs, neural progenitor cells; VPA, valproic acid; FACS, fluorescence-activated cell sorting; VZ, ventricular zone; SVZ, subventricular zone; E, embryonic day; P, postnatal day; c-Myc, myelocytomatosis oncogene; PH3, phospho-histone H3; IWR-1, 4-(1,3,3a,4,7,7a-hexahydro-1,3-dioxo-4,7-methano-2H-isoindol-2-yl)-N-8-quinolinyl-benzamide; ASD, autism spectrum disorder; EGF, epidermal growth factor; FGF, fibroblast growth factors; TDZD-8, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione; SD, Sprague Dawley; RIPA, radio-immunoprecipitation assay; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); ELISA, Enzyme-Linked Immunosorbent Assay; BrdU, bromodeoxyuridine; PBS, phosphate buffered saline; TMB, 3,3′,5,5′-tetramethylbenzidine; FITC, fluorescein isothiocyanate; FBS, fetal bovine serum; PI, propidium iodide; Tuj-1, class beta III tubulin; GFAP, glial fibrillary acidic protein; BSA, bovine serum albumin; TMRE, tetramethylrhodamine methyl ester; IZ, intermediate zone; DAPI, 4′,6-diamidino-2-phenylindole; ICC, immunocytochemistry; IHC, immunohistochemistry; CP, cortical plate; GFP, green fluorescent protein; HDAC, histone deacetylase; ERK, extracellular signal-regulated kinases; PTEN, phosphatase and tensin homolog; PI3K, phosphatidylinositol 3-kinase; iPS, induced pluripotent stem cells; Dvl, disheveled; HDACI, histone deacetylase inhibitor; MAP, mitogen-activated protein; KO, knock out; MZ, marginal zone; mTOR, mammalian target of rapamycin.

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(ASD) (Costa e Silva, 2008). Altered brain development during the neurodevelopmental period is commonly considered the underlying neuropathological cause of ASD, although the causes and symptoms of ASD are variable. Accordingly, knowledge of the correlations between altered features in the autistic brain and autistic behavioral symptoms are required to elucidate the pathophysiological mechanism of ASD.

It has been reported that head circumference of 1–2 year old autistic patients is larger than that of normal subjects (Courchesne et al., 2003). Head circumference is used as a reproducible indicator of relative brain size in ASD (Bartholomeusz et al., 2002). Recently, Courchesne group reported that the number of neuron in the brain of autistic patients is actually higher than normal subjects (Courchesne et al., 2011).

Early brain overgrowth occurs during development and remarkably deviant development has been described in ASD (Landa and Garrett-Mayer, 2006; Wetherby et al., 2004). Although, in autistic patients, an enlarged brain is a well-known phenomenon and may be related to the pathophysiological features of ASD, the cellular and molecular bases of pathological overgrowth in the young autistic brain have not been established.

The induction of ASD by valproic acid (VPA) was firstly indicated by seven case studies of fetal valproate syndrome (Christianson et al., 1994; Williams and Hersh, 1997). As has been reported by us and other researchers, single exposure to VPA during pregnancy induces alterations in behavior reminiscent of ASD that persist throughout life in rats and mice (Kim et al., 2011; Schneider and Przewlocki, 2005). Furthermore, the offspring of rats injected with VPA on the 12th day of gestation have been reported to show brain abnormalities, resembling those found by autopsy and brain-imaging studies in autistic patients (Ingram et al., 2000; Rodier et al., 1996). In addition, abnormal medial prefrontal activation during social tasks (Castelli et al., 2002), altered neocortical microcircuit (Casanova et al., 2002b) and abnormal neocortical structures and functions have been also observed in a VPA animal model (Rinaldi et al., 2008a, 2008b). All together, these results suggest that VPA animal models offer a plausible animal model of ASD (Schneider and Przewlocki, 2005).

In the brain, most neocortical neuron populations are produced from NPCs. The initial step of neuronal development involves cell divisions of NPCs within the VZ. The young neurons produced then migrate radially toward the pial surface. Because differentiated neurons cannot proliferate, the total number of neurons, and therefore, overall brain size, is restricted by the NPC proliferative activity during neurogenesis, and thus, NPC over-proliferation can cause brain enlargement.

This study was designed to examine whether VPA modulates NPC proliferation and differentiation, and if so, to identify the intracellular signaling pathway responsible in NPCs.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium/F12 (DMEM/F12), B27-serum free supplement, and antibiotics were obtained from Gibco BRL (Grand Island, NY). VPA, EGF, and axin stabilizer 4-(1,3,3a,4,7,7a-Hexahydro-1,3-dioxo-4,7-methano-2H-isoindol-2-yl)-N-8-quinolinyl-Benzamide (IWR-1) were purchased from Sigma (St. Louis, MO). FGF and Lipofectamine reagent were from Invitrogen (Carlsbad, CA), and the GSK-3β inhibitor 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) was obtained from Calbiochem-Novabiochem (San Diego, CA).

2.2. Animals

Sprague Dawley (SD) rats (purchased from Daehan Bio-Link, Korea) were used throughout this study. Animal handling was conducted in accordance with approved national guidelines and the Seoul National University Institutional Animal Care and Use Committee (SNUIACUC) guidelines for animal care. The embryonic day is considered as E0 when vaginal plug is detected. For *in vivo* experiments, pregnant SD rats were subcutaneously injected with 0.9% saline or VPA in 0.9% saline (400 mg/kg) at E12. Each brain of offspring was taken at E14, E16, E18 and P2.

2.3. Neural progenitor cell culture

The preparation of cortical NPCs from embryonic day 14 (E14) rat embryos was performed as previously described (Go et al., 2011; Reynolds et al., 1992). Briefly, cortices were dissociated into single cells by mechanical trituration, and cells were incubated in DMEM/F12 supplemented with B27-serum free supplement, 20 ng/ml EGF (Sigma, St. Louis, MO), and 10 ng/ml FGF (Invitrogen, Carlsbad, CA) in a 5% CO_2 incubator. EGF and FGF were added every day and the cells grew into floating neurospheres, which were dissociated into single cells with trypsin-EDTA (GibcoBRL, a subsidiary of Invitrogen, Carlsbad, CA). Cells were re-grown into neurospheres in EGF and FGF containing media. This procedure was repeated two times and neurosphere colonies were dissociated into single cells in DMEM/F12 media containing 20 ng/ml EGF and plated on poly-L-ornithine coated plates. The next day, the EGF containing media was removed and NPCs were incubated in growth factor free fresh media. Test reagents including VPA were added at least 2 h after EGF withdrawal. For the *ex vivo* experiment, VPA-treated or saline-treated control NPCs were prepared from E14 embryos derived from pregnant SD mother rats previously injected at E12 with either 400 mg/kg of VPA or 0.9% saline.

2.4. Preparation of brain lysate

Telencephalons of the offspring of VPA treated and saline-treated control rats were taken on embryonic days 14, 16, and 18, and postnatal days 2, 7, and 28. For Western blotting, homogenized telencephalons were prepared in RIPA buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0). The lysates so obtained were diluted with 2X sample buffer (120 mM Tris–HCl (pH 6.8), 20% glycerol, 4% SDS, 28.8 mM 2-mercaptoethanol, 0.01% bromophenol blue) and adjusted to a protein concentration of 1 μg/μl.

2.5. Transient transfection

To investigate the effects of GSK-3β activation and inhibition, we over-expressed the wild type, the constitutively active (S9A), or the kinase dead form (K85A) of GSK-3β. NPCs in 24 well culture plates were transfected using Lipofectamine reagent with 1 μg of each expression plasmid for 6 h, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). pNASSbtopH2B venus1.5 DNA (a Wnt responsive element-GFP; TOPflash reporter construct) or β-catenin siRNA (Stealth RNAi™, Ctnnb1-RSS331356, Invitrogen), which has been successfully used to knock down β-catenin expression (Zhang et al., 2011), were diluted in OptiMEM (Invitrogen, Carlsbad, CA) and transfected into NPCs for 6 h. After transfection, NPCs were incubated in B27 containing fresh DMEM/F12 medium for 12 h with or without VPA.

2.6. MTT assay

An MTT assay was used to examine the proliferative and toxic effects of VPA on NPCs. Briefly, NPCs were incubated for 20 min with MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 500 μg/ml, Sigma, St. Louis, MO) in the dark. Medium was then removed and the formazan dye formed was extracted with 100% ethanol. Absorbance was read using an ELISA reader (TECAN, Austria) at 590 nm.

2.7. BrdU incorporation

NPCs proliferation was determined by ELISA and immunocytochemistry (ICC) based on BrdU incorporation. Levels of BrdU incorporated were measured using a BrdU incorporation assay kit (Roche Diagnostics, Mannheim, Germany). In brief, cells grown in 96 well plates were incubated with 10 μM of BrdU labeling solution for 3 h in a CO_2 incubator, fixed for 30 min at room temperature, washed, and treated with 100 μl of anti-BrdU solution for 90 min. After washing with PBS 3 times, color was developed using TMB substrate solution and absorbance was read using an ELISA reader (TECAN) at 450 nm. Alternatively, NPCs were incubated in BrdU solution in a CO_2 incubator for 12 h and then fixed with 4% paraformaldehyde for 30 min at room temperature. To denature DNA, NPCs were incubated with 2 N and then with 1 N HCl both for 10 min. After washing 3 times with PBS, cells were incubated with FITC conjugated BrdU antibody (1:500, Abcam, Cambridge, UK), washed 3 times with PBS, mounted using Vectashield (Vector laboratories, Burlingame, CA), and observed under a fluorescence microscope (Carl Zeiss, Germany).

2.8. FACS analysis

NPCs dissociated into single cells were used for *in vitro* cell cycle analysis. Approximately 8×10^5 cells were used for each determination. NPCs were trypsinized and fixed with 70% cold ethanol at −20 °C, washed with PBS, resuspended in PBS containing 1% FBS, 50 μg/ml propidium iodide, and 10 μg/ml RNase A, and kept in this suspension for 30 min at room temperature. Cell cycle distributions were