EFFECT OF ANTIVITAMIN B₆ ON REGIONAL GABA METABOLISM IN MOUSE BRAIN AND ITS RELATION TO CONVULSIONS

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Summary The effects of administration of DL-penicillamine (PeA), thiosemicarbazide (TSC), semicarbazide-HCl (SC) as convulsants and pyridoxine (PN) as anticonvulsant on γ-aminobutyric acid (GABA) content, glutamic acid decarboxylase (GAD) and γ-aminobutyric acid transaminase (GABA-T) activities in cerebral cortex, striatum, diencephalon, mesencephalon, cerebellum and pons/medulla were investigated. The onset of convulsions induced by these convulsants coincides with the fall in GABA content and GAD activity in the mesencephalon area, and in contrast, the cessation of the convulsions by PN supplement coincides with the recovery in both the parameters. Aminooxyacetic acid (AOAA), a potent GABA-elevating agent showed an anticonvulsant property against convulsion by TSC for several hours after the injection of AOAA, but lost this property 16 hr after the treatment. The TSC administration 16 hr after the AOAA pretreatment significantly decreased the GABA content in all the regions, particularly in the mesencephalon and diencephalon areas, which had been elevated by the AOAA pretreatment, together with its ability to induce convulsion. From the above results it may be postulated that the critical drop of GABA level from a plateau to another lower level following the decrease of GAD activity in the mesencephalon area is an important factor in the induction of convulsion.

Keywords antivitamin B₆, convulsion, γ-aminobutyric acid, glutamic acid decarboxylase, γ-aminobutyric acid transaminase, mesencephalon, six regions

Antivitamin B₆ is known to induce tonic or clonic convulsions in mice and vitamin B₆ supplement prevents these seizures in many cases. In the metabolism of
γ-aminobutyric acid (GABA), which has been established to be an inhibitory neurotransmitter in mammals, pyridoxal phosphate works as a coenzyme both in glutamic acid decarboxylase (GAD) [L-glutamate 1-carboxylase; EC 4.1.1.15], a GABA-synthesizing enzyme, and GABA transaminase (GABA-T) [4-aminobutyrate-2-oxoglutarate amino-transferase; EC 2.6.1.19], a GABA-degrading enzyme. From these facts it is naturally expected that the decrease of GABA content in brain induced by antivitamin B₆ would result in the onset of convulsions. However, the correlation between the level of GABA in the brain and convulsions holds true in some instances, but there are many arguments not supporting the correlation. Moreover, data obtained by various investigators has indicated that no correlation exists between any single parameter such as GAD, GABA-T activity or GABA levels and the onset of seizures so far as the whole brain is concerned (1-8). A feature of GABA metabolism which complicates the interpretation of the above results may be its subcellular or regional compartmentation in brain. Recent study in this laboratory has indicated that the decrease of GABA content following the inhibition of GAD in the synaptosomes from mice treated with antivitamin B₆ might be involved in the etiology of the seizures (9,10). It is also known that the GABA content, GAD and GABA-T activities differ among brain regions (11-13). This led us to determine the changes of these parameters of GABA metabolism in various regions of the brain at the onset of seizures.

**MATERIALS AND METHODS**

Male DDY strain mice, 20 g in weight, were used in the experiment. The convulsants (antivitamin B₆) used were DL-penicillamine (PeA, 3.36 mmol/kg), thiosemicarbazide (TSC, 0.22 mmol/kg), semicarbazide-HCl (SC, 2.48 mmol/kg). These drugs were intraperitoneally injected. Pyridoxine (PN, 1.46 mmol/kg) was injected intramuscularly simultaneously with PeA or TSC and 30 min after injection of SC. Aminooxyacetic acid (AOAA, 0.23 mmol/kg) was intraperitoneally given 1 hr or 16 hr prior to injection of TSC. All drugs were dissolved in 0.9% NaCl, the pH being adjusted to 7.0 before use. The mice treated only with convulsant were decapitated at the onset of convulsions (about 139, 58 and 59 min with PeA, TSC and SC, respectively) and the mice treated with both convulsant and PN were killed after the same intervals as those treated only with convulsant. The chilled brains were dissected into six regions: cerebral cortex, striatum, diencephalon, mesencephalon, cerebellum and pons/medulla. The tissue fragments were weighed and immediately homogenized in 9 volumes of 0.25% Triton X-100 and 0.02 M mercaptoethanol. GABA content was measured enzymatically (GABase) as described previously (14,15) after an acid (or ethanol) extract was applied to high voltage paper electrophoresis or Dowex 1 × 8 (CH₃COO⁻ type, 50–100 mesh) column chromatography in order to remove convulsant which might remain in the tissue fragments and inhibit GABase used for the assay. GAD
activity was measured by determining $^{14}$C-GABA synthesis from $^{14}$C-glutamate (16). After the reaction was stopped, the reaction mixture was applied to Dowex 1×8 (CH$_3$COO$^-$ type, 50–100 mesh) and the produced $^{14}$C-GABA was eluted with 5 mM GABA solution. The eluate was collected directly in scintillation vials and the radioactivity was counted in a scintillation counter. GABA-T activity was measured by determining $^{14}$C-glutamate production from $^{14}$C-$\alpha$-ketoglutarate (17, 18). After stopping the reaction by HCl addition, the reaction mixture was applied to Dowex 50×8 (H$^+$ type, 200–400 mesh) and the produced $^{14}$C-glutamate was eluted with 2N NH$_4$OH after washing $^{14}$C-$\alpha$-ketoglutarate with water. The radioactivity of the eluate was counted as above.

RESULTS

Table 1 represents the GABA content, GAD and GABA-T activities in the various regions of mouse brain. The GABA content varies from 3.67 μmol/g in the mesencephalon to 1.54 μmol/g in the cerebellum. The GAD activity also was greatest in the mesencephalon and lowest in the cerebellum. On the other hand, the GABA-T activities were invariably greater than those of GAD, though it was high in the mesencephalon and the pons/medulla and low in the cerebral cortex.

The effect of convulsants and PN (anticonvulsant) on the GABA content, GAD and GABA-T activities in the above six regions was studied in mouse (Table 2). The administration of convulsant dose of PeA, TSC and SC commonly produced a decrease of GABA in every region, although the degree of decrease in GABA content varied with the different drugs used. When the convulsions by these convulsants were prevented by PN supplement, the decreases of GABA in the mesencephalon area were commonly lessened in comparison with those in other regions. In the case of TSC, the PN supplement rather intensified the decreases of GABA in regions other than the mesencephalon and cerebellum. The administration of convulsants significantly inhibited the GAD activities in all the regions, particularly in the mesencephalon and the diencephalon areas, but the effects were quantitatively different, being greatest with SC and least with TSC. The inhibitions were reversed by PN supplement, especially in the mesencephalon and diencephalon, though the degree of the recoveries varied with the different drugs used. The effect of the convulsants on the GABA-T was minimal; a slight decrease in activity being obtained only with SC. The PN + convulsant-treated mice, however, exhibited higher GABA-T activities than did the mice treated with the convulsant alone. In the case of TSC, the PN supplement elevated the activities to higher levels than that of control mice.

Since AOAA has been well known to augment GABA levels by inhibition of GABA-T activity and to be a powerful anticonvulsant, animals treated with AOAA were also included in this study for comparative purposes. The anticonvulsant actions of AOAA, however, were found to be not always coincident with the elevated GABA levels induced by AOAA in whole brain (5). For example,
Table 1. Regional distribution of GABA content, GAD and GABA-T activities in mouse brain.

| Parameter in GABA metabolism | Cerebral cortex | Striatum | Diencephalon | Mesencephalon | Cerebellum | Pons/Medulla |
|-----------------------------|----------------|----------|--------------|---------------|------------|-------------|
| GABA content (μmol/g)       | 1.92 ± 0.17    | 1.95 ± 0.11 | 3.49 ± 0.17  | 3.67 ± 0.19   | 1.54 ± 0.19 | 1.57 ± 0.10 |
| GAD activity (μmol/g/hr)    | 18.0 ± 0.8     | 16.6 ± 0.3 | 30.0 ± 0.3   | 33.6 ± 0.2    | 15.3 ± 0.9  | 19.1 ± 0.3  |
| GABA-T activity (μmol/g/hr) | 25.1 ± 0.7     | 27.3 ± 2.8 | 27.8 ± 1.2   | 32.9 ± 1.8    | 27.9 ± 1.5  | 29.8 ± 1.6  |

Values represent mean ± S.E.M. of 4 mice.
Table 2. Effect of some convulsant agents and PN on GABA metabolism in mouse brain.

| Parameter in GABA metabolism | Treatment | Convulsion | Cerebral cortex | Striatum | Diencephalon | Mesencephalon | Cerebellum | Pons/Medulla |
|-----------------------------|-----------|------------|-----------------|----------|--------------|---------------|-------------|--------------|
| GABA content                | Control   | −          | 100 ± 4         | 100 ± 4  | 100 ± 3      | 100 ± 4       | 100 ± 7     | 100 ± 5      |
|                             | PeA       | +          | 79 ± 3           | 56 ± 16  | 72 ± 5       | 78 ± 4        | 66 ± 2      | 74 ± 6       |
|                             | PeA + PN  | −          | 85 ± 6           | 63 ± 10  | 77 ± 4       | 88 ± 1       | 76 ± 3      | 79 ± 3       |
|                             | TSC       | +          | 108 ± 8          | 77 ± 5   | 68 ± 2       | 70 ± 3       | 79 ± 5     | 73 ± 5       |
|                             | TSC + PN  | −          | 66 ± 2           | 65 ± 3   | 60 ± 3      | 76 ± 4      | 80 ± 5     | 64 ± 2       |
|                             | SC        | +          | 67 ± 8           | 56 ± 6   | 55 ± 4      | 49 ± 3      | 59 ± 9     | 44 ± 5       |
|                             | SC + PN   | −          | 54 ± 9           | 61 ± 6   | 66 ± 3      | 68 ± 3      | 69 ± 6     | 58 ± 7       |

| GAD activity                | Control   | −          | 100 ± 2         | 100 ± 2  | 100 ± 2      | 100 ± 2      | 100 ± 2     | 100 ± 2      |
|                             | PeA       | +          | 68 ± 6          | 71 ± 8   | 58 ± 7      | 60 ± 3      | 72 ± 3     | 62 ± 4       |
|                             | PeA + PN  | −          | 114 ± 24.7      | 114 ± 8.8 | 113 ± 5.4    | 111 ± 21    | 107 ± 3    | 107 ± 3.7    |
|                             | TSC       | +          | 85 ± 22         | 73 ± 4   | 69 ± 3      | 71 ± 2      | 90 ± 3     | 83 ± 5       |
|                             | TSC + PN  | −          | 92 ± 35.4       | 91 ± 36   | 85 ± 33.9   | 85 ± 21.7   | 93 ± 3     | 93 ± 2       |
|                             | SC        | +          | 44 ± 21         | 48 ± 2   | 39 ± 3      | 39 ± 3      | 53 ± 4     | 45 ± 5       |
|                             | SC + PN   | −          | 57 ± 61.1       | 60 ± 57.1 | 63 ± 71.9   | 58 ± 73.10  | 68 ± 72.12 | 65 ± 6.410  |

| GABA-T activity             | Control   | −          | 100 ± 1         | 100 ± 1  | 100 ± 1      | 100 ± 2      | 100 ± 2     | 100 ± 2      |
|                             | PeA       | +          | 103 ± 13        | 97 ± 4   | 103 ± 3   | 84 ± 3       | 87 ± 4     | 92 ± 6       |
|                             | PeA + PN  | −          | 100 ± 3         | 108 ± 8  | 107 ± 3    | 93 ± 3       | 101 ± 5    | 106 ± 6      |
|                             | TSC       | +          | 114 ± 23        | 98 ± 7   | 104 ± 5   | 103 ± 5      | 110 ± 2    | 95 ± 4       |
|                             | TSC + PN  | −          | 144 ± 31.7      | 120 ± 42.9 | 123 ± 31.10  | 129 ± 41.9  | 132 ± 2.7  | 109 ± 5.11   |
|                             | SC        | +          | 88 ± 35         | 88 ± 3   | 92 ± 8     | 90 ± 1.6    | 98 ± 4     | 86 ± 3       |
|                             | SC + PN   | −          | 93 ± 5          | 99 ± 4   | 101 ± 4    | 100 ± 4     | 101 ± 6   | 94 ± 4.5     |

Values represent percent to control of 4–20 mice (mean ± S.E.M.).

1, $p < 0.005$; 2, $p < 0.01$; 3, $p < 0.025$; 4, $p < 0.05$; 5, $p < 0.10$; 6, $p < 0.25$ compared to control group and 7, $p < 0.005$; 8, $p < 0.01$; 9, $p < 0.025$; 10, $p < 0.05$; 11, $p < 0.10$; 12, $p < 0.25$ show the statistical difference between the group treated with convulsant and convulsant + PN.
Table 3. Effect of AOAA and TSC on GABA metabolism in mouse brain.

| Parameter in GABA metabolism | Time after AOAA injection | Treatment | Convulsion | Cerebral cortex | Striatum | Diencephalon | Mesencephalon | Cerebellum | Pons/Medulla |
|-----------------------------|---------------------------|-----------|------------|----------------|----------|--------------|---------------|-------------|--------------|
| GABA content                | 2 hr                      | Control   | –          | 100 ± 4        | 100 ± 4  | 100 ± 3      | 100 ± 4       | 100 ± 7     | 100 ± 5      |
|                             |                           | TSC       | +          | 108 ± 8        | 77 ± 36  | 68 ± 2³      | 70 ± 3¹       | 79 ± 3⁶     | 73 ± 5³      |
|                             |                           | AOAA      | –          | 505 ± 33¹     | 414 ± 31¹| 352 ± 20¹    | 381 ± 13¹     | 694 ± 88¹   | 492 ± 34⁴     |
|                             |                           | AOAA + TSC* | –       | 442 ± 27²,¹²   | 405 ± 39¹| 371 ± 24¹    | 348 ± 22¹     | 632 ± 35¹   | 382 ± 26,¹¹⁴ |
|                             |                           | AOAA      | –          | 282 ± 17¹     | 219 ± 19¹| 251 ± 9¹     | 253 ± 15³     | 250 ± 5¹    | 222 ± 23³     |
|                             |                           | AOAA + TSC** | +       | 239 ± 18³,¹²  | 150 ± 177⁹| 163 ± 4³⁷    | 187 ± 21³,¹²  | 210 ± 2³,¹¹ | 158 ± 6¹¹     |
| GAD activity                | 2 hr                      | Control   | –          | 100 ± 2        | 100 ± 2  | 100 ± 2      | 100 ± 2       | 100 ± 2     | 100 ± 2      |
|                             |                           | TSC       | +          | 85 ± 2²        | 73 ± 4²  | 69 ± 31¹     | 71 ± 2¹       | 90 ± 3⁶     | 83 ± 3⁵       |
|                             |                           | AOAA      | –          | 87 ± 1⁶        | 86 ± 7   | 83 ± 3⁶      | 86 ± 1⁶       | 76 ± 3³     | 92 ± 3        |
|                             |                           | AOAA + TSC | –       | 64 ± 42⁸       | 72 ± 7²  | 57 ± 2²,⁷    | 55 ± 3²,³    | 61 ± 4¹,¹⁰  | 65 ± 3¹,⁷     |
|                             |                           | AOAA      | –          | 69 ± 5²       | 84 ± 5²  | 76 ± 4³      | 70 ± 4²       | 60 ± 5¹    | 75 ± 4³       |
|                             |                           | AOAA + TSC | +       | 55 ± 3²,¹¹    | 62 ± 2⁸  | 56 ± 4¹,¹⁰  | 49 ± 2¹,⁸     | 55 ± 3³    | 60 ± 4¹,¹⁰   |
| GABA-T activity             | 2 hr                      | Control   | –          | 100 ± 1        | 100 ± 2  | 100 ± 1      | 100 ± 2       | 100 ± 2     | 100 ± 2      |
|                             |                           | TSC       | +          | 114 ± 2³       | 98 ± 7   | 104 ± 5      | 103 ± 5       | 110 ± 2⁶    | 95 ± 4        |
|                             |                           | AOAA      | –          | 31 ± 2¹       | 21 ± 1¹  | 29 ± 2¹      | 27 ± 2¹       | 23 ± 2¹     | 26 ± 1¹       |
|                             |                           | AOAA + TSC | –       | 30 ± 1¹       | 25 ± 2¹  | 31 ± 1¹      | 26 ± 1¹       | 26 ± 2¹     | 24 ± 2¹       |
|                             |                           | AOAA      | –          | 47 ± 3¹       | 43 ± 3¹  | 47 ± 1¹      | 38 ± 1¹       | 43 ± 3¹    | 38 ± 1¹       |
|                             |                           | AOAA + TSC | +       | 48 ± 1³       | 43 ± 3¹  | 51 ± 6¹      | 43 ± 4¹,¹²   | 42 ± 5¹    | 38 ± 4¹       |

* Animals were decapitated 1 hr after: TSC supplement (0.22 mmol/kg), corresponding to 2 hr after AOAA injection (0.23 mmol/kg).

** TSC was injected 16 hr after AOAA pretreatment and animals were decapitated at convulsion, corresponding to about 17 hr after AOAA injection.

Values represent percent to control of 4–20 mice (mean ± S.E.M.).

1, p < 0.005; 2, p < 0.01; 3, p < 0.025; 4, p < 0.05; 5, p < 0.10; 6, p < 0.25 compared to control group and 7, p < 0.005; 8, p < 0.01; 9, p < 0.025; 10, p < 0.05, 11, p < 0.10; 12, p < 0.25 show the statistical difference between the group treated with AOAA and AOAA + TSC.
our previous experiment showed that there was a strong anticonvulsant action of AOAA against TSC-induced convulsion during the first 2 hr after AOAA pretreatment, but the action began to decrease at 8 hr, at which time the GABA content was maximal, and completely disappeared at 17 hr, at which time the GABA content was still over twice the normal level (19). In this experiment, therefore, the effect of TSC along with AOAA on the parameters of GABA metabolism was investigated using two groups of mice, one of which was pretreated with AOAA 1 hr prior to TSC treatment and decapitated 1 hr after the TSC treatment (2 hr after the AOAA pretreatment). Another group was pretreated with AOAA 16 hr prior to the TSC treatment and decapitated at the onset of convulsion by the TSC treatment (latent period was about 1 hr), corresponding to 17 hr after the AOAA pretreatment.

Table 3 represents the effects of TSC subsequent to AOAA and of AOAA alone on the parameters of GABA metabolism. The AOAA alone administration elevated GABA levels in all the brain regions 2 hr after the AOAA treatment. GABA-T activities were greatly inhibited in all the regions at the same time, but the inhibited GABA-T activities had recovered significantly in every region 17 hr after the AOAA treatment. While GAD activities were slightly inhibited in all the regions 2 hr after AOAA administration, the inhibition was emphasized in all the regions 17 hr after AOAA administration. On the other hand, TSC administration 16 hr after AOAA pretreatment significantly decreased the GABA content in all regions, particularly in the diencephalon and mesencephalon areas, where it had been elevated by AOAA pretreatment.

DISCUSSION

Both GABA content and GAD activity were richer in the diencephalon and mesencephalon (Table 1). There appears to be a relationship between GAD activities and endogenous GABA concentration; the GABA content of tissues may be a function of GAD activity but is not related to GABA-T activities. These results are consistent with other published data (13, 20, 21).

From the effect of convulsants with PN on GABA content, GAD and GABA-T activities in six regions (Table 2), it may be postulated that the critical drop of GABA level in the mesencephalon area from normal to a lower level, mainly through the inhibition of GAD activity, is an important factor in the induction of convulsions. The onset of convulsions induced by PeA, TSC and SC coincides with the fall in GABA content and GAD activity in the mesencephalon area; in contrast, the cessation of convulsions by PN supplement coincides with a recovery in both parameters. The participation of the mesencephalon area in the onset of convulsions appears to be supported by previous findings in our laboratory that mid-brain (mesencephalon) animals were convulsed with SC treatment, but the medulla animals were not, with the same treatment (22). This postulation agrees well with the opinion of HASSLER et al. that the fast decrease of GABA content in
GABA-rich areas was meaningful in the onset of convulsions (23).

It cannot, however, be ignored that GABA level and GAD activity in the mesencephalon area at the onset of convulsions or at their cessation varied with the kind of convulsants or anticonvulsants used. It might be possible that the decrease of GABA in a critical subcellular location (such as nerve endings) or in a critical basal ganglion (such as substantia nigra) in the mesencephalon area is counteracted by the changes of GABA content in another irrelevant location or ganglion and, therefore, the GABA content in the whole mesencephalon does not reflect the events in the critical location or ganglion of the area. In fact, a recent study in our laboratory indicated that the decrease of GABA content and GAD activity in the synaptosomes possibly is involved in the etiology of seizures induced by antivitamin B6 (9,10). On the other hand, the decrease of GABA content has been found in the substantia nigra of Huntington chorea with involuntary movement (24), showing that the decrease of GABA in the substantia nigra might be associated with excessive involuntary movement. Further experiments are under way to attempt to investigate changes in GABA metabolism in synaptosomal fractions from the mesencephalon or specific ganglion in the same area of animals treated with convulsant drugs.

Table 2 shows that PeA, TSC and SC induced a decrease of GAD and GABA-T activities but the degree of the inhibition in GAD was always more severe than that in GABA-T. The reason for this is not clear, but it may be related to the facts that the binding force between pyridoxal phosphate and apoprotein of GAD is weaker than that of GABA-T and the above drugs can more easily attack the coenzyme of GAD, and that the drugs can hardly penetrate into mitochondria in which GABA-T is localized.

The overall correlation between the changes in GABA levels and the GABA-synthesizing and -catabolizing enzymes is shown in Table 2. Although the correlation suggests that alterations in GAD and GABA-T activities are primarily responsible for the observed changes in GABA levels in each region, the influence of other factors cannot be ignored. For example, the in vivo concentration of ω-ketoglutarate as the substrate of GABA-T may be an important factor in the catabolizing rate of GABA because the brain levels of ω-ketoglutarate are far lower than the $K_m$ value of GABA-T and may be decreased by the above carbonyl trapping agents.

In the case of TSC, the PN supplement rather decreased the GABA content in the four regions other than the mesencephalon and cerebellum to a lower level than that of only TSC-treated mice. This might be due to the activation of GABA-T by PN with TSC, though the mechanism of the elevation remains to be elucidated. PN treatment by itself hardly affected the GABA content, GAD or GABA-T activities (data not shown).

The results shown in Table 3 again appear to support the conclusion obtained above with convulsant and PN, that the decrease of GABA content following the inhibition of GAD in the mesencephalon may be associated with the induction of
convulsion (Table 2), except for the fact that the GABA content observed at 17 hr with AOAA + TSC was still more than that of the control mice. The increased GABA content by AOAA would come from the more severe inhibition in GABA-T than in GAD by this drug. In this respect, AOAA differs from PeA, TSC and SC, although AOAA also is considered to be a carbonyl trapping agent.

From our results, therefore, it may be postulate that the critical drop of GABA level in the mesencephalon area from a plateau to a lower level is associated with the induction of convulsion.

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