Abstract

Protein kinases are responsible for protein phosphorylation and are involved in important signal transduction pathways; however, a considerable number of poorly characterized kinases may be involved in neuronal development. Here, we considered cyclin G-associated kinase (GAK) as a candidate regulator of neurite outgrowth and synaptogenesis by examining the effects of the selective GAK inhibitor SGC-GAK-1. SGC-GAK-1 treatment of cultured neurons reduced neurite length and decreased synapse number and phosphorylation of neurofilament 200-kDa subunits relative to the control. In addition, the related kinase inhibitor erlotinib, which has distinct specificity and potency from SGC-GAK-1, had no effect on neurite growth, unlike SGC-GAK-1. These results suggest that GAK may be physiologically involved in normal neuronal development, and that decreased GAK function and the resultant impaired neurite outgrowth and synaptogenesis may be related to neurodevelopmental disorders.

Keywords: Cyclin G-associated kinase (GAK), SGC-GAK-1, Primary neuron culture, High content screening
inhibition of GAK using SGC-GAK-1 [9–12] affects neurite outgrowth and synaptogenesis in mouse hippocampal neurons, according to the scheme shown in Fig. 1A. For quantification, a high-throughput screening system, the microscope-based CellInsight™ CX5 High Content Screening platform (Thermo Fisher Scientific), was used. First, we compared the total neurite length per number of neurons of samples treated with eight concentrations of SGC-GAK-1 compared to the control as above. Total neurite length per number of neurons was significantly reduced at 5, 10, 20, and 40 μM SGC-GAK-1 compared to the control supplemented with DMSO alone (p < 0.05/8; Fig. 1B, C). Total neurite branch points per number of neurons were also significantly reduced at 10, 20, and 40 μM SGC-GAK-1 compared to the control supplemented with DMSO alone (p < 0.05/8; Additional file 2: Fig. S1). There was no significant difference in the number of neurons from 0.3125 to 10 μM SGC-GAK-1 compared to the control (Additional file 1: Table S1). In addition, we measured synapse formation using double-immunostaining of synaptophysin (presynaptic marker) and SHANK2 (postsynaptic marker), and the total number of synapses per number of neurons was also significantly reduced at 10, 20, and 40 μM SGC-GAK-1 compared to the control (DMSO alone; p < 0.05/8; Fig. 1D–F). There was also no significant difference in the number of neurons from 0.3125 to 5.0 μM SGC-GAK-1 compared to the control (Additional file 1: Table S1).

Next, we immunostained the cultured neurons after exposure to SGC-GAK-1, using NF-200 and SMI-31 antibodies specific to the phosphorylated neurofilament (NF)-200 kDa subunit, and analyzed the immunoreactivity intensity in neurites. In both SMI-31 and NF-200 staining, the neurite intensity decreased in proportion to the increase in SGC-GAK-1 concentration (p < 0.05/8). However, at 2.5 μM, a significant difference was observed only in the intensity of SMI-31 compared to the control (Fig. 1G–J). The result showed that SGC-GAK-1 inhibits the phosphorylation function of GAK even in the concentration range with low neuronal cytotoxicity. Thus, we concluded that phosphorylation of the NF-200 kDa subunit was inhibited by SGC-GAK-1 in growing neurites.

We compared the neurite intensity of neurons treated with SGC-GAK-1 and erlotinib, another protein kinase inhibitor structurally related to SGC-GAK-1 but with lower specificity to GAK [10, 13]. Neurons were treated with erlotinib as described for SGC-GAK-1. In neurons treated with 1.25, 2.5, 5, 10, and 20 μM SGC-GAK-1, the neurite intensity significantly decreased compared to the control (p < 0.05/8). Among them, there were no significant differences in the number of neurons for the 1.25, 2.5, and 5 μM concentrations (Fig. 1K–M). The results showed that SGC-GAK-1 more specifically affected neuronal development involving GAK than did erlotinib. SGC-GAK-1 is a specific inhibitor of GAK, making it an ideal choice for assessing GAK activity. The narrow kinome spectrum and potent cell target engagement make it a better choice for deconvoluting GAK biology than a clinical kinase inhibitor such as erlotinib, which primarily targets EGF receptor tyrosine kinases and has off-target effects on GAK [10, 13]. Erlotinib did not show effects similar to those of SGC-GAK-1 (Fig. 1K–M), indicating that the latter inhibitor affected neurite outgrowth and synaptogenesis via GAK specifically.

Neurodevelopmental disorders, such as autism spectrum disorders, which affect communication, cognition, social interaction, and other patterned behaviors, are currently known to be caused partly by genetic mutations of brain signaling molecules [14] including protein kinases [15]. As shown here, GAK is involved in the normal development of neurons, suggesting the possibility that this or related kinases play a role in the pathogenesis of such diseases. Previously, GAK was reported to be related to neuronal degeneration in Parkinson’s disease by its enhancement of α-synuclein-mediated toxicity [16, 17]; however, no evidence for its relationship to neuronal development has been demonstrated to date.
Fig. 1 (See legend on previous page.)
adapter proteins were reported to be potential substrates for this kinase in in vitro experiments [18], and an ongoing search for other physiological and specific substrates for this kinase is needed.

In conclusion, using the high-throughput imaging quantification system, we showed that axon outgrowth and synaptogenesis are impeded by inhibiting the phosphorylation function of GAK. It was also demonstrated that SGC-GAK-1 inhibits the substrate phosphorylation function of GAK, and affects neurodevelopment to a greater extent than erlotinib. The novel findings of the present study demonstrate that reduced GAK function is associated with neurodevelopmental disorders as well as α-synuclein-mediated neurodegeneration.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13041-022-00951-6.

Additional file 1: Table S1. The number of neurons
Additional file 2: Figure S1. Neurite branches were inhibited by SGC-GAK-1.

Acknowledgements
The authors are grateful to Professor John L. Bixby (Miami University Miller School of Medicine) for the informative discussions.

Author contributions
JE planned and designed the experiments, designed and directed the project, performed the experiments, and wrote the manuscript. RKA performed the experiments. YS analyzed the data. MI planned and designed the experiments, performed the experiments, and wrote the manuscript. TS directed the project, and wrote the manuscript. VPL planned and designed the experiments, designed and directed the project, and wrote the manuscript. AMED-CREST (#21gm1210007s0103 and #22gm1210007s0104 to MI), NIH (R01NS100531 to VPL), the State of Florida (to VPL), the Miami Project to Cure Paralysis (to VPL), and the W.G. Ross Foundation (to VPL).

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations
Ethics approval and consent to participate
All animal experiments were performed following approval from the Animal Resource Center of Niigata University.

Consent for publication
All authors consent to publication.

Competing interests
The authors declare no competing interests.

Author details
1Department of Psychiatry, School of Medicine, and Graduate School of Medical and Dental Sciences, Niigata University, 757 Asahimachi Dori-Ichibancho, Chuo-ku, Niigata 951-8510, Japan. 2Department of Neurochemistry and Molecular Cell Biology, School of Medicine, and Graduate School of Medical and Dental Sciences, Niigata University, 757 Asahimachi Dori-Ichibancho, Chuo-ku, Niigata 951-8510, Japan.

1Miami Project to Cure Paralysis, University of Miami Miller School of Medicine, Miami, FL, USA. 2Institute for Data Science and Computing, University of Miami Miller School of Medicine, Miami, FL, USA.

Received: 8 April 2022 Accepted: 9 July 2022
Published online: 26 July 2022

References
1. Graves LM, Duncan JS, Whittle MC, Johnson GL. The dynamic nature of the kinase. Biochem J. 2013;450:1–8.
2. Bayer KU, Schulman H. CaM kinase: still inspiring at 40. Neuron. 2019;103:380–94.
3. Kawasaki A, Okada M, Tamada A, Okuda S, Nozumi M, Ito Y, Kobayashi D, Yamazaki T, Yokoyama R, Shibata T, Nishina H, Yoshida Y, Fuji Y, Takeuchi K, Igarashi M. Growth cone phosphoproteomics reveals that GAP-43 phosphorylated by JNK is a marker of axon growth and regeneration. iScience. 2018;4:190–203.
4. Dzamko N, Zhou J, Huang Y, Halliday GM. Parkinson's disease-implicated kinases in the brain: insights into disease pathogenesis. Front Mol Neurosci. 2014;7:57.
5. Zhang CX, Engqvist-Goldstein AE, Careno S, Owen DJ, Smythe E, Drubin DG. Multiple roles for cyclin G-associated kinase in clathrin-mediated sorting events. Traffic. 2005;6:1103–13.
6. Lee DW, Zhao X, Yim YI, Eisenberg E, Greene LE. Essential role of cyclin-G-associated kinase (Auxilin-2) in developing and mature mice. Mol Biol Cell. 2008;19:2766–76.
7. Asquith CRM, Bennett JM, Su L, Laitinen T, Elkins JM, Pacheco CD, Strathearn KE, Latourelle JC, Goldwurm S, Pezzoli G, Rochet JC, Lindquist S, Myers RH. Cyclin-G-associated kinase (GAK). J Med Chem. 2019;62:2830–6.
8. Cohen P, Cross D, Jänne PA. Kinase drug discovery 20 years after imatinib: progress and future directions. Nat Rev Drug Discov. 2021;20:351–69.
9. Asquith CRM, Bennett JM, Su L, Laitinen T, Elkins JM, Pacheco CD, Wells CI, Li Z, Willson TM, Zuercher WJ. Towards the development of an in vivo chemical probe for Cyclin G Associated Kinase (GAK). Molecules. 2019;24:4016.
10. Asquith CRM, Naegeli KM, East MP, Laitinen T, Havener TM, Wells CI, Johnson GL, Drewry DH, Zuercher WJ, Morris DC. Design of a Cyclin G-associated kinase (GAK)/epidermal growth factor receptor (EGFR) inhibitor set to interrogate the relationship of EGFR and GAK in Chordoma. J Med Chem. 2019;62:4772–8.
11. Asquith CRM, Treiber DK, Zuercher WJ. Utilizing comprehensive and mini-kinome panels to optimize the selectivity of quinoline inhibitors for cyclin G associated kinase (GAK). Bioorg Med Chem Lett. 2019;29:1277–31.
12. Asquith CRM, Laitinen T, Bennett JM, Wells CI, Elkins JM, Zuercher WJ, Tizard GJ, Poso A. Design and analysis of the 4-anilinoquinazoline kinase inhibition profiles of GAK/SLK/STK10 using quantitative structure-activity relationship. ChemMedChem. 2020;15:26–49.
13. Addeo R, Zappavigna S, Parlato C, Caraglia M. Erlo tinib: early clinical development in brain cancer. Expert Opin Investig Drugs. 2014;23:1027–37.
14. Li X, Zou H, Brown WT. Genes associated with autism spectrum disorder. Brain Res Bull. 2012;88:543–52.
15. Boksha IS, Prokhorova TA, Tereshkina EB, Savushkina OK, Bubueva GS. Protein phosphorylation signaling cascades in autism: the role of mTOR pathway. Biochem Mosc. 2021;86:577–96.
16. Belина A, Rudenko IN, Kaganovich A, Civiero L, Chau H, Kalia SK, Kalia LV, Lobbestael E, Chia R, Ndukwie K, Ding J, Nalls MA, International Parkinson's Disease Genomics Consortium, North American Brain Expression Consortium, Otleszewski M, Hauser DR, Kumaran R, Lozano AM, Baecklandt V, Greene LE, Taymans JM, Greggio E, Cokson MR. Unbiased screening for interactors of leucine-rich repeat kinase 2 supports a common pathway for sporadic and familial Parkinson disease. Proc Natl Acad Sci USA. 2014;111:2626–31.
17. Dumitriu A, Pacheco CD, Wilk JB, Strathearn KE, Latourelle JC, Goldwurm S, Pezzoli G, Rochet JC, Lindquist S, Myers RH. Cyclin-G-associated kinase modifies alpha-synuclein expression levels and toxicity in Parkinson's disease: results from the Gene PD Study. Hum Mol Genet. 2011;20:1478–87.
18. Umeda A, Meyerholz A, Ungewickell E. Identification of the universal cofactor (auxilin 2) in clathrin coat dissociation. Eur J Cell Biol. 2000;79:336–42.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.