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Evaluation of Isonicotinoyl-γ-Aminobutyric Acid (GABA) and Nicotinoyl-GABA as Pro-drugs of GABA

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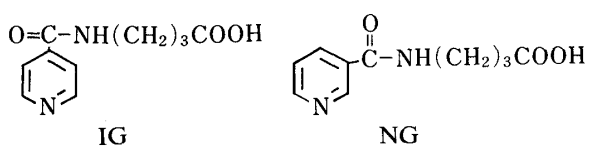
Isonicotinoyl-γ-aminobutyric acid (GABA) (IG) and nicotinoyl-GABA (NG), candidate pro-drugs of GABA, were assessed by measuring various pharmacological responses such as anti-convulsant effect, prolongation of pentobarbital sleeping time and depressive effect on rearing or ambulation in general behavior, in relation to the GABA level in the mouse brain. The GABA level after the intraperitoneal administration of IG at a dose of 1000 mg/kg increased significantly from $2.30 \pm 0.02\,\mu$ mol/g wet wt. in the control to $2.93 \pm 0.05\,\mu$ mol/g wet wt., while NG caused only a slight increase in GABA level. IG showed a stronger anticonvulsant effect, greater prolongation of pentobarbital sleeping time and greater depressive effect on rearing in general behavior than NG did. The pharmacological effect of IG or NG corresponded well to the GABA level in the brain.

Keywords——nicotinoly-GABA; isonicotinoyl-GABA; GABA pro-drug; GABA level; pharmacological response; GC-MS

Since Elliot and Gelder reported that γ-aminobutyric acid (GABA) might be an inhibitory neurotransmitter in the brain,[1] there has been a rapidly increasing interest in the pharmacological effects of GABA. In recent years, the biochemical link between the function of the GABA system and the symptoms of Huntington's disease,[2–4] parkinsonism,[5] and epilepsy[6] has been investigated, and agents which act on the GABA system have been developed by many researchers.[7–10] However, there are many obstacles to the design of potential therapeutic agents acting through the GABA system. The most important problem is the existence of the blood-brain barrier (BBB).

In the present study, the synthesis and purification of GABA derivatives were carried out with the aim of developing GABA pro-drugs with a hydrophobic character stronger than that of GABA, together with greater ease of administration and good susceptibility to conversion back to GABA by amidase in the brain. The GABA level in the brain after intraperitoneal administration of isonicotinoyl-GABA (IG) or nicotinoyl-GABA (NG) was investigated using mice. The effects of these compounds on picrotoxin-induced seizure, pentobarbital-induced hypnosis, and general behavior were also examined in relation to GABA levels after the administration of these compounds. The structures of the compounds examined are shown in Fig. 1.



IG NG

Fig. 1. Chemical Structures of IG and NG

Experimental

Materials and Reagents——Reagent-grade isoniazid (INH) and GABA were purchased from Wako Pure Chemical Ind. Co., Ltd. Reagent-grade nicotinic acid hydrazide was obtained from Aldrich Chemical Company, Inc., and sodium pentobarbital from Dainippon Pharmaceutical Co., Ltd. 4-Aminobutyric-2,2-d_2 acid (d_2-GABA; 98 atom%) was purchased from Merck Sharp & Dohme Canada Ltd., and picrotoxin from Nakarai Chemical Ind. Co., Ltd. Pentafluoropropionic anhydride (PFPA) and 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) were obtained from Gasukuro Kogyo Co., Ltd. All other common reagents used were special reagent grade products purchased from Wako Pure Chemical Ind. Co., Ltd.

Preparation of IG and NG——IG: An aqueous solution of $NaNO_2$ (45 g in 100 ml of water) was added dropwise with stirring to an ice-cooled solution of INH (20 g) in 13% HCl (130 ml). When the reaction was complete, the reaction mixture was basified to pH 7.5 with dil. NaOH solution, then extracted with ether. The resultant viscous syrup was dissolved in 300 ml of 0.01 N NaOH solution, then GABA (16 g) was added. After standing for 48 h at room temperature, the mixture was concentrated to dryness at 80 °C *in vacuo*. The residue was chromatographed on silica gel with a mixture of $CHCl_3$–MeOH–AcOEt–H_2O (7 : 3 : 1.5 : 0.5) and recrystallized from ether–MeOH to give 3.5 g (11.5%) of colorless needles. mp 151—152 °C. *Anal.* Calcd for $C_{10}H_{12}N_2O_3$: C, 57.68; H, 5.81; N, 13.46. Found: C, 57.44; H, 5.83; N, 13.60. Mass spectra (MS) *m/z*: 208 (M^+). ^{1}H-NMR (DMSO-d_6) δ: 1.76 (2H, quint, J=6.8 Hz, $-CH_2\underline{CH_2}CH_2-$), 2.27 (2H, t, J=6.8 Hz, $-\underline{CH_2}COOH$), 3.28 (2H, br q, J=5.6, 6.8 Hz, $-NH\underline{CH_2}-$; t on D_2O exchange, J=6.8 Hz), 7.72 (2H, d, J=6.4 Hz, C_3– and C_5–H of pyridine ring), 8.69 (2H, d, J=6.4 Hz, C_2– and C_6–H of pyridine ring), *ca.* 8.78 (*ca.* 1H, m, NH; disappeared on D_2O exchange).

NG: NG was prepared in a similar manner by using 20 g of nicotinic acid hydrazide as the starting material. When the reaction mixture with GABA was allowed to stand, crystals of NG were precipitated, which were filtered off, washed with cold water and dried. Yield, 10 g (33%), colorless needles, mp 210—212 °C. *Anal.* Calcd for $C_{10}H_{12}N_2O_3$: C, 57.68; H, 5.81; N, 13.46. Found: C, 57.54; H, 5.79; N, 13.52. MS *m/z*: 208 (M^+), ^{1}H-NMR (DMSO-d_6) δ: 1.80 (2H, quint, J=6.8 Hz, $-CH_2\underline{CH_2}CH_2-$), 2.30 (2H, t, J=6.8 Hz, $-\underline{CH_2}COOH$), 3.29 (2H, br q, J=5.6, 6.8 Hz, $-NH\underline{CH_2}-$; t on D_2O exchange, J=6.8 Hz), 7.49 (1H, dd, J=4.8, 8.0 Hz, C_5–H of pyridine ring), 8.17 (1H, ddd, J=1.6, 2.4, 8.0 Hz, C_4–H of pyridine ring), 8.69 (1H, dd, J=1.6, 4.8 Hz, C_6–H of pyridine ring), *ca.* 8.68 (1H, m, NH; disappeared on D_2O exchange), 8.99 (1H, d, J=2.4 Hz, C_2–H of pyridine ring).

Determination of GABA Levels in Mouse Whole Brain after IG or NG Administration——Male ddY mice weighing 20 to 25 g from Shizuoka Experimental Animals Cooperative were used. The mice were kept on a commercial diet (Oriental Yeast Co., Ltd.) but fasted for 12 h prior to experimentation. Water was given *ad libitum*. Solutions of IG or NG were prepared using 5% (w/v) Na_2CO_3. One g/kg of IG or NG was administered intraperitoneally in a volume of 0.1 ml/10 g body weight. Mice were sacrificed by decapitation, and the brains were rapidly removed, and immediately frozen in acetone with dry ice within 30 s after sacrifice. The frozen brains were weighed and homogenized in three volumes of chilled 80% aqueous ethanol solution containing d_2-GABA (200 μg) as an internal standard for the determination. The homogenate was centrifuged at 9000 ***g*** for 30 min at 4 °C and the supernatant was further filtered to remove protein using a membrane filter (Centriflo CF-25, Amicon). The filtrate was concentrated in an evaporator at 70 °C. The residue was further dried in a nitrogen gas stream. For the derivatization of GABA, the amino group was acylated with PFPA and the carboxyl group was esterified with HFIP. Mass fragmentography was employed in the analysis of GABA derivatives by using a Shimadzu GC-MS 7000 instrument. The peak height ratios were calculated by dividing the peak height of the PFPA-HFIP derivative of GABA at *m/z* 232 (base ion peak) by that of d_2-GABA at *m/z* 234 (base ion peak). GABA levels in the brain were calculated by comparison of the peak height ratios with calibration curves obtained on the same day. Column conditions and mass spectrometer conditions were the same as those of the previous paper.[11]

Effects of IG or NG on Picrotoxin-Induced Seizure——Male ddY mice weighing 20 to 25 g were used in the experiment. One hour after IG or NG administration (1000 mg/kg), picrotoxin was administered to mice (5 mg/kg, *s.c.*) and behavioral changes were observed for 60 min. Estimation of the intensity of convulsions was carried out by the method of Satoh *et al.*[12] Seizure severity was scored as follows: no seizure, 0; tremor, 1; tremor and mild clonic seizure, 2; tremor, mild and severe clonic seizure, 3; tremor, mild clonic, severe clonic and tonic extensor, 4; death, 5. Seizure intensity data were statistically analyzed by means of the Mann–Whitney U test.[13]

Effect of IG or NG on Pentobarbital-Induced Hypnosis——Male ddY mice weighing 20 to 25 g were divided into groups each consisting 10 animals. One h after the intraperitoneal administration of 1000 mg/kg of NG or IG, 45 mg/kg of pentobarbital was intraperitoneally administered to mice. The hypnotic effect was evaluated by measuring the sleeping time, *i.e.* the time elapsed from loss to recovery of the righting reflex.

Open-Field Test——The experiment was performed on male ddY mice weighing 17 to 23 g. For observation of general behavior, the modified open-field apparatus of Hall,[14] was used. This apparatus was made of metal and consisted of a round floor with a diameter of 60 cm surrounded by wall 47 cm in height and with a diameter of 80 cm at the upper edge, giving the overall appearance of a truncated cone. The inside was completely painted a gray-white color. The floor was divided into 19 blocks of approximately equal width by means of black lines. At 80 cm above the center of the floor, a white electric lamp (100 W) was provided as an illumination.

The mouse was placed at the center of the floor of the apparatus and the frequencies of crossing the dividing lines on the floor and of rearing by the mouse during 1 min were determined; the former was designated as ambulation. An open-field test was performed immediately prior to drug administration and the animals were divided into 7 groups of 10 mice in such a way as to minimize the difference in mean frequencies of ambulation among the groups. Then the test was performed at 0.5, 1, 2 and 4 h after intraperitoneal administration of IG or NG.

In order to test the significance of differences between the groups, the Mann–Whitney U-test and Fisher's exact probability test[14] were used.

Results

In order to assess the effectiveness of IG or NG as a pro-drug of GABA, GABA levels in the mouse whole brain were determined by mass fragmentography after the intraperitoneal administration of 1000 mg/kg of IG or NG. Figure 2 shows the time course of GABA levels in the mouse brain after IG or NG administration. The shadowed area represents the mean ± S.E. for the control level of GABA. IG caused a significant increase in GABA levels from 0.5 to 4 h. GABA levels increased significantly from 2.30 ± 0.02 μmol/g wet wt. in the control to 2.93 ± 0.05 μmol/g wet wt. at 2 h after IG administration ($p < 0.001$ by the two-tailed Student's t-test). On the other hand, NG resulted in only a brief elevation of GABA

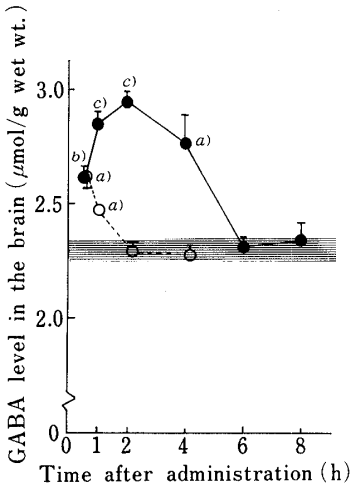


Fig. 2. Time Course of GABA Levels in Mouse Whole Brain after Intraperitoneal Administration of 1000 mg/kg of IG (—●—) and NG (---○---)

The shadowed area represents the mean ± S.E. for the control level of GABA ($n = 5$). Vertical bars indicate standard errors of the mean of 5 to 8 animals. Statistical significance in the two-tailed Student's t-test: *a*) $p < 0.05$; *b*) $p < 0.01$; *c*) $p < 0.001$.

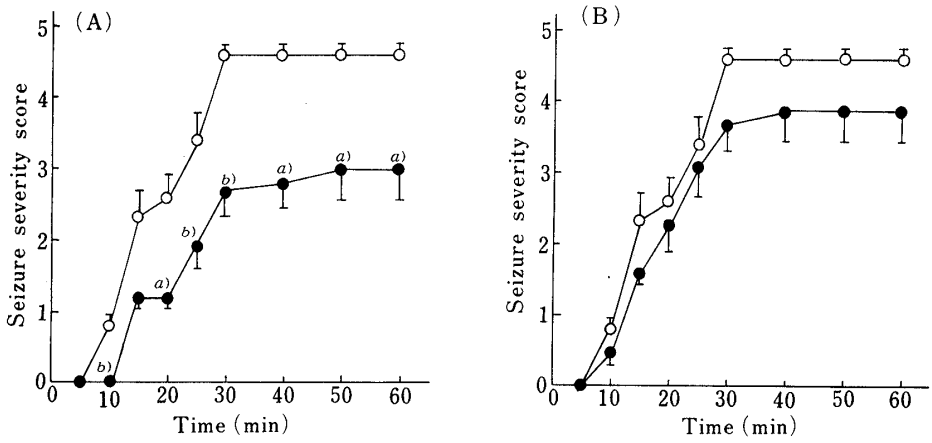


Fig. 3. Effects of IG (A) and NG (B) on Picrotoxin-Induced Seizure in Mice

IG or NG (1000 mg/kg) was intraperitoneally administered 60 min before the injection of picrotoxin (5 mg/kg), *s.c.*). Open and closed circles represent control and IG- or NG-treated groups, respectively. Values are the means of 10 mice and the vertical bars indicate standard errors. Statistical significance in the Mann–Whitney U test: *a*) $p < 0.05$; *b*) $p < 0.01$.
○—○, control; ●—●, IG in (A), NG in (B).

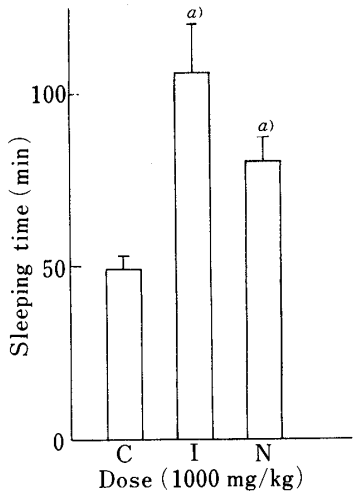


Fig. 4. Effects of IG and NG on Pentobarbital-Induced Hypnosis in Mice

IG or NG (1000 mg/kg) was intraperitoneally administered 60 min before the injection of pentobarbital (45 mg/kg, *i.p.*).

C, control; N, NG; I, IG. Values are the means of 10 mice and the vertical bars indicate standard errors. Statistical significance in the two-tailed Student's *t*-test: *a*) $p<0.001$.

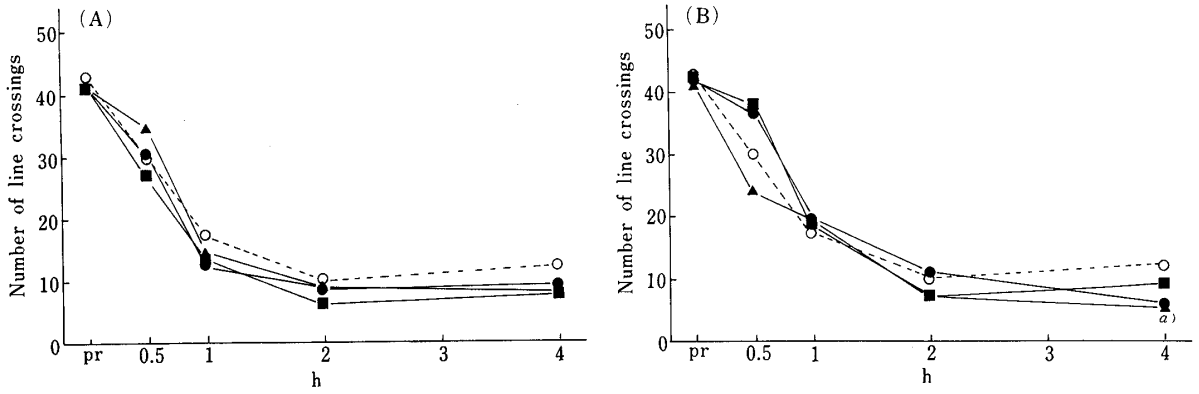


Fig. 5. Effects of IG (A) and NG (B) on Ambulation of Mice in the Open-Field Test

The number of blocks traversed on the floor during a 1 min test period was counted; each value represents the mean of 10 mice. Statistical significance in the Mann–Whitney U-test: *a*) $p<0.05$.

○---○, vehicle; (A), ●—●, IG 100 mg/kg *i.p.*; ■—■, IG 300 mg/kg *i.p.*; ▲—▲, IG 1000 mg/kg *i.p.*; (B), ●—●, NG 100 mg/kg *i.p.*; ■—■, NG 300 mg/kg, *i.p.*; ▲—▲, NG 1000 mg/kg *i.p.*

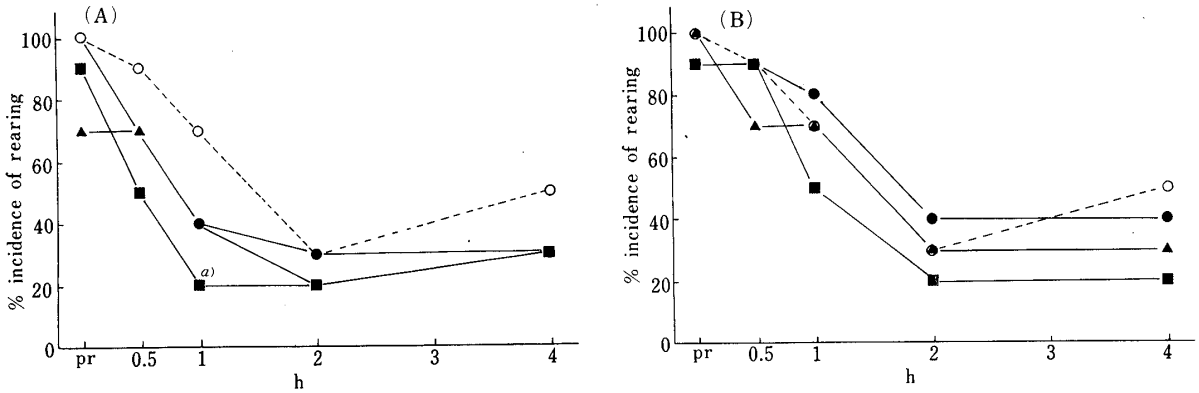


Fig. 6. Effects of IG (A) and NG (B) on Rearing of Mice in the Open-Field Test

The % incidence of animals standing up (rearing) during a 1 min test period was counted; each value represents the mean of 10 mice. Statistical significance in Fisher's exact probability test: *a*) $p<0.05$.

○---○, vehicle; (A), ●—●, IG 100 mg/kg *i.p.*; ■—■, IG 300 mg/kg *i.p.*; ▲—▲, IG 1000 mg/kg *i.p.*; (B), ●—●, NG 100 mg/kg *i.p.*; ■—■, NG 300 mg/kg *i.p.*; ▲—▲, NG 1000 mg/kg *i.p.*

level in mouse whole brain. In the case of NG administration, GABA increased from 2.30 ± 0.02 μmol/g wet wt. in the control to 2.64 ± 0.04 μmol/g wet wt. at 0.5 h. The increased GABA level began to decline by 1 h and reached the control level at 2 h.

Next, the anti-convulsant actions of IG and NG were examined. Figure 3A shows the effects of IG on the seizure after injection of 5 mg/kg of picrotoxin. IG significantly reduced the score until 60 min after picrotoxin. On the other hand, NG showed only a slight reduction in the score (Fig. 3B). There was no significant difference between the NG-treated group and the control. There were significant differences between the scores of IG and NG at 15 and 30 min.

Figure 4 shows the effects of IG or NG on the pentobarbital-induced hypnosis. Both IG and NG resulted in a significant increase in the sleeping time. However, IG was more potent than NG. In the case of IG, the sleeping time increased from 49.21 ± 2.44 min in the control to 106.18 ± 13.17 min. On the other hand, NG increased the sleeping time from 49.21 ± 2.44 min to 79.90 ± 7.19 min.

Next, the effects of IG or NG on general behavior were examined by means of the open-field test. The effects of IG or NG on ambulation in the open-field test are shown in Fig. 5. IG at all doses caused slight decrease of ambulation from 1 to 4 h after intraperitoneal administration (Fig. 5A). On the other hand, NG at doses of 100 and 300 mg/kg showed slight increases of ambulation at 30 min after administration, whereas at a dose of 1000 mg/kg, NG showed a slight decrease of ambulation and there was a significant decrease ($p < 0.05$) at 4 h after administration (Fig. 5B).

Figure 6 shows the effects of IG or NG on rearing in the openfield test. IG at a dose of 300 mg/kg showed a significant inhibition ($p < 0.05$) of rearing 1 h after administration (Fig. 6A). In the case of NG, no significant difference was observed at doses of 100 to 1000 mg/kg (Fig. 6B).

Discussion

It is generally believed that in fully developed animals the blood-brain barrier prevents the passage of GABA from circulating blood to the brain parenchyma.[15-17] Levi *et al.*[18] confirmed low penetration of GABA from the blood to the brain in practice. They concluded that the injection more than 3 g/kg of GABA (29.1 mmol/kg) was necessary in order to produce a significant increase in brain GABA level. However, two types of GABA carriers (the isonicotinoyl group and the nicotinoyl group) enable GABA to pass through blood-brain barrier at a dose of 1000 mg/kg (4.8 mmol/kg). From the present data, it should be stressed that the slight difference in structure resulted in a considerable difference in GABA levels in the mouse brain. The present study suggests that the isonicotinoyl group is superior to the nicotinoyl group as a carrier of GABA.

Callery *et al.* have synthesized a series of candidate pro-drugs of GABA which are susceptible to conversion back to GABA in the brain.[19-21] They performed quantitative determination of GABA by using gas chromatography-mass spectrometry after intravenous administration of 250 mg/kg of 2-pyrrolidinone. The increased GABA was found to be only 17 nmol/g wet wt. at 30 min.[19] As regards the apparent low degree of conversion of pyrrolidinone to GABA, they suggested that a slow rate of hydrolysis limited GABA formation in the brain.

Since the turnover rate of GABA is rapid in mammalians,[22] IG appears to be far more effective than pyrrolidinone as a pro-drug of GABA.

γ-Acetylenic GABA and γ-vinyl GABA, two catalytic irreversible inhibitors of GABA transaminase, increase brain GABA levels by approximately 500% at 4 h after treatment.[23] However, no significant alteration in seizure frequency or mortality induced by picrotoxin was

observed after pretreatment with 200 mg/kg of γ-acetylenic GABA (*i.p.*) or 1500 mg/kg of γ-vinyl GABA (*i.p.*).[24] In contrast, 1000 mg/kg of IG showed a significant anti-convulsant action with low mortality. The present observations indicate that the pharmacological responses after IG or NG administration correspond to the GABA levels in the brain. The prolonged effect of IG or NG on pentobarbital-induced hypnosis and the depressive effect on general behavior after IG or NG administration may also be explained on the basis of increased GABA levels in the brain.

Olsen proposed that the GABA receptor was associated with receptors for picrotoxin, barbiturates and benzodiazepine as well as chloride ionophore.[25] According to this hypothesis, it is to be expected that increased GABA afffects the seizure induced by picrotoxin and the pentobarbital-induced hypnosis, as well as showing a depressive effect on general behavior. The present observations support the GABA receptor hypothesis proposed by Olsen.

Perry *et al.*[26] tried to use INH in the therapy of Huntington's disease. They observed a significant improvement in half of the patients who were given INH in dosages three to five times greater than normally used in tuberculosis treatment. However, a later study demonstrated improvement in only one of nine patients with Huntington's disease.[27] The therapeutic effect of INH cannot certainly be attributed to its GABAergic action. Furthermore, γ-acetylenic GABA,[7] γ-vinyl GABA[9] and sodium valproate,[28] which are potent GABA inducers, showed no effect in the treatment of Huntington's disease. To account for this, Sober *et al.*[29] suggested that the GABA transaminase inhibitors described above produce an inhibition of glutamic acid decarboxylase (GAD), the enzyme synthesizing GABA. It is possible that if GABA levels are increased directly by using a pro-drug of GABA, the symptoms of Huntington's disease might be mitigated. We are now planning to test the utility of IG in patients with Huntington's disease.

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