carry multiple HBV genomic inserts, only PLC/PRF/5 and GTC2 cells treated with 5′-azacytidine grown in LHC4 medium are known to express the *HBc* gene in vitro (*8*). Hepatocellular carcinoma cells containing HBV express the *HBs* gene constitutively, producing the HBV surface antigen, HBsAg, without cytopathologic effects (*4*). The sequences coding for HBcAg in PLC/PRF/5 cells are highly methylated, whereas HBV virion DNA and HBV DNA extracted from liver samples of infected patients is unmethylated (*10*). The contrasting methylation pattern of the *HBc* gene in cells carrying HBV integrated as a provirus in the human genome and in HBV DNA extracted from virions and tissues of acutely infected patients suggests that methylation of $HpaII^{-280}$ in the 5′-flanking region of the *HBc* gene may be important to the viability of host cells. Thus, host factors regulating *HBc* gene expression may have a pivotal role in establishing the intricacies of HBV host range and the variations in susceptibility that are related to nutrition, sex, age, and immunologic abnormalities.

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19 November 1984; accepted 14 March 1985

Endogenous Anticonvulsant Substance in Rat Cerebrospinal Fluid After a Generalized Seizure

Abstract. *Cerebrospinal fluid taken from rats subjected to electroshock-induced seizures and injected into the cerebral ventricles of rats that had not been shocked increased the seizure threshold of the recipients. The anticonvulsant activity of the donor cerebrospinal fluid was antagonized by opioid antagonists and enhanced by peptidase inhibitors. These results suggest the existence of an endogenous anticonvulsant substance in rat cerebrospinal fluid, possibly opioid in nature, which is activated as a consequence of a seizure and which may play a critical role in postseizure inhibition.*

Postseizure inhibition has been shown both experimentally (*1, 2*) and clinically (*3, 4*) to be a physiological regulator in the self-limitation of seizures. Although the clinical significance of the postseizure refractory state for preventing the continuous recurrence of seizures in the epileptic is clear, the mechanism underlying this state is not known. Because opioid peptides, endogenous ligands for opiate receptors, have marked anticonvulsant activity against a variety of experimentally induced seizures (*2, 5–7*) and are activated as a consequence of seizures (*5, 7, 8*), it was suggested that seizure-activated endogenous opioid systems function to decrease the probability or severity of subsequent seizures (*9, 10*). Working under this hypothesis, we demonstrated that endogenous opioid systems play a selective role in the postictal rise in seizure threshold (*3*) and that the opioid antagonist naloxone stereoselectively attenuates and morphine tolerance prevents the postseizure protection produced by repeated maximal electroshock (MES) seizures in rats (*11*). We suggested that the brain's opioid systems may act postictally as endogenous anticonvulsants and thus play an important role in maintaining a refractory state after a seizure. More recently, it was reported that the anticonvulsant effects of electroconvulsive shock or foot-shock stress against kindled seizures are mediated by the activation of endogenous opioid systems (*12*). We report here the presence of an endogenous "anticonvulsant substance" in the cerebrospinal fluid (CSF) of rats subjected to MES seizures, whose bioactivity is completely antagonized by opioid antagonists and enhanced by peptidase inhibitors. We suggest that a seizure activates in the central nervous system (CNS) an endogenous substance, possibly opioid in nature, that acts as an endogenous modulator of CNS excitability, particularly in the regulation of postseizure inhibition.

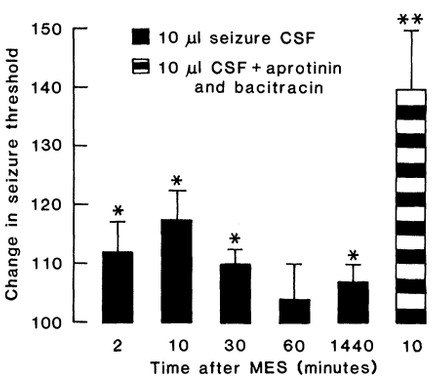

Fig. 1. Effects of seizure CSF collected at various times after MES on the seizure thresholds of recipient rats challenged with flurothyl. Data (means + standard errors) are expressed as the percentage of increase in seizure threshold beyond the baseline threshold for the control CSF–treated rats. The filled bars represent the response to seizure CSF not protected by protease inhibitors. The banded bar represents the response to seizure CSF taken 10 minutes after MES, with aprotinin and bacitracin added to the pooled sample. Control groups were included with each experimental treatment group. Since there was no significant difference among the control seizure measurements made in each trial, they were combined into a single control group and used for all comparisons (the same was done with the data in Fig. 2). (*) Significantly different from the result for control CSF ($P < 0.05$, Student's t-test). (**) Significantly different from the result for control CSF plus aprotinin and bacitracin ($P < 0.01$).

Male Sprague-Dawley rats (275 to 350 g; Zivic-Miller Laboratories) were randomly designated as donor or recipient animals. Donors were subjected to single transauricular MES (2 seconds at 60 Hz and 50 mA), causing a generalized seizure characterized by an initial tonic extension of the limbs followed by clonic jerking. At various times after an MES seizure, donor rats were anesthetized with ketamine HCl (100 mg/kg intraperitoneally), and CSF (referred to as seizure CSF) was collected with a 21-gauge needle inserted into the cisternal space. Seizure CSF from three to six donor animals was pooled and kept on ice throughout the experiment; control CSF was also collected from donor rats given sham MES.

In recipient animals (n = 12 to 15 per group) a 30-gauge cannula was stereotaxically implanted in the right lateral cere-

bral ventricle. Control CSF (10 μl) or seizure CSF (10 μl) was injected through the cannula, and 15 minutes later flurothyl (Indoklon; hexafluorodiethyl ether), a volatile convulsant, was infused into a 1-gallon sealed glass jar as a 10 percent solution (by volume) in 95 percent ethanol and the time until the onset of the clonic convulsion (seizure threshold) was determined (*9*). All intracerebroventricular injections were followed by a 2-μl flush with the vehicle.

Mean seizure thresholds after injection of control CSF ranged from 325 to 365 seconds. Administration of seizure CSF resulted in significant increases in seizure threshold (Fig. 1). The magnitude of the anticonvulsant effect of seizure CSF was dependent on the time of its collection after MES. The maximum increase in seizure threshold (approximately 18 percent) was seen with seizure CSF obtained 10 minutes after MES. Seizure CSF obtained as long as 24 hours after MES continued to produce significant, albeit smaller, increases in the seizure threshold of recipient animals.

To gain some insight into the chemical nature of the anticonvulsant substance, we assessed the bioactivity of control and seizure CSF in the presence of the protease inhibitors aprotinin and bacitracin (756 kallikrein units and 1 mg per milliliter of CSF, respectively; Sigma). Compared to unprotected seizure CSF, seizure CSF with added aprotinin and bacitracin resulted in a twofold increase in seizure threshold to 40 percent above the control level (banded bar in Fig. 1). No changes in seizure thresholds were noted with control CSF in the presence of aprotinin or bacitracin.

In keeping with our original hypothesis, we tested the possibility that endogenous opioid systems are involved in the anticonvulsant action of seizure CSF by treating recipient animals with doses of the opioid antagonists naloxone or ICI 174,864 which, by themselves, had no influence on flurothyl seizure thresholds. Groups of 8 to 15 rats were treated with saline (1 ml/kg, subcutaneously or 5 μl, intracerebroventricularly), naloxone (1 or 10 mg/kg, subcutaneously), or ICI 174,864 (1 or 5 μg, intracerebroventricularly) 10 minutes before intracerebroventricular injection of control CSF or seizure CSF. High (10 mg/kg) but not low (1 mg/kg) doses of naloxone completely antagonized the ability of seizure CSF to raise the flurothyl seizure threshold in these rats (Fig. 2). Furthermore, the selective δ opioid receptor antagonist ICI 174,864 (*13*) antagonized the anticonvulsant activity of the seizure CSF at doses as low as 1 to 5 μg, intracerebroventricularly. Doses of ICI 174,864 greater than 5 μg were not tested since this dose alone (but not the 1-μg dose) caused transient behavioral effects characterized by a splayed limb posture and turning behavior, which often progressed to rolling.

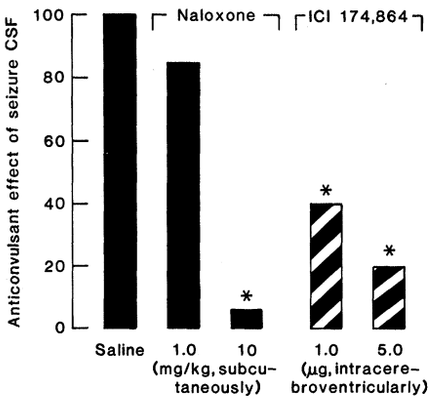

Fig. 2. Effect of treatment with saline, naloxone, or ICI 174,864 on the anticonvulsant action of seizure CSF taken 10 minutes after MES. Anticonvulsant activity of seizure CSF in drug-treated animals is expressed as the percentage of the total anticonvulsant effect of seizure CSF in saline-treated rats (normalized to 100 percent). (*) Significantly different from the respective control groups ($P < 0.05$, Student's t-test performed with the data before normalization to 100 percent).

Rats receiving control or seizure CSF were behaviorally alert during the flurothyl challenge. There were no signs of sedation, ataxia, or respiratory depression in these animals. Furthermore, seizure CSF (10 μl) from the same pooled samples that markedly increased seizure threshold (broken bar in Fig. 1) did not reduce nociception in tail-flick and hot-plate tests.

The results suggest that an endogenous anticonvulsant substance is present in the CSF of rats after a generalized seizure. The activity of this substance was enhanced in the presence of protease inhibitors, suggesting that the endogenous material may be a peptide. Furthermore, since the anticonvulsant activity of seizure CSF was antagonized by high doses of naloxone or low doses of ICI 174,864, the active substance may be opioid in nature. Consonant with the δ-receptor mediation of these anticonvulsant actions, the δ receptor–directed opioid agonist D-Ala^2-D-Leu^5-enkephalin has been reported to be a potent anticonvulsant in the rat flurothyl test, actions completely antagonized by high doses of naloxone or the δ-receptor antagonist ICI 154,129, but not by the irreversible μ-receptor antagonist β-funaltrexamine (*14*).

Many investigators have reported increases in endogenous opioid peptides in the CNS after electroconvulsive shock or kindled seizures (*8*). In preliminary experiments we have also observed increases in β-endorphin–like immunoreactivity in seizure CSF. However, there is no evidence that the anticonvulsant substance is β-endorphin, enkephalin, or dynorphin. The combined properties of the anticonvulsant substance described herein—in particular its apparent activity at the δ opioid receptor subtype, its sensitivity to enzyme degradation, and the fact that it is not analgesic at doses that are anticonvulsant—do not completely fit the biological profiles of classically defined opioid peptides. Furthermore, while it is possible that γ-aminobutyric acid may be involved, the results seen with low doses of the δ-receptor antagonist strongly support a participation of endogenous opioid systems. Consequently, an as yet unrecognized endogenous ligand with affinity for and anticonvulsant bioactivity at δ opioid receptor binding sites may exist.

The isolation and identification of an endogenous anticonvulsant factor could have major implications for the understanding and treatment of epilepsy. Such a factor may play an important physiological role in the mechanism of postseizure inhibition. If, indeed, our results represent the identification of an endogenous modulator of CNS excitability, then the characterization of this substance might provide novel therapeutic approaches not only to epilepsy but to other disturbances of mental function as well.

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15. We thank L. Robles and E. Echevarria for their technical assistance and Endo DuPont and Imperial Chemical Industries for their generous gifts of naloxone and ICI 174,864, respectively. In conducting this research we adhered to the recommendations of the Committee on Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council.

3 December 1984; accepted 30 January 1985

Functional Coupling of γ-Aminobutyric Acid Receptors to Chloride Channels in Brain Membranes

Abstract. *γ-Aminobutyric acid (GABA), the major inhibitory neurotransmitter in mammalian brain, is believed to act by increasing membrane conductance of chloride ions. In this study it was found that GABA agonists increased the uptake of chloride-36 by cell-free membrane preparations from mouse brain. This influx was rapid (less than 5 seconds), and 13 micromolar GABA produced a half-maximal effect. The GABA antagonists (bicuculline and picrotoxin) blocked the effect of GABA, whereas pentobarbital enhanced the action. This may be the first demonstration of functional coupling among GABA and barbiturate receptors and chloride channels in isolated membranes. The technique should facilitate biochemical and pharmacological studies of GABA receptor–effector coupling.*

Understanding the actions of neurotransmitters and hormones requires knowledge of coupling between receptors and effectors. During the past 10 years we have learned much about neurotransmitter receptors in the central nervous system. Studies of the coupling of some of these receptors to adenylate cyclase have been particularly fruitful (*1*). However, most neurotransmitter receptors are not linked to adenylate cyclase, but regulate ion channels. The major inhibitory neurotransmitter in the mammalian brain, γ-aminobutyric acid (GABA), appears to produce its effects by enhancing the flux of chloride ions across nerve membranes (*2*). The importance of this system is supported by behavioral, receptor-binding, and electrophysiological studies. The electrophysiological approach has provided strong evidence that GABA receptors regulate chloride conductance, but to our knowledge there has been no neurochemical demonstration of a GABA-regulated chloride flux in a cell-free membrane preparation (*3*).

Our experience with the voltage-activated channels of brain membranes suggested that the receptor-mediated chloride flux would be very rapid in brain membrane vesicles. Accordingly, we measured the influx of $^{36}Cl^-$ by a procedure in which GABA and $^{36}Cl^-$ are added simultaneously to membrane vesicles. Uptake can be stopped within 2 seconds by a quench solution and rapid filtration (*4*). The brain "microsac" preparation (*5*) was chosen as a source of membrane vesicles. This cell-free preparation, first devised to study coupling between brain receptors and adenylate cyclase, contains sealed vesicles derived from pre- and postsynaptic elements.

Addition of GABA to brain microsacs stimulated a rapid influx of $^{36}Cl^-$ (Fig. 1) (*6*). There was appreciable uptake after 2 seconds, the shortest period measured, and accumulation was near-maximal by 5 seconds. In view of these kinetics, we chose an uptake time of 3 seconds for the remainder of the experiments. The effect of GABA was concentration-dependent, with 13 μ*M* GABA giving a half-maximal effect (Fig. 2). The concentration-response relation for the GABA-stimulated uptake of chloride was consistent with first-order kinetics for receptor occupation and channel activation (inset in Fig. 2).

Chloride uptake was also stimulated by two GABA agonists, muscimol and 3-amino-1-propanesulfonic acid (APS) (Table 1). Concentration-response curves indicated that muscimol was more potent and APS less potent than GABA. The agonistic action of muscimol, which is not a substrate for uptake at these concentrations (*7*), indicates that the chloride flux is not related to the GABA transport system. These results are consistent with the relative potencies of these drugs in studies of GABA receptor binding (*8*).

The GABA receptor antagonist bicuculline completely blocked GABA-stimulated chloride uptake (Table 1). The sensitivity to bicuculline, together with the lack of an effect of baclofen on chloride uptake, suggests that only the $GABA_A$ subtype of receptor (*9*) is coupled with chloride channels. Picrotoxin, a drug that blocks GABA responses by acting on the chloride channel rather than the GABA receptor, also inhibited GABA-stimulated chloride uptake. Neither picrotoxin nor bicuculline decreased

Table 1. Effects of GABA, GABA-mimetic drugs (muscimol and APS), and GABA antagonists (picrotoxin and bicuculline) on the influx of $^{36}Cl^-$ in mouse brain microsacs. Uptake was calculated as flux in the presence of drug (total flux) minus flux in the absence of drug. Values are means ± standard errors.

Drug	Number of preparations tested	Chloride uptake (nanomoles of Cl^- per milligram of protein)
Muscimol (5 μ*M*)	4	9.6 ± 0.4*
APS (30 μ*M*)	4	4.3 ± 0.4*
GABA (10 μ*M*)	16	6.4 ± 0.4
GABA (10 μ*M*) + picrotoxin (100 μ*M*)	4	1.2 ± 0.1†
GABA (10 μ*M*) + bicuculline (100 μ*M*)	4	0.6 ± 0.9†
Pentobarbital (10 μ*M*)	16	0.7 ± 0.5
Pentobarbital (300 μ*M*)	16	3.3 ± 0.6*
GABA (10 μ*M*) + pentobarbital (10 μ*M*)	16	8.9 ± 0.8†
GABA (10 μ*M*) + pentobarbital (100 μ*M*)	16	14.5 ± 0.9†

*Significantly different from value for no drug addition ($P < 0.01$, paired *t*-test). †Significantly different from value for GABA alone ($P < 0.01$).