Direct Evidence that 4'-*O*-methylpyridoxine Induces Hyperactivity and Convulsions due to Pyridoxal Phosphate Deficiency in the Brain

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Ginkgo biloba seed (GBS) poisoning is caused by the toxic substance 4'-*O*-methylpyridoxine (MPN). Gamma-aminobutyric acid (GABA) in the brain is suggested to be involved in the convulsion-inducing effect of MPN.

To clarify the direct effects of MPN in the brain on behavior and changes in vitamin B_6 and GABA concentration, we intrathecally administered MPN (5-80 nmol) to mice. Some of them developed hyperactivity and seizures. Incidence of behavioral abnormalities were correlated with the MPN dose/body weight. In the analysis of the brain, mice with hyperactivity shown decreased Pyridoxal-5'-phosphate (PLP) and decreased GABA/Glutamae (Glu) ratio. Administered MPN dose/body weight was negatively correlated with PLP ($p<0.001$) and GABA/Glu ratio ($p=0.004$) in the brain. MPN concentration in the brain negatively correlated with PLP ($p<0.001$) and GABA/Glu ratio ($p<0.001$), and a positive correlation between concentration of MPN and phosphorylated MPN (4'-O-methylpyridoxine-5'-phosphate, MPNP) was found ($p=0.01$). We report for the first time that MPNP is produced from MPN *in vivo* and MPN decreases PLP concentrations in the brain, resulting in the decrease of GABA/Glu ratio and this change leads to behavioral changes. *Shinshu Med J 72 : 159—167, 2024*

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Key words : 4'-*O*-methylpyridoxine, 4'-*O*-methylpyridoxine-5'-phosphate, gamma-aminobutyric acid, Ginkgo biloba seed, pyridoxal-5'-phosphate

Abbreviations : BW, body weight ; GABA, gamma-aminobutyric acid ; GBS, ginkgo biloba seed ; Glu, glutamate ; GAD, glutamate decarboxylase ; HPLC, high-performance liquid chromatography ; MPN, 4'-*O*-methylpyridoxine ; MPNP, 4'-*O*-methylpyridoxine-5'-phosphate ; PBS, phosphate-buffered saline ; PK, pyridoxal kinase ; PL, pyridoxal ; PLP, pyridoxal-5'-phosphate

I Introduction

Ginkgo biloba seed (GBS) is a popular food in Asia. It can be eaten after heating them and are often included as an ingredient in stir-fries and other dishes. However, children have been reported to suffer poisoning after consuming even small amounts of GBS and are warned against eating them[1)-3)]. Case of GBS poisoning in adults have been described as well[4)5)].

Present in GBS, 4'-*O*-methylpyridoxine (MPN) and MPN-5'-glucoside (MPNG) causes hyperactivity and seizures[6)7)]. Research into the mechanism of convulsion by MPN in mice suggests the involvement of gamma-aminobutyric acid (GABA), which is produced from glutamate (Glu) by glutamate decarboxylase (GAD), to exert an inhibitory effect on brain activity[8)]. GABA and Glu are respectively two major inhibitory and excitatory neurotransmitters in the central nervous system[9)]. In complex partial epilepsy patients, decrease of GABA/Glu ratio in intrahippocampal microdialysates was linked to seizures[10)]. When GAD generates GABA from Glu, pyridoxal-5'-phosphate (PLP), an active form of vitamin B_6, is needed as a cofactor[11)].

Decreases in plasma PLP concentration and PLP/Pyridoxal (PL) ratio were found in rats receiving MPN[8)]. Lower PLP/PL ratios have been detected in

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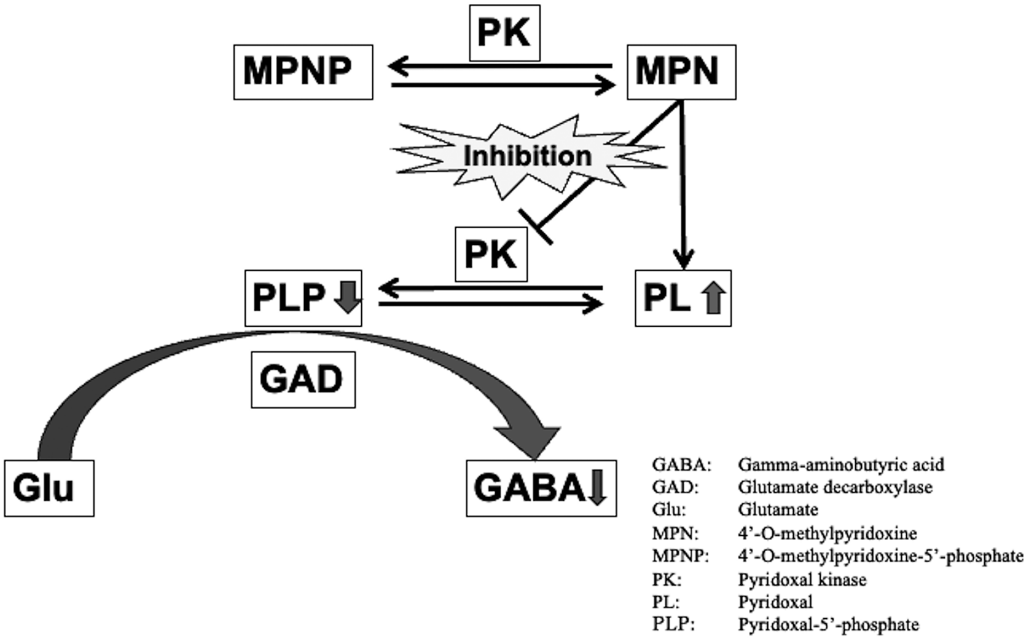


Fig. 1 The pathway by which MPN lowers GABA production. PK is needed to convert PL to PLP, and PLP is needed as a cofactor to convert Glu to GABA. MPN is phosphorylated to MPNP by PK.

the serum and cerebrospinal fluid of patients with GBS poisoning[2]. The PLP/PL ratio in blood is a biomarker for pyridoxal kinase (PK)[8], which is involved in the production of PLP, as well as for the alkaline phosphatase that is needed to produce PL from PLP[12]. *In vitro* studies have described an inhibitory effect of MPN on PK[13)14].

Based on the above reports, the inhibition of PK by MPN is thought to cause decreases in PLP, PLP-dependent GAD activity, and GABA concentration, thereby leading to convulsions **(Fig. 1)**[1)8)15]. However, reports on the relationships among MPN concentration, vitamin B_6 concentration, GAD activity, and GABA concentration in the brain after MPN administration are lacking. The present study on mice intrathecally administered MPN was conducted to clarify the direct effects of MPN on neurological symptoms, brain vitamin B_6, GAD activity, and GABA concentration. We opted for intrathecal administration because intraperitoneal and oral administration of MPNs may cause phosphorylation of MPNs before they enter the intrathecal space[14], and there is a possibility that individual differences could be observed. Since Kästner et al.[14] found that PK generated 4'-*O*-methylpyridoxine-5'-phosphate (MPNP) from MPN, we also evaluated MPNP along with vitamin B_6 in the mouse brain.

Ⅱ Materials and Methods

A Chemicals

MPN-HCl and MPNP were chemically synthesized as reported previously[13)16]. All other reagents used were of analytical grade.

B Animals

The study was submitted to the Ethics Committee in Shinshu University with the stipulation that administered MPN doses that do not cause convulsions should be used, and that in the event of convulsions, the mice should be euthanized immediately. During intrathecal MPN administration, the mice were anesthetized with isoflurane to avoid causing them pain. All experiments were approved by the Institutional Animal Care Committee of Shinshu University (Ethical committee approval number : 021064) in accordance with the US National Research Council 2011 (Guide for the Care and Use of Laboratory Animals). Reporting of animal testing experiments comply with the ARRIVE guidelines. In this investigation, 4-5-week-old male ddY mice were obtained from Japan SLC, Inc (Shizuoka, Japan). Mean ± standard deviation body weight was 29.2 ± 3.1 g. All mice had free access to

food and water throughout the study period.

C Intrathecal MPN administration

The mice were first anesthetized with isoflurane, and then head position was fixed with positioning devices. Insertion of a needle into the right lateral ventricle was performed by incision with a scalpel and advancement of the needle tip 3 mm into the brain from a position 1 mm to the right and 0.5 mm caudal to the small fontanelle[17]. MPN was dissolved in phosphate-buffered saline (PBS), and 5 μl of MPN (5-80 nmol) solution was injected into the right lateral ventricle using a microinjector (5 nmol for 2 mice, 10 nmol for 2 mice, 20 nmol for 5 mice, 40 nmol for 9 mice, and 80 nmol for 5 mice). In control mice, 5 μl of PBS was administered to 6 animals. The mice were observed for 30 minutes after injection, during which time the experiment was stopped in mice exhibiting seizures. After either 30 minutes of observation or seizure onset, all mice were anesthetized with isoflurane and sacrificed by cervical dislocation for harvesting brain tissue. Brain specimens were immediately frozen with dry ice and cryogenically stored at -70 ℃.

D Sample preparation

A 10-fold volume of 5 mM Tris-HCl buffer (pH 7.4) containing 1.15 % KCl, 0.1 mM EDTA, and homoserine as an internal standard was added to each brain sample, which were subsequently homogenized with a homogenizer, and stored at -70 ℃ until analysis. A reaction solution containing semicarbazide and glycine was added to the sample for vitamin B_6 concentration measurement. After 30 minutes of reaction to produce a semicarbazone derivative, the sample was deproteinized with perchloric acid and centrifuged. The supernatant was then neutralized with potassium hydroxide, centrifuged again, and analyzed by high-performance liquid chromatography (HPLC) after filtration[8]. To measure amino acids and GABA, homogenized samples were deproteinized with methanol, centrifuged, and filtered. The samples were injected to HPLC 5 minutes after derivatization by ortho-phthalaldehyde.

E Chromatographic conditions

HPLC conditions were optimized from a previously reported method[8)18)]. The HPLC apparatus comprised a Shimadzu LC-10Avp system equipped with a RF-10A spectrofluorometer (Shimadzu, Kyoto, Japan). In vitamin B_6 measurements, chromatographic separation was performed in an Inertsil ODS-3 column (5 μm, 150×4.6 mm, GL Sciences, Tokyo, Japan) at a flow rate of 1.0 mL/min and column temperature of 25 ℃. Mobile phase A was 60 mM disodium hydrogen phosphate containing 400 mg/L ethylenediaminetetraacetic acid disodium salt adjusted to pH 6.5 with concentrated phosphoric acid. Mobile phase B was methanol. The ratio of mobile phases A to B was 90 : 10. For determining the concentrations of vitamin B_6 analogues apart from the aldehyde types of vitamin B_6, PL and PLP, fluorescence measurement was performed at 420 nm emission wavelength and 320 nm excitation wavelength. To identify PL and PLP, fluorescence measurement was conducted at 450 nm emission wavelength and 380 nm excitation wavelength after derivatization by semicarbazide. The concentration of vitamin B_6 analogues were calculated using the absolute calibration curve method. Linearity for each compound was confirmed from 6 nM to 900 nM. In GABA and Glu measurements, chromatographic separation was performed in an Eicompak SC-5ODS column (3.0 μm, 150×4.6 mm, EICOM, Kyoto, Japan) at a flow rate of 0.5 mL/min and column temperature of 30 ℃. Mobile phase A was 100 mM disodium hydrogen phosphate containing 28 % methanol and 0.5 % tetrahydrofuran after adjustment to pH 6.0 with concentrated phosphoric acid. GABA and Glu concentrations were determined by fluorescence measurement at 455 nm emission wavelength and 340 nm excitation wavelength. GABA and Glu concentrations were calculated by the internal standard method using homoserine as an internal standard.

F Statistical analysis

The relationship between MPN dose/body weight and abnormal behavior in mice was analyzed by Student's *t*-test, as were the relationships of behavioral abnormalities with the concentrations of vitamin B_6 and amino acid analogues. The relationships of MPN dose/body weight with vitamin B_6 and amino acid analogue concentrations in the brain were evaluated with Pearson's chi-squared test. This test was

Table 1　Relationship between MPN dose/body weight (BW) ratio and abnormal behavior in mice

	MPN dosage/ BW (mg/kg)
Group 1 : No hyperactivity, no seizure (n = 8)	0.22 ± 0.18
Group 2 : Hyperactivity, no seizure (n = 12)	0.28 ± 0.14
Group 3 : Hyperactivity and seizure (n = 3)	0.50 ± 0.08

Values represent the mean ± SEM of each group. Statistically significant differences were found between group 1 and group 3 (p = 0.007) as well as between group 2 and group 3 (p = 0.013).

also employed to assess the correlations between vitamin B_6 and amino acid analogue concentrations. All analyses were performed using SPSS software (version 29.0.0.0). A p-value of less than 0.05 was considered statistically significant.

Ⅲ　Results

A　Reactions of experimental animals

After intrathecal injection, the test mice required 10–15 minutes to awaken from the isoflurane-induced anesthesia. Hyperactivity was defined as obvious abnormal behavior, such as jumping around in a beaker or spinning in the same direction and was observed in groups administered 20 nmol or more of MPN. The percentages of mice that became hyperactive were 5 nmol : 0 %, 10 nmol : 0 %, 20 nmol : 100 %, 40 nmol : 67 % , and 80 nmol : 80 % . No hyperactivity was seen in the control mice. Obvious seizures were observed in the 40 nmol and 80 nmol MPN administration groups. The percentages of mice displaying seizures were 5 nmol : 0 %, 10 nmol : 0 %, 20 nmol : 0 %, 40 nmol : 11 %, and 80 nmol : 20 %. In terms of MPN dose/body weight ratio, the lowest dose that caused seizures was 0.39 mg/kg (1.79 nmol/kg). All mice that suffered seizures were hyperactive immediately beforehand. We observed a significant difference in MPN dose/body weight between mice with (0.50 ± 0.08 mg/kg) and without (0.22 ± 0.18 mg/kg) hyperactivity and seizures ($p < 0.05$). In addition, a significant difference was seen between mice with hyperactivity (0.28 ± 0.14 mg/kg) and those with seizures (0.50 ± 0.08 mg/kg) ($P < 0.05$) **(Table 1)**.

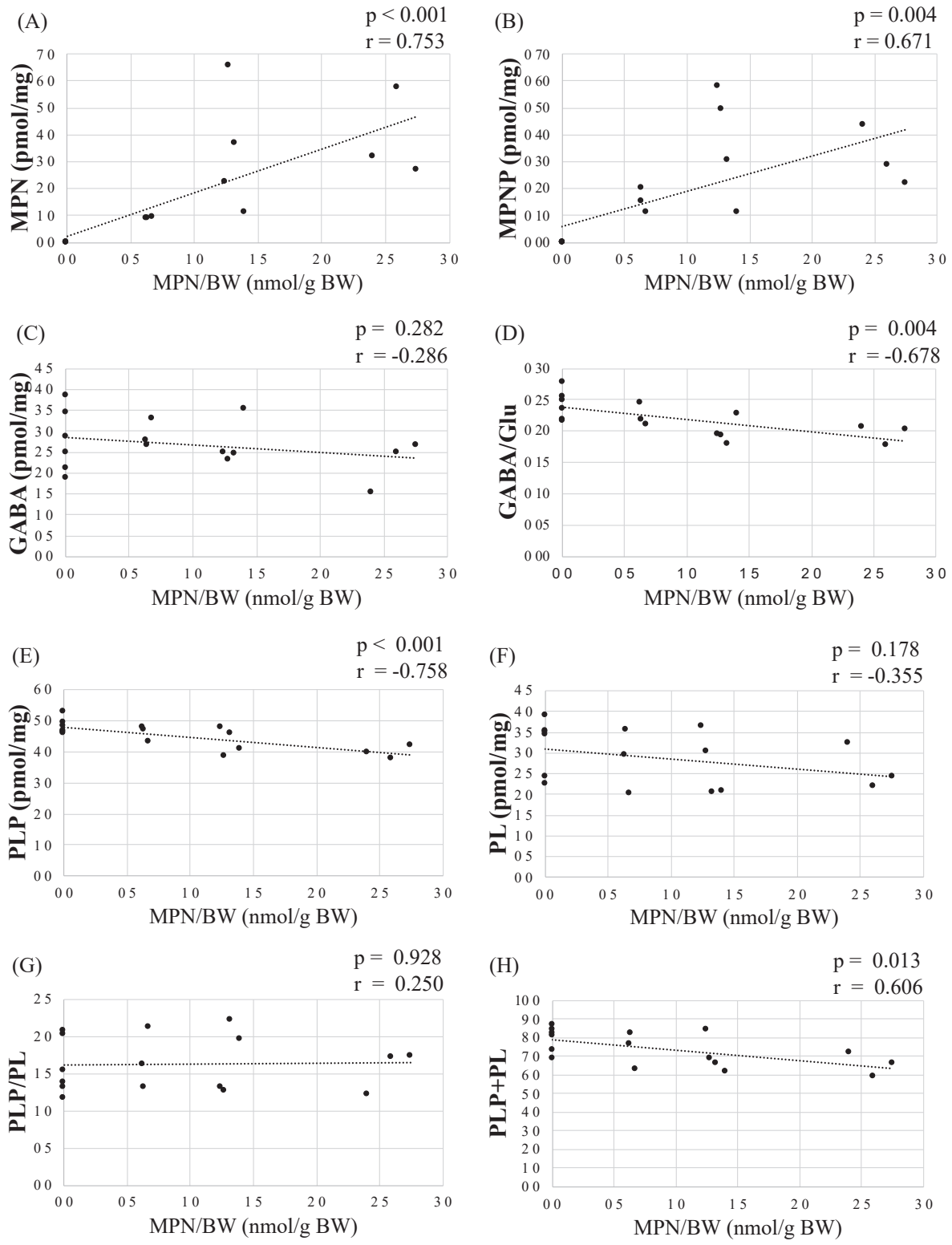
B　Measurement results of vitamin B_6 and amino acid analogues

During the process of sample preparation and analysis, only data that are considered sufficiently suitable for the analysis are included in this study. In total, 18 mice brain were analyzed. MPN dose/body weight had positive correlations with MPN and MPNP concentrations **(Fig. 2A, B)**. MPN dose/body weight was negatively correlated with the GAD biomarker GABA/Glu ratio, PLP concentration, and total vitamin B_6 (PLP + PL) concentration **(Fig. 2D, E, H)**. In **Fig. 2**, two mice that had a seizure within 30 minutes were excluded. Comparisons between control mice and hyperactive mice showed that GABA/Glu ratio, PLP concentration, and total vitamin B_6 (PLP + PL) concentration were significantly lower in the hyperactive group **(Fig. 3A, C, E)**. The GABA/Glu ratios of the two mice that suffered seizures were 0.19 and 0.16, respectively, showing a decreasing trend compared with the control group. In the analysis of vitamin B_6 and amino acid analogues in the brain, MPN concentration was significantly negatively correlated with GABA/Glu ratio and PLP concentration while being positively correlated with MPNP concentration **(Fig. 4B, C, G)**. GABA/Glu ratio displayed a significant positive correlation with PLP concentration and a negative correlation with MPNP concentration **(Fig. 4H, J)**. GABA concentration and PLP/PL ratio showed a significant positive correlation **(Fig. 4K)**.

Ⅳ　Discussion

MPN intrathecally administered to mice induced behavioral abnormalities, including hyperactivity and convulsions, which were closely related to increases in MPN dose/body weight. By comparable amounts of GBS intake, children with lower body weight may experience increased MPN concentration in the brain, which is thought to contribute to the higher propensity of GBS poisoning in youths[8].

The significant difference in GABA/Glu ratio, a



BW: Body weight, GABA: Gamma-aminobutyric acid, Glu: Glutamate,
MPN: 4'-*O*-methylpyridoxine, MPNP: 4'-*O*-methylpyridoxine-5'-phosphate,
PL: Pyridoxal, PLP: Pyridoxal-5'-phosphate

Fig. 2 Relationship of vitamin B_6 and amino acid analogues with MPN dose/body weight (BW) were shown. Only the mice that could be observed for 30 minutes after MPN administration were analyzed.

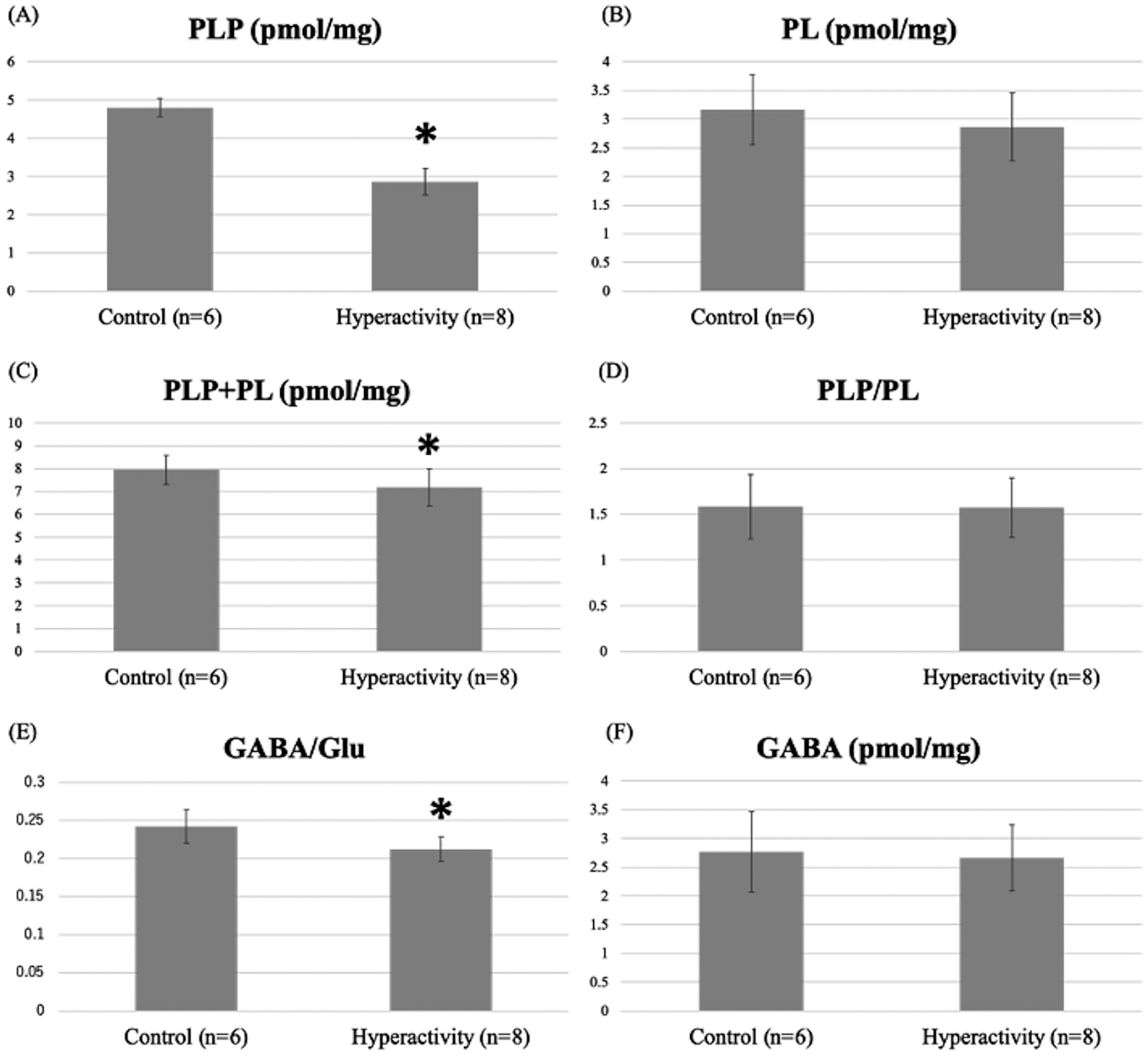
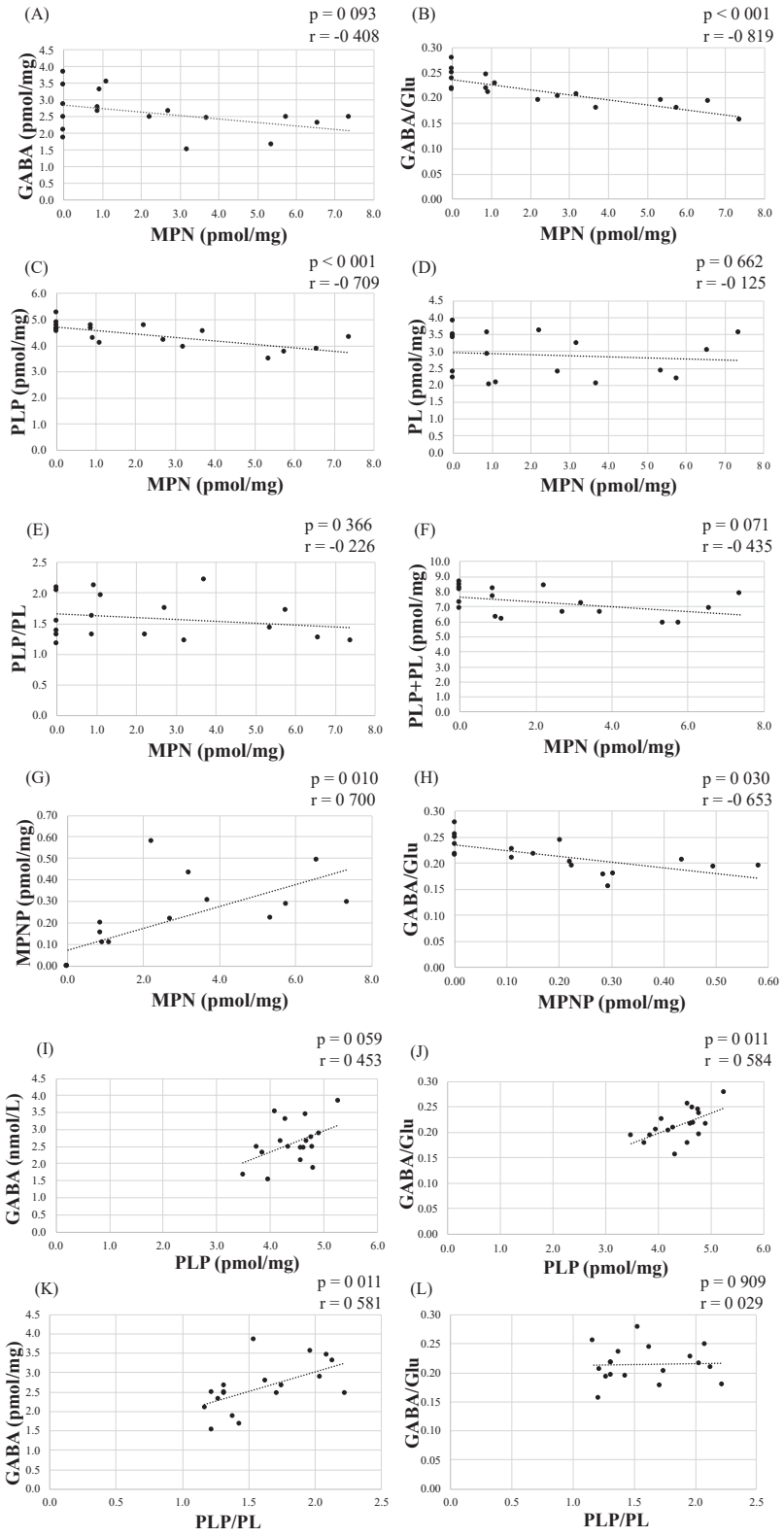


Fig. 3 Vitamin B_6 and amino acid analogues in mice with or without abnormal behavior were shown. The graphs contain the data of control mice. Data was expressed as mean ± SEM. $^*p<0.05$ versus control mice.

biomarker of GAD, between the hyperactive group and control group indicated a major influence on the behavioral abnormalities of the mice. We witnessed significant differences in brain GABA/Glu ratio, PLP concentration, and PLP＋PL concentration between controls and hyperactive mice. As the given MPN dose or MPN concentration in the brain increased, PLP concentration and GABA/Glu ratio in the brain fell, likely since PK inhibition by MPN reduced PLP production and prevented GABA production from Glu by GAD.

Although we observed a tendency for GABA to decline, no significant difference or correlation was found. Due to ethical considerations, the mice in this investigation were euthanized at the time seizures occurred. As their brains were removed earlier than in other mice, they were excluded from the correlation studies in **Fig. 2** and **3**. Accordingly, mice without convulsions were analyzed, suggesting that GABA might be more important in seizures than in hyperactivity. Another possible explanation for the non-significance of GABA was individual differences in the control mice and the limited sample size ; further research is required on the impact of GABA concentration after MPN administration. Considering our results, it can be proposed that not only a decrease in GABA concentration in



GABA: Gamma-aminobutyric acid, Glu: Glutamate, MPN: 4'-*O*-methylpyridoxine,
MPNP: 4'-*O*-methylpyridoxine-5'-phosphate, PL: Pyridoxal, PLP: Pyridoxal-5'-phosphate

Fig. 4 The concentrations of vitamin B_6, amino acid analogue, MPN, and MPNP in all mice brain was measured. The relationship between MPN and amino acid analogue, vitamin B6, and MPNP were shown in (A – G), and (H) shows the relationship between MPNP and GABA/Glu ratio. The relationship between vitamin B6 and amino acid analogues were shown in (I – L).

the brain, but also a lower GABA/Glu ratio, play a role in the behavioral changes caused by GBS poisoning. GABA suppresses brain activity, while Glu works to activate brain activity[19]. Stable global neural function is achieved by a coordinated and dynamically regulated balance between these excitatory (glutamatergic) and inhibitory (GABAergic) inputs[20]. Experiments on rats have shown that decreased GABA in the brain causes hyperactivity[21]. The present study demonstrated an importance of GABA/Glu ratio in behavioral changes involving the balance between excitatory and inhibitory neurotransmitters.

We report for the first time the changes in vitamin B_6 in the brain after MPN administration to animals. Thirty minutes after intravenous MPN administration in an earlier rat experiment, blood PL concentration was significantly increased and PLP concentration was decreased, resulting in an increase in PLP + PL[8]. A marked decrease in PLP/PL ratio was also observed in the blood and cerebrospinal fluid of patients with GBS poisoning[2]. Those extracellular fluid results were due to PL production from MPN in the liver as well as MPN inhibiting PK[8]. However, in the intrathecal administration of MPN in mice, both PLP and PLP + PL decreased in the brain, with no increase in PL. This suggests that the metabolic activity from MPN to PL is low in brain, and that PL may have leaked out of cells owing to its good membrane permeability.

To the best of our knowledge, this is the first investigation to detect MPNP *in vivo*. Although MPNP could decrease GAD activity at such non-physiologically high concentrations as 0.5–3.5 mM[22], the MPNP concentration measured in this experiment was insufficient to inhibit GAD. A significant positive correlation was seen between MPN and MPNP concentrations in our study. In other animal experiments, MPNP was not detected in the plasma when MPN was intravenously injected into rats[8]. These differences are since MPNP is produced in cells but cannot be exported extracellularly owing to its hydrophilicity. Our results indicate that after MPN enters the spinal cavity, part of it is phosphorylated in the brain to become MPNP by PK.

V Conclusion

Intrathecal administration of MPN to mice induced seizures and abnormal behavior. MPN concentration increases were significantly correlated to decreases in PLP concentration and GABA/Glu ratio. In the pathology of GBS poisoning, lower GABA/Glu ratio causes the brain to become more excitatory, resulting in hyperactivity and seizures. We report for the first time that MPNP is produced in the brain in a dose-dependent manner upon MPN administration. Additional intrathecal MPN administration studies are needed to understand the chemical changes occurring in the brain during GBS poisoning.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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