Specific inhibition of $\alpha 5$ subunit-containing GABA_A receptors enhances locomotor activity and neuronal activity in the motor cortex

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(Received 10 February 2021; and accepted 22 February 2021)

ABSTRACT

Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the central nervous system (CNS). This study examined the effect of specific inhibition of $\alpha 5$ subunit-containing GABA_A receptors ($\alpha 5$ GABA_AR) on the behavioral profile and neuronal activity of the CNS using a compound called L-655,708, which is a selective negative allosteric modulator of $\alpha 5$ GABA_AR. L-655,708 administration significantly increased locomotor activity without anxiety-related behavior. Furthermore, L-655,708 administration significantly increased c-Fos mRNA expression (a neuronal activity marker) in motor area of the cerebral cortex, whereas it hardly altered c-Fos mRNA expression in the sensory cortex, hippocampus, and spinal cord. This study revealed for the first time that alteration of neuronal activity with specific inhibition of $\alpha 5$ GABA_AR differs depending on each CNS region. $\alpha 5$ GABA_AR could be a potential target for modulating CNS excitability and behavioral activity.

Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the central nervous system (CNS). In general, GABA-mediated inhibition is divided into synaptic phasic inhibition and extrasynaptic tonic inhibition. In particular, accumulating evidence indicates that excessive tonic inhibition, which reduces the excitability of neurons, is associated with several functional impairments in CNS disorders (*e.g.*, sensorimotor and cognitive impairments after stroke, motor symptoms in Parkinson's disease, and memory impairment in Alzheimer's disease) (Clarkson *et al.*, 2010; Jo *et al.*, 2014; Orfila *et al.*, 2019; Heo *et al.*, 2020). Most extrasynaptic GABA_A receptors, which primarily induce tonic inhibition, contain $\alpha 5$ subunits (Walker and Semyanov,

2008). Thus, researchers are currently focusing on specific inhibition of $\alpha 5$ subunit-containing GABA_A receptors ($\alpha 5$ GABA_AR) to restore reduced excitability and enhance activity-dependent synaptic plasticity, such as long-term potentiation, in CNS disorders.

A compound called L-655,708 is a selective negative allosteric modulator of $\alpha 5$ GABA_AR. L-655,708 can specifically reduce GABA-mediated tonic inhibition and facilitate long-term potentiation by shifting toward less inhibitory activity, which was supported by previous *in vitro* studies (Atack *et al.*, 2006; Clarkson *et al.*, 2010). Indeed, L-655,708 administration enhances sensorimotor and cognitive function recovery in animal model of CNS disorders such as stroke (Clarkson *et al.*, 2010; Lake *et al.*, 2015; Orfila *et al.*, 2019). Therefore, it is assumed that specific inhibition of $\alpha 5$ GABA_AR (*i.e.*, reducing GABA-mediated tonic inhibition) could beneficially contribute to functional impairment in CNS disorders. Despite its growing clinical importance, the fundamental effect of L-655,708 administration on the behavioral profile and neuronal activity of the

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CNS remains poorly understood. Furthermore, no study has assessed neuronal activity after L-655,708 administration in several CNS regions. Therefore, this study aimed to examine the effect of specific inhibition of $\alpha 5\text{GABA}_A\text{R}$ on the behavioral profile and neuronal activity using L-655,708. In order to compare region-dependent effects, we exploratory assessed neuronal activity of the CNS, including the cerebral cortex, hippocampus, and spinal cord.

Twelve 7-week-old male Wistar rats (300.3 $\pm$ 12.0 g, mean $\pm$ SD) were included. Rats were randomly divided into two groups: a control group (CON, $n = 6$) and an L-655,708 group (L655, $n = 6$). All rats were housed in a temperature- and humidity-controlled room on a 12-hour light/dark cycle with food and water available *ad libitum*. All study procedures were approved by the ethics committee for animal research of Hokkaido University in Japan (# 19-0101) and conducted in accordance with the guidelines of the committee. To inhibit $\alpha 5\text{GABA}_A\text{R}$, we intraperitoneally administered L-655,708 (Molecular weight: 341.37, product #: 1327; R&D Systems, Inc., USA) to the L655 group at a dose of 0.50 mg/kg. Similarly, we intraperitoneally administered physiological saline to the CON group to achieve the same conditions. L-655,708, which has a 30- to 70-fold higher affinity for $\alpha 5\text{GABA}_A\text{R}$ than for other GABA_A receptor subtypes, is rapidly absorbed into the blood and thereafter the brain 5 min after intraperitoneal injection (Atack *et al.*, 2005). It was estimated that 30 minutes after intraperitoneal injection, L-655,708 at a dose of 0.50 mg/kg would cause 50–65% occupancy of the $\alpha 5\text{GABA}_A\text{R}$, with minimum occupancy of the other receptor subtypes (Atack *et al.*, 2005).

The open field test (OFT) was performed to evaluate spontaneous locomotor activity and anxiety-related behavior. The OFT was performed twice: 1 week before drug administration (Baseline) and 30 min after drug administration (Post-injection), as shown in Fig. 1a. Briefly, for 5 min, each rat was allowed to freely explore the test arena (60 cm $\times$ 60 cm) and videotaped. The coordinates of the rat's position were captured using MATLAB R2019b software (MathWorks, USA). The total distance, which was used to indicate locomotor activity, was calculated by tracing the movement of each rat during free exploration (Fig. 1b). In addition, we measured the time spent in the corner and peripheral areas (filled areas of each y-axis label, Fig. 1d–e) to evaluate anxiety-related behavior. In general, rats with anxiety prefer to stay in the corner or peripheral area of the test arena (Kraeuter *et al.*, 2019).

Immediately after the Post-injection OFT, the cerebral cortex (motor and sensory areas), hippocampus, and spinal cord (4th to 6th cervical vertebrae (C4–6) level) were carefully removed under sterile DNase/RNase-free conditions. Total RNA extraction and reverse transcription were performed by using the RNeasy Lipid Tissue Mini Kit (QIAGEN, Hilden, Germany) and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), respectively, as described in our previous study (Inoue *et al.*, 2020). Subsequently, real-time quantitative RT-PCR was performed using a StepOnePlus real-time PCR system (Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems), as described in our previous study (Inoue *et al.*, 2020). The relative RNA expression level of each target gene (c-Fos, Rn02396759_m1; BDNF, Rn02531967_s1; GABRA5, Rn00568803_m1, Applied Biosystems) was determined relative to that of β -actin (internal control gene, 4352340E, Applied Biosystems) by using the comparative Ct ($2^{-\Delta\Delta C_t}$) method. Transcript levels were normalized to the average levels in the CON group.

Statistical analyses were performed by using SPSS Statistics software (v26.0, IBM, USA). Comparisons between the CON and L655 groups were analyzed by using a two-tailed, unpaired *t*-test. The associations between numeric variables were determined by using Pearson's correlation analysis, and *r* represented the correlation coefficient. The associations were examined in the entire subject group, including both the CON and L655 groups. The statistical significance threshold was set at $P < 0.05$.

The effect of L-655,708 administration on behavior profile is shown in Fig. 1c–e. Regarding the Baseline OFT, there were no significant differences between the CON and L655 groups concerning total distance (Fig. 1c), time in the corner area (Fig. 1d), and time in the peripheral area (Fig. 1e). These results indicated that there was no significant difference in the behavioral profile between the two groups before the intervention. By contrast, in the Post-injection, total distance of the L655 group was significantly higher than that of the CON group ($df = 10$, $t = -2.832$, $P = 0.018$, Fig. 1c). There were no significant differences between the CON and L655 groups concerning either time in the corner area (Fig. 1d) or time in the peripheral area (Fig. 1e). In addition, we observed each rat's behavior for 10 min after drug administration, and no seizures were noted after L-655,708 administration on the Racine scale, which is commonly used as a seizure assessment score in rodents (Macias *et al.*, 2013).

Fig. 1 (a) Experimental timeline. OFT: open field test. (b) Representative rat movements and distribution pattern during free exploration for 5 min in the OFT. (c) Effects of L-655,708 administration on locomotor activity. L-655,708 administration significantly increased locomotor activity in the L655 group compared with the CON group ($P = 0.018$). (d, e) Effects of L-655,708 administration on anxiety-related behavior. Data are shown as the mean $\pm$ SE and each value. * indicates $P < 0.05$.

Although it is well-recognized that the use of non-specific GABA antagonists has serious limitations of clinical application because of their known adverse effects, including convulsant, pro-convulsant, and anxiogenic effects (Braudeau *et al.*, 2011), the present study demonstrated that specific inhibition of $\alpha 5$ GABA_AR can enhance locomotor activity without obvious adverse effects reflected in behavioral outcomes. Furthermore, our findings also suggest that $\alpha 5$ GABA_AR could be a potential target for enhancing spontaneous locomotor activity.

Subsequently, the effect of L-655,708 administration on c-Fos mRNA expression is shown in Fig. 2a–d. Detection of the expression of immediate early genes, such as c-Fos, is commonly used as a reliable method to confirm neuronal activity because expression of such genes is rapidly induced by intracellular signaling, such as Ca^{2+} influx into neurons

accompanied by evoked action potentials (Okuno, 2011). The expression of c-Fos mRNA in the motor cortex of the L655 group was significantly higher than that in the CON group ($df = 10$, $t = -3.261$, $P = 0.009$, Fig. 2a). There were no significant differences between the CON and L655 groups concerning c-Fos mRNA expression in the sensory cortex (Fig. 2b), hippocampus (Fig. 2c), and spinal cord (Fig. 2d). These results demonstrated that the inhibition of $\alpha 5$ GABA_AR increased neuronal activity specifically in the motor cortex. Considering that tonic inhibition mediated by $\alpha 5$ GABA_AR sets an excitability threshold for neurons (Bonin *et al.*, 2007; Walker and Semyanov, 2008), it is reasonable that pharmacological inhibition of $\alpha 5$ GABA_AR enhanced neuronal activity by the downregulation of neuronal excitability threshold.

It is well-recognized that brain-derived neuro-

Fig. 2 Effects of L-655,708 administration on c-Fos (a–d) and BDNF mRNA expressions (e–h) in the motor cortex (a, e), sensory cortex (b, f), hippocampus (c, g), and spinal cord (d, h). L-655,708 administration significantly increased the expression of c-Fos mRNA specifically in the motor cortex ($P = 0.009$, a). In addition, there was a significant positive correlation between c-fos mRNA and BDNF mRNA expression levels in the motor cortex ($r = 0.609$, $P = 0.036$, i). These mRNA expression levels were normalized to the average levels in the CON group. The correlation coefficient determined by Pearson correlation analysis. Data are shown as the mean $\pm$ SE and each value. ** indicates $P < 0.01$.

trophic factor (BDNF), a member of neurotrophin family, mediates many activity-dependent processes in the mammalian brain and beneficially contributes to neuroplasticity, neurogenesis, and neuroprotection (Park and Poo, 2013). Therefore, we further examined the effect of L-655,708 administration on the expression of BDNF. Although there were no significant differences between the CON and L655 groups in BDNF mRNA expression in any region (Fig. 2e–h), there was a significant positive correlation between c-Fos and BDNF mRNA expressions in the motor cortex ($r = 0.609$, $P = 0.039$, Fig. 2i), indicating that the higher neuronal activity in the motor cortex was, the higher the BDNF mRNA expression in the motor cortex. This relationship between c-Fos and BDNF mRNA expression was consistent with the feature of neuronal activity-dependent BDNF expression (Vermehren-Schmaedick *et al.*, 2015).

The present study revealed that specific inhibition of $\alpha 5$ GABA_AR increases locomotor activity accompanying the enhancement of neuronal activity. Interestingly, the enhancement of neuronal activity with L-655,708 was limited to the motor cortex, whereas the major expression site of GABA_A receptor $\alpha 5$ subunit (GABRA5) is the hippocampus (Fritschy and Brünig, 2003; Engin *et al.*, 2018). We did in

fact confirm that the expression of GABRA5 mRNA in the hippocampus was higher than that in the cerebral cortex and spinal cord (see the Supporting data, a). In addition, L-655,708 administration did not affect GABRA5 mRNA expression in any region (see the Supporting data, b). Therefore, neuronal activity enhancement in motor area of the cortex with L-655,708 administration did not correspond to regional differences in the expression of the GABRA5.

Alternatively, it was also considered that the inhibition of $\alpha 5$ GABA_AR might have altered cortical neuronal activity indirectly via behavioral change, but not directly. There was in fact a significant positive correlation between locomotor activity in the Post-injection OFT and c-Fos mRNA expression in the motor cortex ($r = 0.589$, $P = 0.044$, Fig. 3a), suggesting that there was a relationship between behavioral activity and neuronal activity in the motor cortex. This study thus provides two hypotheses that i) the inhibition of $\alpha 5$ GABA_AR might induce neuronal activity followed by behavioral change and ii) the inhibition of $\alpha 5$ GABA_AR might increase locomotor activity followed by neuronal activity change. Although this study could not identify the causal link between the behavioral activity and neuronal activity, further study using animals lacking normal

Fig. 3 Associations between locomotor activity and gene expressions in the motor cortex. There were significant positive correlations between locomotor activity in the Post-injection OFT and c-Fos mRNA expression level ($r = 0.589$, $P = 0.044$, a), and cortical BDNF mRNA expression level in the motor cortex ($r = 0.610$, $P = 0.035$, b), respectively. These associations were examined in the entire subject group, including both the CON and L655 groups. The correlation coefficients were determined by Pearson correlation analysis.

neuronal activity and real-time measurements of neuronal activity is expected to corroborate the crucial role of $\alpha 5$ GABA_AR in the behavior profile and neuronal activity. However, given the fact that L-655,708 administration certainly increased neuronal activity and locomotor activity (Fig. 1c and 2a), we showed that the inhibition of $\alpha 5$ GABA_AR could be effective in facilitating activity-dependent neuroplasticity in the motor cortex, because both neuronal activity and locomotor activity positively correlated with the expression of BDNF mRNA in the motor cortex (Fig. 2i and 3b). Altogether, this study highlights the importance of the interaction between the behavioral activity and neuronal activity after reducing GABA-mediated tonic inhibition.

In conclusion, this study revealed that specific inhibition of $\alpha 5$ GABA_AR increases locomotor activity accompanying the enhancement of neuronal activity, specifically in motor area of the cerebral cortex. These results suggest that $\alpha 5$ GABA_AR could be a potential target for modulating CNS excitability and behavioral activity. As a treatment of patients with CNS disorders, specific inhibition of $\alpha 5$ GABA_AR could make a neuronal platform to facilitate activity-dependent plasticity in the motor cortex without adverse effects. However, except for motor and sensory areas, the effect of L-655,708 on neuronal activity in the other cortical area remained unclear in this study. Further research using an alternative technical approach such as *in vivo* imaging, which can detect whole-brain neuronal activity, could elu-

cidate the limitation of this study.

Acknowledgements

This study was supported by JSPS KAKENHI Grant JP17H02117, 19J23508 and 20H04048.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supporting data (a) Regional differences in GABRA5 mRNA expression. These data were based on the CON group to eliminate the effect of L-655,708 administration. One-way ANOVA showed a significant group effect ($F_{(3, 20)} = 104.991$, $P < 0.001$), and Tukey's post hoc analyses demonstrated that GABRA5 mRNA expression in the hippocampus was significantly higher than that in the other region. In addition, GABRA5 mRNA expression in the motor cortex was significantly higher than that in the sensory cortex and spinal cord. *** indicates $P < 0.001$. (b) Effect of L-655,708 administration on the expression of GABRA5 mRNA. L-655,708 administration did not affect the expression of GABRA5 mRNA in any region. These mRNA expression levels were normalized to the average levels in the motor cortex or the CON group. Data are shown as the mean $\pm$ SE and each value.