POSTNATAL DEVELOPMENT OF THE GABA SYSTEM IN THE RAT SPINAL CORD

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Abstract—To assess the postnatal development of the GABA system in the rat spinal cord, GABA levels and GAD activity, as well as specific $[^{3}H]$-muscimol binding, were determined in the dorsal and ventral areas. GABA levels and GAD activities did not vary in parallel from 1 to 8 postnatal days since the former decreased and the latter increased. After 8 days, however, increases in GABA levels and GAD activities were observed in tissues from the dorsal area and decreases were observed in tissues from the ventral area. Specific $[^{3}H]$-muscimol binding was unexpectedly high in both areas at 1 and 8 days but decreased linearly up to 22 days. Our findings show that there are distinct differences in the development of the GABA system in the rat spinal cord and brain.

\[ \gamma \text{-Aminobutyric acid (GABA)} \text{ is considered to be an inhibitory neurotransmitter in the mammalian central nervous system. This substance is present in a high concentration in the dorsal area of the rat spinal cord, whereas the content is lower in the ventral area (1). Glutamic acid decarboxylase (GAD), the rate limiting enzyme for GABA formation, is also highly distributed in the dorsal area (1–3). Evidence obtained in electrophysiological studies supports the view that GABA may be involved in presynaptic inhibition in the spinal cord (4, 5). Evans (6) reported that GABA depolarized motoneurons in the isolated spinal cord of newborn rats, whereas electrophoretically administered GABA produced hyperpolarization of spinal motoneurons in mature cats (7). The major objective of the present work was to investigate the development of GABAergic neurons by comparing the postnatal changes in the levels of GABA and GAD activity in the dorsal and ventral areas of the rat spinal cord.}

Recent findings on the GABA receptors suggested that the density of these receptors in the rat spinal cord is rather low (8–11). As there is no documentation of the precise distribution of these receptors in the spinal cord, we also investigated the development of GABA receptor levels in the dorsal and ventral areas of the rat spinal cord by measuring the specific binding of $[^{3}H]$-muscimol, a potent GABA agonist (8, 10, 12).

MATERIALS AND METHODS
Tissue preparation: Experiments were performed on male Wistar rats, but for the study on 1 day-old rats, both sexes were used. The pups were reduced to a maximum of ten per liter within 24 hr after birth. Spinal cords were studied at 1, 8, 15, 22 and 60 days after birth of the rats. These animals were exsanguinated, and spinal cords quickly removed and frozen in liquid nitrogen. These spinal cords were hemisected sagi-
tally and then dissected into dorsal and ventral halves.

GABA and GAD assay: GABA content was determined according to the enzymatic fluorometric method of Graham and Aprison (13). GAD activity was assayed by the rate of formation of $^{14}$CO$_2$ from L-[1-$^{14}$C]-glutamic acid according to a modification of the method of Wilson et al. (14). Briefly, the reaction was carried out for 45 min at 37°C and stopped by adding 0.1 ml of 3 N H$_2$SO$_4$. The $^{14}$CO$_2$ evolved from the reaction mixture was absorbed by Hyamine solution for 30 min at 37°C.

$[3H]$muscimol binding: The spinal cords were pooled at −70°C until there was sufficient material to perform the assay. Crude synaptic membranes were prepared by the method of Zukin et al. (11) and frozen at −70°C at least 18 hr prior to use. The frozen pellet was resuspended in distilled water, incubated at 37°C for 30 min, and centrifuged at 48,000×g for 20 min. This pellet was resuspended in 50 mM Tris-citrate buffer (pH 7.1, 4°C) and recentrifuged at 48,000×g for 20 min, after which this procedure was repeated to remove the endogeneous GABA. The binding assay was conducted as follows: 0.2 ml aliquots of membrane suspension in Tris-citrate buffer were incubated for 30 min at 4°C with 2 nM $[3H]$muscimol in the presence and absence of 1 mM unlabeled GABA. The amount of protein in each assay mixture was 0.6–3.5 mg. After incubation, the samples were rapidly filtered under vacuum through Whatman GF/B filters, which were then rapidly washed twice with 4 ml of ice-cold 50 mM Tris-citrate buffer. Filtration and washing took less than 10 sec. The radioactivity remaining on the filter was measured by liquid scintillation spectrometry (Packard TRI-CARB 3255) in 8 ml of scintillator (2,5-diphenyloxazole, 4.0 g; 1,4-bis-2-(5-phenyloxazol)-benzene, 0.1 g; toluene up to 1 liter and Triton X-100, 500 ml). Specific binding was defined as the total binding minus the binding that could not be displaced by 1 mM of unlabeled GABA. The concentration of proteins was determined by the method of Lowry et al. (15).

Statistical analysis was performed using the Student's t-test (two-tailed).

Materials: Drugs used were γ-amino-butyric acid (Wako Pure Chem. Co.), hydroxide of Hyamine 10-X (Packard Co.), L-[1-$^{14}$C]glutamic acid (48 mCi/mmol, New England Nuclear), and $[methylamine-3H]$muscimol (19 Ci/mmol. The Radiochemical Centre, Amersham).

RESULTS

Postnatal changes of GABA content and GAD activity in rats: The results are shown in Table 1. In the dorsal area of 1 day-old rats, GABA content, expressed as nmol/mg protein, was as high as in the mature rats but decreased slightly until 8 days, and then gradually increased to levels seen in the adult animals. In the ventral area of 1 day-old rats, GABA levels were 2.5 times as high as those in the adult rats and decreased in the course of development. No significant difference in GABA levels was noted in the dorsal and ventral areas on the first postnatal day. After 8 days, however, the content in the dorsal area increased considerably. In agreement with the findings of others (1), at 60 days, the concentration of GABA was about twofold higher in the dorsal region than in the ventral area.

In the dorsal area of 1 day-old rats, GAD activity showed a relatively low level, that is approximately 25% of the level in the adults, followed by a rapid increase, and adult levels were attained by 15 days (Table 1). In the ventral area of 1 day-old rats, GAD activity was higher than that in the dorsal area and increased to a peak at 8 days, at which time the activity was twice that seen
Table 1. Postnatal development of GABA content and GAD activity in rats

| Age (days) | GABA content (nmol/mg protein) | GAD activity ($^{14}$CO$_2$ nmol/mg protein/hr) | GABA/GAD |
|------------|-------------------------------|-----------------------------------------------|----------|
|            | dorsal                        | ventral                                      | dorsal   | ventral |
| 1          | 9.93±0.78 (108.4)             | 8.79±1.11** (245.5)                         | 18.9±1.8** (26.7) | 27.2±2.2 (114.8) | 0.53 | 0.32 |
| 8          | 7.52±0.18† (82.1)             | 5.53±1.11 (154.5)                          | 55.7±4.6* (78.6) | 47.0±2.8** (198.3) | 0.14 | 0.12 |
| 15         | 8.64±0.68†† (94.3)            | 4.79±0.50 (133.8)                          | 69.5±4.2†† (98.0) | 35.8±2.1** (151.1) | 0.12 | 0.13 |
| 22         | 8.17±1.00†† (89.2)            | 3.62±1.02 (101.1)                           | 78.5±7.4†† (110.7) | 28.5±2.9 (120.3) | 0.10 | 0.13 |
| 60         | 9.16±0.79†† (100.0)           | 3.58±0.49 (100.0)                          | 70.9±2.3†† (100.0) | 23.7±2.1 (100.0) | 0.13 | 0.15 |

GABA content was determined by the enzymatic fluorometric assay (13). GAD activity was determined by the rate of formation of $^{14}$CO$_2$ from L-[1-$^{14}$C]glutamic acid (14). Each point represents the mean of 6–10 values, with the S.E.M. indicated. GABA/GAD was the calculated ratio of GABA content and GAD activity on each day. Values in parentheses are percentages of adult levels. *P<0.05, **P<0.01, compared to the adult (60 days) rats. †P<0.05, ††P<0.01, compared to the ventral area on each day.

Table 2. Kinetics of GAD during postnatal development of rats

| Age (days) | Km (mM) | Vmax ($^{14}$CO$_2$ nmol/mg protein/hr) |
|------------|---------|----------------------------------------|
|            | dorsal  | ventral                                | dorsal   | ventral |
| 1          | 2.28±0.50 (100.0) | 1.70±0.22 (100.0)                 | 28.3±2.7 (100.0) | 42.0±4.2 (100.0) |
| 8          | 1.23±0.28 (100.0) | 0.92±0.46 (100.0)                 | 84.1±13.9 (100.0) | 80.1±35.6 (100.0) |
| 15         | 1.46±0.16 (100.0) | 1.48±0.33 (100.0)                 | 104.5±14.5 (100.0) | 49.3±5.7 (100.0) |
| 22         | 1.43±0.15 (100.0) | 1.45±0.15 (100.0)                 | 117.7±23.3 (100.0) | 57.1±8.9 (100.0) |
| 60         | 1.88±0.20 (100.0) | 1.63±0.18 (100.0)                 | 92.3±30.6 (100.0) | 26.2±5.6 (100.0) |

Km and Vmax values were determined by a Lineweaver-Burk plot. Concentrations of L-[1-$^{14}$C]glutamic acid added were 0.5, 0.75, 1.0, 1.5, 2.0, 4.0 and 5.0 mM. The data represent the mean of 3–4 values, with the S.E.M. indicated.

in the adults. A progressive decrease towards the adult level was seen by the 22nd day. As in the case of the GABA levels, regional differences in GAD activity became evident from 8 days. The ratio between GABA content and GAD activity in both regions became constant from 8 days (Table 1). In the adult rat, the distribution of GAD activity paralleled the GABA content.

**Kinetics of GAD during postnatal development:** We also investigated whether or not the affinity of GAD for glutamic acid is related to the development of GAD activity, and the results are shown in Table 2. Km values of GAD for glutamic acid obtained from a Lineweaver-Burk plot were the same in both dorsal and ventral areas. There were no significant changes in Km values during development. The postnatal development of Vmax in both regions was similar to the alterations in GAD activity, thereby suggesting that the postnatal development of GAD activity was not due to the change of affinity for glutamic acid, but rather to changes in the concentrations of GAD.

**Specific [3H]muscimol binding in**
The results are shown in Table 3. In the dorsal area of 1 day-old rats, specific [3H]muscimol binding was twice as high as that in the adults, and increased to a peak at 8 days. Linear decreases followed until 22 days when the levels reached those seen in adult rats. Specific [3H]muscimol binding in the ventral area was also high on 1 day, decreased from 8 to 22 days and then reached adult levels. There was no significant difference in the specific [3H]muscimol binding between the dorsal and ventral areas on day 1, whereas from 8 days, the binding in the dorsal area was double that seen in the ventral area. In adult rats, the distribution of [3H]muscimol binding was compatible with that of the GABA content and GAD activity. The amounts of non-specific binding of [3H]muscimol in the dorsal and ventral areas were not significantly different, and they did not change during development.

**DISCUSSION**

We confirmed herein that the GABA levels are high in the rat spinal cord at very early stages of postnatal development as has already been reported (16–19). The endogenous GABA content in the rat brain reportedly increases after birth; and at about three weeks, the level is twofold higher than that at birth (18). In contrast, the developmental pattern of the GABA content in the rat spinal cord differed as follows: The GABA content in the dorsal area was high on day 1, decreased by 8 days and then increased gradually, the increment being minute. The high GABA content in the ventral area on day 1 decreased continuously to reach the low levels seen in the adults.

Coyle and Enna (18) reported that GAD activity in brain regions such as the hypothalamus, striatum and cerebellum increased in a linear fashion after birth. In the present study, GAD activity in the dorsal area of the spinal cord increased in a similar manner. In the ventral area, however, the developmental pattern of GAD activity differed from that in the brain: GAD activity in the ventral area reached a peak at 8 days, and then gradually declined to levels seen in the adult rats.

Differences in the postnatal development of GABA content and GAD activity in the rat spinal cord and brain might be due to the fact that the results were expressed on a
Table 4. Protein content of the spinal cord during postnatal development in rats

| Age (days) | Protein content (mg/g tissue) |
|-----------|-------------------------------|
|           | dorsal                        | ventral                     |
| 1         | 104.4±6.8                     | 104.1±3.7**                 |
| 8         | 114.2±3.3                     | 109.0±3.2*                  |
| 15        | 129.8±3.7*                    | 146.9±6.8*                  |
| 27        | 133.8±4.7*                    | 139.6±6.9                   |
| 60        | 117.8±3.4                     | 123.6±4.7                   |

Tissues were homogenized in phosphate buffer and the protein content determined. Each datum represents the mean of 6 values, with the S.E.M. indicated. *P<0.05, **P<0.01 compared to the adult (60 days) rats.

protein concentration basis, whereas other workers made assessments based on a tissue weight basis. However, the protein content per g tissue weight (Table 4) changed little after birth. Thus, the possibility can be excluded that the characteristic pattern of the development of GABA content and GAD activity in the rat spinal cord may be an artifact.

The level of bound \(^{3}H\)muscimol was high in the dorsal and ventral areas on day 1 and decreased after 8 days. Developmental changes in GABA receptors in the spinal cord differ from changes which occur in the brain in which the amount of bound \(^{3}H\)GABA increases in a linear fashion after birth (18). Thus, the regional differences in the receptor binding during development may be masked due to the receptor binding in the large brain regions. Decrease in the amount of bound \(^{3}H\)muscimol after 8 days might reflect the loss of excessive GABA receptors in synaptogenesis (20) or neuron death. Other factors such as GABA-modulin (21) which regulates GABA receptors could account for the decrease of \(^{3}H\)muscimol binding. At present, however, we have no definite explanation for the observed decrease.

An interesting point in this study is that the regional differences in the GABA levels and GAD activity, as well as specific \(^{3}H\)muscimol binding, became evident from 8 days in the dorsal and ventral areas. GABA is also an intermediate in energy metabolism and immunohistochemical studies confirmed that GAD was located only in GABA synaptic terminals (2, 3, 22). In the rat brain, the high affinity uptake of GABA was present at early postnatal stages (18, 23). The K\(^{+}\)-stimulated, Ca\(^{2+}\)-dependent release of radioactive GABA from rat cortical slices was reported to be present in the 1 day-old rats (24). On the other hand, Redburn et al. (25) and Levi et al. (26) indicated that the GABA release was not observed in synaptosomes prepared from the brain of 1 week-old rats. Although the studies on high affinity uptake of GABA, its release, and metabolism in the spinal cord will be necessary to elucidate a neuronal role of GABA in early stages of spinal development, it may be considered that a large part of GABA on day 1 is not responsible for the neurotransmission since the GAD activity is low while GABA content is high.

The GABA levels, GAD activity, and specific \(^{3}H\)muscimol binding in the ventral area decreased during development. These findings may suggest that the role of GABA as a neurotransmitter is small in the ventral area of adult rats. Stelzner (27) reported that the large increase in the synaptic density in the dorsal area of the rat spinal cord took place at 6–8 days and that the pattern was
mature by 21 days of age. Considering the rapid increase in GAD activity and of $[^3]H$ muscimol binding in the dorsal area at about 8 postnatal days, these 8 days may be the "critical period" for the formation of the GABA system in the rat spinal cord, and the GABA system may be mature by 22 days.

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