Changes in $^3$H-Spiperone, $^3$H-WB 4101 and $^3$H-Dihydroalprenolol Bindings to Brain Membranes Produced by Postnatal Pretreatment with Chlorpromazine in Adult Rats

Tetsu HAYASHI, Mineo KUNIHARA and Sakutaro TADOKORO
Behavioral Research Institute, Gunma University School of Medicine, Maebashi 371, Japan
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Abstract—In order to elucidate possible mechanisms of the learning deficit produced by postnatal pretreatment with chlorpromazine (CPZ), changes in catecholamine receptors in the rat brain were investigated. Male neonates of Wistar strain rats were given s.c. 2 mg/kg/day of CPZ for 7 successive days from days 6 to 12 after birth. Effect of the postnatal pretreatment with CPZ on saturation constants for specific bindings of $^3$H-spioperone, $^3$H-WB 4101 and $^3$H-dihydroalprenolol, respectively, in 8 brain regions was investigated at 60 days after birth. Significant decreases in $B_{\text{max}}$ values of $^3$H-WB 4101 binding sites in the cortex, thalamus, hypothalamus, mid brain and medulla oblongata/pons and decreases in $K_d$ values of the binding sites in thalamus, hypothalamus and mid brain were observed in CPZ-pretreated rats when compared with corresponding $B_{\text{max}}$ and $K_d$ values obtained in saline-pretreated rats. Furthermore, significant decreases in both $B_{\text{max}}$ and $K_d$ values of $^3$H-DHA binding sites in the thalamus were detected in CPZ-pretreated rats when compared with those obtained in saline-pretreated rats. However, no alterations in $^3$H-spioperone binding sites in all brain regions were found between CPZ- and saline-pretreated rats. These results suggest that the learning deficit observed in CPZ-pretreated rats may be produced by a functional disorder of catecholaminergic, in particular $\alpha_1$-noradrenergic neurons in the brain.

Previously, we reported that postnatal pretreatment with chlorpromazine (CPZ) frequently produced a deficit in discriminated lever-press avoidance learning after maturation in rats (1-4). In CPZ-pretreated animals, hyperirritability was observed showing unstable lever-pressing, vocalization, running or hopping and sometimes biting the grids in the experimental chamber when foot shock was delivered. However, when shock intensity was diminished, the lever-pressing was stabilized, and the acquisition processes of the avoidance learning were rather improved. Furthermore, in CPZ-pretreated animals, hypersusceptibility to several psychotropic drugs, such as methamphetamine or CPZ, was observed. These results suggested the possibility that functional disorder of catecholaminergic neurons might be implicated in the mechanisms underlying the learning deficit. However, details in the mechanisms of the learning deficit have not been elucidated.

The present study was designed to elucidate possible mechanisms of the learning deficit on the bases of receptor binding experiments.

Materials and Methods
Animals and drug treatment
Forty-eight male neonates obtained by 2 matings of 5 females with 2 male Wistar strain rats, which were supplied by the Institute of Experimental Animal Research of the Gunma University School of Medicine, were used. Twenty-four rats were given s.c. 2 mg/kg/day of CPZ (Contomin* Inj., Takeda) for 7 successive days from 6 to 12
days after birth, and the other 24 rats were given s.c. saline solution under the same conditions. Doses were made up to the volume of 1 ml/kg of body weight by diluting chlorpromazine hydrochloride with saline solution.

Groups of 5 or 6 animals were housed in stainless steel wire mesh cages of 38(D) x 25(W) x 20(H) cm after weaning at 21 days of age and had free access to a solid diet of MF (Oriental Yeast Co., Tokyo) and tap water. The animals were kept in a room at 24±2°C with a 12 hr light-dark cycle (fluorescent illumination on 7:00–19:00 hr). However, the humidity was not controlled.

**Receptor binding**

**3H-Spiperone (SPP) binding assay:** The animals were killed by decapitation at 60 days after birth, and brains were rapidly removed. Each brain was divided into 8 regions: the cerebral cortex, striatum, hippocampus, hypothalamus, thalamus, midbrain, medulla oblongata/pons and cerebellum according to the method of Glowinski and Iversen (5) with a minor modification. 3H-SPP (18 Ci/mmol, Amersham Japan) binding tests to these regions were performed by the method of Usdin et al. (6). Fresh tissues were homogenized in 50 mM Tris-HCl buffer (pH=7.7) using an ultrasonic homogenizer (US-150, Nissei Co., Ltd., Tokyo) and then centrifuged at 50,000 g for 20 min. Each of the resultant pellets was resuspended in 50 mM Tris-HCl buffer (pH=7.7) and centrifuged again at 50,000 g for 20 min. Finally, the pellet was resuspended in 50 mM Tris-HCl buffer (pH=7.1) containing 0.1% ascorbic acid. The reaction mixture (1 ml) containing the membrane suspension (about 0.5 mg protein), 3H-SPP (final concentrations, 0.11–2 nM) and d-Ala2-Enkephalinamide (final concentration, 1 nM) was incubated at 37°C for 20 min. Then the mixture was filtered through a Whatman GF/B filter under vacuum. The filter was rinsed three times with 5 ml ice cold Tris-HCl buffer (pH=7.7).

**3H-WB 4101 binding assay**

3H-WB 4101 {2-[(2′,6′-dimethoxy)phenoxyethylamino]methyl benzodioxan} (27 Ci/mmol, Amersham Japan) binding was performed by the method of U'Prichard et al. (7). Fresh tissues were homogenized in 50 mM Tris-HCl buffer (pH=7.7) using the ultrasonic homogenizer, and then each membrane suspension obtained was centrifuged at 50,000 g for 20 min. The resulting pellet was resuspended in the same buffer and centrifuged as before. The reaction mixture (1 ml) consisted of the membrane suspension (about 0.5 mg protein), 3H-WB 4101 (final concentrations, 0.11–2 nM) and phentolamine (final concentration, 0.5 mM). The mixture was incubated at 25°C for 20 min and then filtered through a Whatman GF/B filter under vacuum. The filter was rinsed three times with 5 ml ice cold Tris-HCl buffer (pH=7.7).

**3H-dihydroalprenolol binding assay**

3H-DHA (44 Ci/mmol, Amersham Japan) binding was performed by the method of Bylund and Snyder (8). Fresh tissues were homogenized in 50 mM Tris-HCl buffer (pH=8.0) and centrifuged at 50,000 g for 20 min. The pellet obtained was rehomogenized in the same buffer and centrifuged as before. The reaction mixture (1 ml) consisted of the membrane suspension (about 0.5 mg protein), 3H-DHA (final concentrations, 0.11–2 nM) and propranolol (final concentration, 10 μM). The mixture was incubated at 23°C for 20 min and was then filtered through a Whatman GF/B filter under vacuum. The filter was rinsed three times with 5 ml ice cold Tris-HCl buffer (pH=8.0).

Kd and Bmax values were determined by Scatchard analysis only for each case where significant difference in specific binding was observed between CPZ- and saline-pretreated rats. Protein concentration was determined by the method of Lowry et al. (9).

**Statistical analysis**

Differences between groups were assessed statistically by one way ANOVA followed by Student's t-test. They were considered to be significant when P was equal or less than 0.05.

**Results**

There were no significant differences in specific bindings of 3H-SPP in all brain regions between CPZ- and saline-pretreated rats. However, as shown in Table 1, significant
Table 1. Effects of postnatal pretreatment with chlorpromazine on specific bindings of $^3$H-sperone, $^3$H-WB 4101 and $^3$H-DHA in rat brain

|                  | Cerebellum | Medulla +pons | Mid brain | Thalamus | Hypothalamus | Hippocampus | Striatum | Cortex |
|------------------|------------|---------------|-----------|----------|--------------|-------------|----------|--------|
| Saline-pretreated| 14.1±3.7   | 12.8±5.6      | 33.6±4.3  | 44.9±5.5 | 61.2±3.9     | 35.6±3.5    | 268.6±11.8| 97.1±4.2|
| CPZ-pretreated   | 15.5±3.8   | 10.5±3.3      | 33.4±4.9  | 45.8±5.6 | 55.0±3.3     | 36.8±7.3    | 250.2±17.1| 94.1±6.1|

|                  | Cerebellum | Medulla +pons | Mid brain | Thalamus | Hypothalamus | Hippocampus | Striatum | Cortex |
|------------------|------------|---------------|-----------|----------|--------------|-------------|----------|--------|
| Saline-pretreated| 31.1±3.2   | 39.4±2.3      | 44.5±1.2  | 54.5±3.0 | 47.4±2.3     | 88.4±4.9    | 41.7±3.8 | 70.4±4.0|
| CPZ-pretreated   | 31.7±3.1   | 30.6±0.9***   | 34.0±2.1***| 45.2±1.8**| 36.1±2.4*** | 84.5±4.8    | 33.5±2.2 | 54.5±4.0*|

|                  | Cerebellum | Medulla +pons | Mid brain | Thalamus | Hypothalamus | Hippocampus | Striatum | Cortex |
|------------------|------------|---------------|-----------|----------|--------------|-------------|----------|--------|
| Saline-pretreated| 14.1±2.6   | 16.1±2.2      | 17.4±2.9  | 17.9±2.8 | 22.7±2.8     | 34.0±4.1    | 35.4±3.8 | 33.2±1.7|
| CPZ-pretreated   | 15.8±2.6   | 12.9±1.9      | 14.5±1.6  | 9.6±1.5**| 17.6±3.6     | 28.0±1.1    | 35.0±9.6 | 33.4±4.6|

Binding tests were done at a fixed ligand concentration (final concentration about 1.5 nM in all cases)

*P<0.05, **P<0.01, ***P<0.001 vs. controls
differences in specific bindings of $^3$H-WB 4101 in the cortex, thalamus, hypothalamus, mid brain and medulla oblongata/pons were observed, while significant decrease in the binding of $^3$H-DHA in the thalamus was observed between CPZ and saline-pretreated rats.

Table 2. Effect of postnatal pretreatment with chlorpromazine on saturation constants for specific bindings of $^3$H-WB 4101 and $^3$H-DHA in rat brain

| Ligands     | Regions         | Constants          | Saline-pretreated (1 ml/kg/day×7) (N=5) | CPZ-pretreated (2 mg/kg/day×7) (N=5) |
|-------------|-----------------|--------------------|----------------------------------------|-------------------------------------|
|             |                 | $K_d$ (nM)         | 0.87±0.05                              | 0.76±0.07                           |
|             |                 | $B_{max}$ (fmol/mg prot) | 119.16±3.91                               | 86.33±4.12***                       |
| Cortex      |                 | $K_d$              | 1.16±0.10                               | 0.85±0.06*                          |
|             |                 | $B_{max}$          | 109.08±5.65                             | 69.58±2.60***                       |
| Thalamus    |                 | $K_d$              | 0.65±0.08                                | 0.41±0.03*                          |
|             |                 | $B_{max}$          | 131.22±6.90                             | 64.82±1.77***                       |
| $^3$H-WB 4101 | Hypothalamus   | $K_d$              | 1.67±0.08                                | 0.75±0.10***                       |
|             |                 | $B_{max}$          | 104.26±3.63                             | 55.91±3.86***                       |
| Mid brain   |                 | $K_d$              | 0.48±0.06                                | 0.41±0.03                           |
|             |                 | $B_{max}$          | 57.65±2.58                              | 46.65±1.68**                        |
| Medulla-Pons|                 | $K_d$              | 0.49±0.03                                | 0.33±0.04**                         |
|             |                 | $B_{max}$          | 25.62±0.87                              | 14.94±0.74***                       |

*P<0.05, **P<0.01, ***P<0.001 vs. controls

The primary action of CPZ is considered to block both dopamine and noradrenaline receptors in the brain, while HPD blocks more selectively dopamine receptor than noradrenaline receptor (10, 11). On the other hand, amphetamines release both central and peripheral catecholamines and inhibit reuptake of the amines at the synaptic sites (12-14). A small dose of AMOK is thought to stimulate the autoreceptor (15, 16), while a large dose causes a stimulation of the postsynaptic dopamine receptor (17). It is, therefore, suggested that the learning deficit observed in CPZ-pretreated rats may be produced by a functional disorder of catecholaminergic, in particular noradrenergic neurons in the brain.

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Table 2 represents $B_{max}$ and $K_d$ values of $^3$H-WB 4101 and $^3$H-DHA binding sites in the regions where significant differences in the specific bindings were observed between CPZ- and saline-pretreated rats.

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**Discussion**

Previously, we reported that postnatal pretreatment with CPZ frequently produced behavioral abnormalities, in particular irreversiblelearning deficit, and that its intensity and incidence rates were observed in a dose-dependent manner (1-4). Furthermore, hypersusceptibility to methamphetamine (MAP) and CPZ was observed in CPZ-pretreated rats, but that to apomorphine (AMOR) and haloperidol (HPD) was not detected in the same rats (2-4). It was also shown that there was a critical period for the administration of CPZ to produce such abnormalities.

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$^3$H-SPP has been successfully used as a
dopamine D₂-receptor labeler (6). However, several biochemical studies indicated that ³H-SPP labeled 5-HT₂ receptor as well as dopamine receptor in frontal cortex (18, 19). Therefore, it is recommended to use ³H-SPP in D₂-receptor binding studies in the presence of ketanserin, a S₁₂-serotonin blocker. ³H-WB 4101 and ³H-DHA label α₁- and β-adrenergic receptors, respectively, in animal brains (7, 8).

In the present experiment, marked decreases in the maximum density of ³H-WB 4101 and ³H-DHA binding sites (Bₘₐₓ) with increases in the ligand affinity (1/Kₐ) were observed, particularly in ³H-WB 4101 binding tests, while no alterations in ³H-SPP binding sites were found when CPZ was pretreated. Consequently, the results obtained in the present experiment suggest that CPZ-pretreatment during early postnatal periods produces functional disorders of noradrenergic neurons in the brain, in particular those of α₁-noradrenergic neurons in adult rats. Furthermore, these results well support the previous results obtained on the bases of behavioral and pharmacological experiments (2-4), although it is difficult to explain in detail the correlation between the receptor changes and the brain function.

It has been reported by many researchers that central catecholaminergic mechanisms are implicated in the avoidance learning (20-23). Fuxe and Hanson (21) investigated the correlation between central catecholaminergic neurons and conditioned avoidance response in rats using a histochemical fluorescence technique, and they reported that the noradrenergic neurons played an important role as an arousal system essential for the establishment of the avoidance response. On the other hand, Tonge (24) reported that CPZ given to female Wistar strain rats during pregnancy and suckling periods produced a decrease in noradrenaline and an increase in its metabolite contents in the brain of the offspring.

It is concluded that the changes in central noradrenergic neurons are one of the possible mechanisms of the learning deficit observed in CPZ-pretreated rats.

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