Cannabinoid suppressed bicuculline-induced convulsion without respiratory depression in the brainstem-spinal cord preparation from newborn rats

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ABSTRACT
Previous studies have suggested that cannabinoid compounds are anticonvulsants and that these compounds depress respiratory activity. However, the anticonvulsant potential of cannabinoids and their depressive effect on respiration have not been evaluated simultaneously. In the present study, we used a brainstem-spinal cord preparation model to investigate changes in inspiratory activity and the anticonvulsant effects of a cannabinoid receptor agonist, WIN55, 212-2, in bicuculline-induced convulsion. Application of 10 μM WIN55, 212-2 caused no change in inspiratory activity (6.9 ± 0.89 bursts/min vs. 8.0 ± 1.3 bursts/min, not significant) and decreased bicuculline-induced seizure-like nerve activity (number of seizure-like activities in 10 min, 11 ± 7.4 bursts vs. 1.5 ± 1.6 bursts, P < 0.01; average duration of seizure-like activity, 8.9 ± 4.0 sec vs. 4.7 ± 2.1 sec, P < 0.01). Our results suggest that administration of an appropriate dose of cannabinoid receptor agonist WIN55,212-2 has an anticonvulsant effect but does not cause respiratory depression.

Epilepsy is one of the most common neurological disorders and is characterized by spontaneously recurrent seizures (10). Recently, several cannabimimetic compounds have been evaluated for effects on seizures. Wallace et al. (35) determined that tetrahydrocannabinol and the CB1-cannabinoid receptor agonist WIN55, 212-2 have anticonvulsant effects in the rat pilocarpine model of epilepsy, likely via a CB1-receptor-dependent mechanism that serves to decrease hyperexcitability. Wallace et al. (37) also showed that cannabinoid compounds block seizure spread via a CB1-receptor-dependent mechanism in the maximal electroshock model of short-term seizure (36, 37).

Studies with brainstem-spinal cord preparations from newborn rats have provided substantial information regarding the neuro-physiology, -pharmacology, -anatomy of the respiratory center, including mechanisms of respiratory rhythm generation and development of a respiratory center or respiratory reflex (19, 20, 22, 32, 33). Bicuculline is a [gamma]-aminobutyric acid (GABA) receptor antagonist that blocks Cl⁻-mediated inhibition. Treatment of brainstem-spinal cord preparations with bicuculline has been used as a model of convulsion; bicuculline evokes epileptiform discharges in the respiratory active spinal nerve roots (6–8, 21). It has also been reported that inspiration-related spinal nerve activity persists after block of Cl⁻-mediated inhibition with bicuculline in these preparations (6, 8, 21). Thus, this brainstem-spinal cord preparation may be useful for simultaneous evaluation of the anticonvulsant and respiratory effects of cannabinoids.

The anticonvulsant activity and respiratory effects of cannabimimetic compounds have not been evaluated in the brainstem-spinal cord preparation model, and therefore, we performed this analysis in the...
present study.

MATERIALS AND METHODS

Preparation. This study was performed in accordance with the protocol approved by the Animal Care and Use Committee of Nippon Medical School, Japan. Brainstem-spinal cord preparations from 0- to 2-day-old Wistar rats (40 preparations) were used (3, 12, 22). Brainstem and spinal cord were isolated under deep ether anesthesia (33). The brainstem was rostrally decerebrated between the sixth cranial nerve roots and the lower border of the trapezoid body. Most of the pons was removed. The preparation was then placed in a perfusion chamber (2 mL) with its ventral surface up and was super-fused continuously at 3.0–3.5 mL/min with artificial cerebrospinal fluid (aCSF) (124 mM NaCl, 5.0 mM KCl, 1.2 mM KH₂PO₄, 2.4 mM CaCl₂, 1.3 mM MgCl₂, 26 mM NaHCO₃, and 30 mM glucose), equilibrated with 95% oxygen and 5% CO₂ and maintained at 26–27°C and a pH of 7.4.

Recording. Discharges of respiratory motor activity were recorded extracellularly with suction electrodes applied to the proximal ends of cut ventral roots of spinal (C4 or C5) nerves. Signals were fed through a high-pass filter with a 0.3-sec time constant. During the experiments, neuronal activity was displayed on a chart recorder, monitored with an oscilloscope, digitized (Digidata 1200B; Axon Instruments, Foster, CA, USA), and stored on a hard disk for offline analysis with a personal computer and data acquisition software (Axoscope; Axon Instruments).

Drugs. Bath application was used for all drugs. Stock solutions were diluted with aCSF just before application. GABA receptor antagonist bicuculline and CB₁-receptor agonist WIN55, 212-2 were purchased from Sigma (St. Louis, MO, USA). CB₁-receptor antagonist AM-251 was purchased from Tocris (Ellisville, MO, USA). WIN55, 212-2 and AM-251 stock solutions were made in dimethyl sulfoxide (DMSO). Aliquots were then diluted in aCSF so that the final maximal concentration of DMSO in the perfusate was 0.2%. DMSO had no significant effect per se on the excitatory transmission (18). All solutions were adjusted to a pH of 7.4 and were applied to the recording chamber through the perfusion system.

Evaluation of the effects of cannabinoid agonist (Protocol 1, Fig. 1A). aCSF was applied for 10 min as a control. aCSF containing 10 μM WIN55, 212-2 was then applied for 30 min (n = 5, Fig. 1A).

Evaluation of the effects of cannabinoid agonist after application of bicuculline (Protocol 2, Fig. 1B). aCSF was applied for 10 min as a control. The specimens were divided into two groups; a bicuculline group (n = 15) and a cannabinoid group (n = 15). In both groups, 10 μM bicuculline was applied for 15 min. After application of bicuculline, the cannabinoid group was treated with 10 μM WIN55, 212-2 and 10 μM bicuculline for 30 min. The bicuculline group continued application of 10 μM bicuculline for 30 min (Fig. 1B). The number of seizure-like activities and the average duration of these activities were measured during four periods. The first period was 5–15 min after application of bicuculline, the second period was 0–10 min after application of each drugs (bicuculline group, bicuculline; cannabinoid group, WIN55, 212-2 with bicuculline) and the third and fourth periods were 10–20 min and 20–30 min after application of drugs, respectively.

Effects of cannabinoid antagonist pretreatment

After application of bicuculline and AM-251, seizure-like activity was observed in C4 inspiratory activity (Figs. 2 and 4). In the presence of AM-251, the number of seizure-like activities did not change after the application of WIN55, 212-2 (Table 4). The average duration of seizure-like activities did not change (Table 4).

DISCUSSION

The major findings of our investigation were that application of 10 μM WIN55, 212-2 suppressed bicuculline-induced convulsions without changing inspiration-related spinal nerve activity in a brainstem-spinal cord preparation model. In the presence of CB1-receptor antagonist AM-251, the anticonvulsant effect of WIN55, 212-2 was absent.

In the present study, we used a bicuculline-in-

### Table 1

| Effects of 10 μM WIN55, 212-2 on Burst Rate of C4 inspiratory activity |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Control                     | 0–10 min after WIN55, 212-2 | 10–20 min after WIN55, 212-2 | 20–30 min after WIN55, 212-2 |
| Burst Rate (bursts/min)     | 6.9 ± 0.89                  | 7.2 ± 0.89                  | 7.6 ± 0.86                  | 8.0 ± 1.3                  |

Values are mean ± SD.

Figure 2. C4 respiratory motor activity. (A) Normal waves of C4 discharges. (B) Appearance of bicuculline-induced seizure-like activities in C4 discharges. (C) Representation of recordings in (B) on an expanded time scale.
noid actions are believed to involve attenuation of glutamate release. Because modulation of presynaptic neurotransmitter release is believed to be a primary result of CB1-receptor activation, it is possible that this mechanism underlies the anticonvulsant properties of cannabinoids (35). CB1-receptor activation is known to decrease calcium influx (15), the result of which is decreased Ca\(^{2+}\)-dependent glutamate release. Glutamate is the primary excitatory neurotransmitter of the central nervous system. Although glutamate is critical for normal neurotransmission, elevated levels of glutamate are associated with excitotoxicity, and excessive glutamatergic transmission is a hallmark of epilepsy (14). With elevated levels of glutamate detected in epileptic tissue, decreased release of this neurotransmitter would be a logical mechanism for the anticonvulsant activity of cannabinoids (14).

The respiratory effects of bicuculline in this preparation have been discussed. Recently, it was reported that hyperpolarizing GABAergic inhibitory produced convulsion model in brainstem-spinal cord preparations. Bicuculline is a GABA receptor antagonist, and its pharmacological effect is blockade of Cl\(^{-}\)-mediated inhibition. In the brainstem-spinal cord preparation, bicuculline evokes epileptiform discharge in the respiratory active spinal nerve roots (6–8, 21). This seizure-like spinal motor output is caused by disinhibition of spinal neuronal networks with afferent connections to the ventral respiratory group (7). This seizure-like activity in spinal cord resembles the epileptiform activity observed in cortical brain regions after blockade of GABAergic inhibition (31).

CB1-receptor agonist WIN55, 212-2 had an anticonvulsant effect in our study. The anticonvulsant mechanism of cannabinoids has been discussed. Recent studies have revealed that cannabinoids ameliorate symptoms were associated with neuronal hyperexcitability. In a pilocarpine model of convulsion, the endogenous cannabinoid system regulates seizure frequency and duration (35). These cannabinoid actions are believed to involve attenuation of glutamate release. Because modulation of presynaptic neurotransmitter release is believed to be a primary result of CB1-receptor activation, it is possible that this mechanism underlies the anticonvulsant properties of cannabinoids (35). CB1-receptor activation is known to decrease calcium influx (15), the result of which is decreased Ca\(^{2+}\)-dependent glutamate release. Glutamate is the primary excitatory neurotransmitter of the central nervous system. Although glutamate is critical for normal neurotransmission, elevated levels of glutamate are associated with excitotoxicity, and excessive glutamatergic transmission is a hallmark of epilepsy (14). With elevated levels of glutamate detected in epileptic tissue, decreased release of this neurotransmitter would be a logical mechanism for the anticonvulsant activity of cannabinoids (14).

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![Fig. 3](image-url) Effects of cannabinoid agonist WIN55, 212-2 on C4 respiratory motor activity. Bicuculline-induced seizure-like activities appeared in the 1st period. Cannabinoid agonist WIN55, 212-2 decreased the number of seizure-like activities in the 4th period.
postsynaptic potentials determine the pattern of membrane potential fluctuations in respiratory neurons in vivo and in reduced in vitro preparations from newborn and adult rodents (1–8, 21, 23, 24, 25).
28, 29, 32). It is obvious that increased interstitial levels of GABA block the in vitro respiratory rhythm due to activation of Cl− conductance of the ventrolateral medulla (VLM)-ventral respiratory group (VRG) neurons and of a Gi/o protein-coupled K+ conductance that is presumably characteristic of neurons providing excitatory drive to the network (1, 3, 6, 7, 23). It is also shown that blockade of GABA receptors does not affect the primary respiratory rhythm despite considerable changes in discharge characteristics of several classes of VLM-VRG neurons in the reduced preparations (3, 6, 7, 21, 23, 24). Inspiration-related spinal nerve activity also persisted after blockade of Cl−-mediated inhibition with bicuculline in brainstem-spinal cord preparations (6, 8, 21). Thus, this bicuculline-induced convulsion model in brainstem-spinal cord appears to be appropriate for simultaneous evaluation of the anticonvulsant and respiratory effects of cannabinoids and other pharmacologic agents.

Application of WIN55, 212-2 depressed respiratory rate and respiratory minute volume and slightly increased tidal volume in an in vitro model system (13, 26, 27, 30). It is not known which brainstem respiratory center (ventral medullary respiratory group, dorsal medullary respiratory group or pontine respiratory group) was the primary target of cannabinoids for lowering respiratory rate. CB1-receptor mRNA and protein are expressed in several nuclei of these respiratory centers, including the nucleus of the solitary tract, dorsal motor nucleus of the vagus, area postrema, rostroventrolateral reticular nucleus, ambiguus nucleus and parabrachial nucleus (9, 11, 16, 17, 25, 34). In our study, respiratory rate was not changed after application of WIN55, 212-2. The discrepancy between results may be due to different concentrations of cannabinoid at the target site and/or differences in the model systems (in vivo vs. in vitro). The brainstem-spinal cord preparation from newborn rats preserves a basic neuron network and the function of the respiratory center in the medulla oblongata (3, 22, 32). This in vitro preparation, maintained under anesthesia-free conditions, is suitable for detailed pharmacological studies of the respiratory center because drugs can be applied at defined concentrations into regions of interest by superfusion (22). Although this reduced preparation has these technical advantages, extrapolation of results to the mature and intact animal should always be made with a degree of caution. Our results suggest that administration of an optimal dose of cannabinoid has an anticonvulsant effect but does not cause respiratory depression. Further studies are needed to evaluate the anticonvulsant and respiratory effects of cannabinoids in various models.

In conclusion, application of CB1-receptor agonist WIN55, 212-2 suppressed bicuculline-induced convulsion without respiratory depression in the brainstem-spinal cord preparation from newborn rats.

### REFERENCES

11. Herkenham M, Lynn AB, Johnson MR, Melvin LS, de Costa

### Table 4  Effects of Cannabinoid Antagonist Pretreatment on Number and Duration of Seizure-like Activities

<table>
<thead>
<tr>
<th></th>
<th>1st period</th>
<th>2nd period</th>
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<tr>
<td>Number of Seizure-like Activities (bursts/10 min)</td>
<td>1.3 ± 0.50</td>
<td>1.6 ± 0.42</td>
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<tr>
<td>Average Duration of Seizure-like Activities (sec)</td>
<td>11 ± 7.0</td>
<td>11 ± 6.9</td>
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Values are mean ± SD. Values are mean ± SD.


