Neuroscience Research xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Neuroscience Research

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

Identification of genes regulating GABAergic interneuron maturation☆

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ARTICLE INFO

Article history:
Received 27 November 2017
Received in revised form
29 November 2017
Accepted 30 November 2017
Available online xxx

Keywords:
GABAergic interneuron
FACS-array
Neurite outgrowth
Dgkg
Diacylglycerol kinase

ABSTRACT

During embryonic development, GABAergic interneurons, a main inhibitory component in the cerebral cortex, migrate tangentially from the ganglionic eminence (GE) to cerebral cortex. After reaching the cerebral cortex, they start to extend their neurites for constructing local neuronal circuits around the neonatal stage. Aberrations in migration or neurite outgrowth are implicated in neurological and psychiatric disorders such as epilepsy, schizophrenia and autism. Previous studies revealed that in the early phase of cortical development the neural population migrates tangentially from the GE in the telencephalon and several genes have been characterized as regulators of migration and specification of GABAergic interneurons. However, much less is known about the molecular mechanisms of GABAergic interneurons-specific maturation at later stages of development. Here, we performed genome-wide screening to identify genes related to the later stage by flow cytometry based-microarray (FACS-array) and identified 247 genes expressed in cortical GABAergic interneurons. Among them, *Dgkg*, a member of diacylglycerol kinase family, was further analyzed. Correlational analysis revealed that *Dgkg* is dominantly expressed in somatostatin (SST)-expressing GABAergic interneurons. The functional study of *Dgkg* using GE neurons indicated alteration in neurite outgrowth of GABAergic neurons. This study shows a new functional role for *Dgkg* in GABAergic interneurons as well as the identification of other candidate genes for their maturation.

1. Introduction

In the cerebral cortex, there are two major types of neurons divided by function and the migration mode during brain development (Lodato and Arlotta, 2015). Glutamatergic pyramidal neurons in the cerebral cortex are produced in the ventricular zone and radially migrate on the radial glial processes. On the other hand, progenitors of GABAergic interneurons arise from the embryonic subcortical progenitor zone, called the ganglionic eminence (GE), and migrate to the cortex tangentially in streams located above and below the cortical plate (CP) during embryonic development in mice (Marin and Rubenstein, 2001). After termination of tangential migration at the postnatal stage, GABAergic interneurons switch to a mode of radial migration, invade the CP, and extend their neurites to construct local neural circuits (Hatanaka et al., 2016; Marin, 2013; Marin and Rubenstein, 2001). Within the cortex, the GE gives rise to several types of interneurons, including the parvalbumin (PV)-expressing fast-spiking interneurons, the somatostatin (SST)-expressing interneurons and the vasointestinal peptide (VIP)-expressing multipolar interneurons (Bandler et al., 2017; Kepecs and Fishell, 2014; Kessaris et al., 2014; Tremblay et al., 2016). GABAergic interneurons are an extremely heteroge-

Abbreviations: GABA, gamma-aminobutyric acid; GE, ganglionic eminence; Gad67, glutamic acid decarboxylase-67; FACS, fluorescence activated cell sorting; Dgkg, diacylglycerol kinase gamma; SST, somatostatin; PV, parvalbumin; Vstm2a, V-set and transmembrane domain containing 2A; Dlx, distal less homeobox; DIV, day in vitro; PA, phosphatidic acid; DGK, diacylglycerol kinase; GEO, gene expression omnibus; RPKM, reads per kilobase per million.

☆ Database: The gene expression data are available in the GEO database under the accession number GSE93295.

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https://doi.org/10.1016/j.neures.2017.11.010
0168-0102/

neous group of neurons (Lodato and Arlotta, 2015). Previous studies suggest that disruption of GABAergic neuronal function leads to a variety of neurological and psychiatric disorders such as epilepsy, schizophrenia and autism spectrum disorders (Chu and Anderson, 2015 ; Le Magueresse and Monyer, 2013 ; Marin, 2012). Therefore, proper migration and neurite outgrowth are critical for normal brain function and behavior.

During embryonic development, neurogenesis, migration and specification of cortical GABAergic interneurons are regulated by a number of transcription factors and their targets. For example, distal-less homeobox (*Dlx*) transcription factors, *Dlx1* and *Dlx2*, function at multiple stages of GABAergic maturation, NK2 homeobox 1, *Nkx2-1* acts as master regulator in promoting GE-derived interneuron fates, and combinatorial binding of NKX2-1 and LIM homeobox protein (*Lhx*), Lhx6, activates genes expressed in cortical migrating interneurons (Bandler et al., 2017; Kepecs and Fishell, 2014; Kessaris et al., 2014; Lodato and Arlotta, 2015; Marin, 2012). After reaching their proper position at the postnatal stage, GABAergic interneurons begin to mature and extend their neurites for generating axon and dendrites (Hatanaka et al., 2016; Marin, 2013; Yamasaki et al., 2010). However, much less is known about the signaling molecules involved in the postnatal migration and maturation of GABAergic interneurons.

To identify the genes involved in maturation of GABAergic interneurons, we performed fluorescence activated cell sorting (FACS)-array analysis at postnatal day 0 (P0) by using GAD67-GFP knock-in mice (Tamamaki et al., 2003), in which a cDNA-encoding enhanced GFP (EGFP) is targeted to the locus encoding glutamic acid carboxylase 67 (GAD67). We identified 247 genes (132 up-regulated and 115 down-regulated) expressed in cortical GABAergic interneurons. Further analyses of candidate genes revealed that *Dgkg*, a member of diacylglycerol kinase family, regulates neurite outgrowth of GABAergic interneurons.

2. Materials and methods

2.1. Animals

Female mice for primary culture were purchased (ICR; CLEA Japan, Inc., Tokyo, Japan). The vaginal plug day was assigned as day 0.5 (E0.5) for mouse. For FACS-array analysis, neonatal pups were obtained by crossing GAD67-GFP$^{+/GFP}$ (Tamamaki et al., 2003) and WT mice (C57BL/6J). Mice were housed in a room with a 12 h light/dark cycle (light on at 8:00 a.m. and off at 8:00 p.m.) and provided with *ad libitum* access to water and food. All of the protocols for animal experiments were approved by the Animal Research Committee of Osaka Bioscience Institute, Hiroshima University and RIKEN Brain Science Institute, and performed in accordance with institutional guidelines and regulations.

2.2. DNA constructs

For overexpression of *Dgkg*, *Vstm2a* and *AW551984*, PCR products containing each coding region were subcloned into a pcDNA3-3xFLAG vector. For subcellular localization analysis of *Dgkg*, a coding sequence of *Dgkg* was subcloned into C-terminus of pβActin-EGFP (β-actin promoter driven expression of EGFP, gifted from S. Okabe, The University of Tokyo). To silence the expression of *Dgkg* (GenBank Accession No. NM_138650.2), 4 types of shRNA were designed by BLOCK-iT RNAi Designer (Thermo Fisher Scientific, Waltham, USA). Each sequence is shown in Supplementary Table 1. The annealed oligonucleotide was subcloned into pENTR-U6 vector (Thermo Fisher Scientific). All plasmids were purified by NucleoBond Xtra Midi kit (Takara Bio Inc., Shiga, Japan) and verified with DNA sequencing (3130 Genetic Analyzer; Thermo Fisher Scientific, Waltham, USA).

2.3. Primary neuronal culture

E14.5 pups were transferred from pregnant ICR into ice-cold Hank's balanced salt solution (HBSS) and the GE was dissected out following previously published procedures (Olsson et al., 1995). Neurons were dissociated using SUMITOMO Nerve-Cell Culture System (SUMITOMO BAKELITE CO., LTD., Tokyo, Japan) and plated on a poly-L-lysine coated glass bottom dish (MATSUNAMI GLASS IND., LTD., Osaka, Japan) at 1×10^6 cells/35 mm dish for imaging and 3×10^6 cells/35 mm dish (IWAKI, Chiba, Japan) for RNA extraction. Cells were treated with 10 μM Ara C (Sigma-Aldrich, Missouri, USA) to inhibit the growth of non-neuronal cells. Neurons were cultured in B27 supplement minus (Thermo Fisher Scientific)/minimum essential medium (MEM) (Thermo Fisher Scientific) with 5% fetal bovine serum (FBS) (Biowest, Nuaille, France), 0.5 mM L-glutamine (Thermo Fisher Scientific), 100 U/ml penicillin and 100 μg/ml streptomycin (Nacalai tesque, Kyoto, Japan) at 37 °C, 5% CO_2.

2.4. Cell line culture

HEK293T and NIH3T3 cells were cultured in Dulbecco's modified eagle medium (DMEM) (Thermo Fisher Scientific) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C, 5% CO_2.

2.5. Transfection

HEK293T and NIH3T3 cells were plated on 6 well plates (Becton, Dickinson and Company, New Jersey, USA) at 1×10^6 and 2×10^5 cells/well 1 day before transfection, respectively. HEK293T cells and GE primary neurons were transfected by the calcium phosphate method (Sambrook and Russell, 2006). At DIV1, 3 μg of each 3 x FLAG tagged plasmid and 1 μg of pβActin-DsRed (gifted from S. Okabe) were used in this experiment. Following 4 h of incubation, the medium was changed to fresh one. For GE primary culture in Fig. 5A and C, 0.5 μg of pβActin-Dgkg-EGFP and pβActin-EGFP were transfected by Lipofectamine 2000, according to manufacturer's protocols (Thermo Fisher Scientific). Similarly, 3 μg of shRNA for *LacZ* and *Dgkg* were transfected by Lipofectamine 2000 in Fig. 5C. In the case of shDgkgALL, 0.75 μg of 4 types shDgkg was used. In Fig. 5B, each shRNA was transfected to NIH3T3 cells by Lipofectamine LTX according to manufacturer's protocols (Thermo Fisher Scientific).

2.6. Western blotting

Two days after transfection, cells were washed with phosphate buffered saline (PBS) and lysed in radio-immunoprecipitation assay (RIPA) buffer contained 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1% Na-deoxycholate, 0.1% SDS, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. After sonication and centrifugation at 17,000 x g for 30 min, the supernatant was used for SDS-PAGE. Cell lysates (20 μg) were separated by using 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Merck Millipore, Massachusetts, USA). The membranes were blocked with 5% skim milk (Thermo Fisher Scientific) and incubated with primary antibody in 2% skim milk for overnight at 4 °C. Secondary antibodies were applied the next day for 1 h at room temperature. The antibodies used in this study were as follows: monoclonal anti-FLAG M2 antibody (F3165, 1:1000; Sigma-Aldrich) and IRDye800CW Goat anti-mouse IgG

Please cite this article in press as: Fukumoto, K., et al., Identification of genes regulating GABAergic interneuron maturation. Neurosci. Res. (2017), https://doi.org/10.1016/j.neures.2017.11.010