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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 ($P < 0.01$).

chloride uptake in the absence of exogenous GABA, suggesting that the membrane preparation did not contain sufficient endogenous GABA to activate the channel.

To investigate the possibility that the chloride flux was secondary to a GABA-stimulated cation influx or increased vesicle permeability, we measured the effects of GABA agonists on uptake of $^{45}Ca^{2+}$, $^{22}Na^{+}$, $^{86}Rb^{+}$, and $^{35}SO_4^{2-}$. Permeability of these ions was not changed by concentrations of GABA that produced maximum increases in chloride influx (*10*). Pharmacological specificity is further supported by the observation that 100 μ*M* baclofen, inosine, taurine, glutamate, or aspartate or 1 m*M* diaminobutyric acid failed to stimulate chloride influx. Glycine (100 μ*M*) produced a slight stimulation of chloride influx, consistent with suggestions that glycine receptors are coupled with chloride channels (*11*). The chloride transport inhibitor 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid inhibited both GABA-dependent and -independent chloride influx. These results agree with those of electrophysiological studies of the effects of this inhibitor on GABA action (*12*). The GABA-stimulated chloride flux appears to be passive rather than active, as it was affected little by temperature changes within the range 20° to 40°C.

Electrophysiological evidence (*13*) indicates that barbiturate receptors in the brain are coupled with GABA receptors and chloride channels, such that low concentrations of barbiturates augment the effects of GABA and high concentrations have direct GABA-mimetic effects. This complex relation between GABA and barbiturate receptors and chloride channels was preserved in our isolated microsacs, as 10 μ*M* pentobarbital augmented the effect of GABA but did not alter chloride flux alone (Table 1). Pentobarbital at 300 μ*M* stimulated uptake in the absence of GABA. These results are consistent with those of electrophysiological studies of intact cells (*14*).

Our findings indicate that isolated brain membranes retain functional coupling between the GABA, barbiturate, and picrotoxin receptors and the chloride channel and provide an opportunity to study the function of these receptors. This is likely to provide information that cannot be obtained from studies of receptor binding. For example, the concentrations of GABA required to stimulate chloride flux and to produce electrophysiological effects in intact tissue are 10 to 100 μ*M* (*14*). However, investigators have identified two populations of GABA receptors in the brain with affinities of about 0.02 and 0.1 μ*M* (*8*). This discrepancy between binding and function may exist because binding studies detect only a high-affinity, desensitized form of the receptor, while the functional, nondesensitized form can be detected in the flux assay (*15*). Desensitization of the GABA receptor on exposure to agonists would also explain the rapidity of the chloride influx (Fig. 1). An analogous situation has been observed for the nicotinic acetylcholine receptor (*15*).

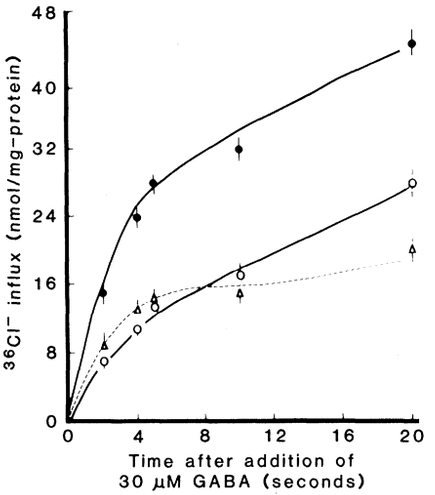

Fig. 1. Time course of resting flux and GABA-stimulated flux of chloride into brain membrane vesicles. Symbols: (●) chloride flux in the presence of GABA, (○) flux in the absence of GABA, and (△) GABA-stimulated (net) influx. Values are means ± standard errors (n = 4).

In addition to allowing rigorous kinetic studies, our in vitro assay of GABA receptor–chloride channel coupling should provide a new approach to questions about the role of GABA in epilepsy and anticonvulsant drug action (*16*), in ethanol dependence (*17*), and in the action of intoxicant-anesthetic drugs (*16*). Finally, the technique may facilitate the purification and functional reconstitution of the GABA receptor–effector complex.

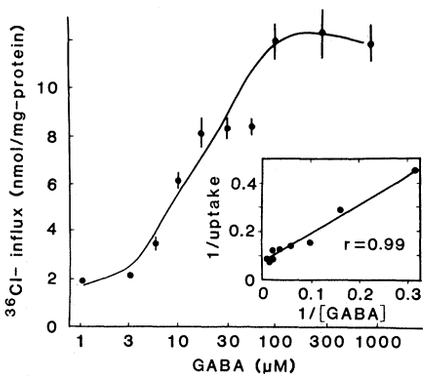

Fig. 2. Concentration dependence of GABA-stimulated flux of $^{36}Cl^-$ into brain membrane vesicles. Values are means ± standard errors (n = 8 to 12) (for some values, the standard error was smaller than the data point). (Inset) Reciprocal plot of GABA concentration versus net flux.

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Late Pleistocene Faunal Extinctions in Southern Patagonia

Abstract. *Major environmental changes recorded in pollen records from various sites in southern Patagonia and Tierra del Fuego are also reflected in pollen and cuticle data from dung of the late Pleistocene groundsloth. The most prominent change was the large-scale reduction of steppe environment about 10,000 years ago, which coincides with the latest dates for extinctions of many large grazers such as the giant groundsloth. Stress on food resources for all the large grazers may well have hastened their extinction. Hunting pressure by paleoindians may have been the final blow.*

Darwin's (*1*) gloomy description of the environment and aboriginal life-style in the southernmost part of South America provided a background for the much publicized find in 1895 of the nearly complete skin of a giant groundsloth (*Mylodon*) in a cave near the Ultima Esperanza inlet in southern Chile (*2, 3*). Because of its fresh appearance and presumed butcher marks, researchers assumed that the animal might still roam and be hunted by the Indians in some remote areas (*2*), and a British newspaper mounted an expedition to attempt to capture the animal (*4*). Since the discovery of the original Mylodon Cave, which yielded groundsloth skin, bones, and dung, many more such sites have been found in southern Patagonia (Fig. 1). Remains of other extinct taxa, such as native horse (*Onohippidium*), fox (*Dusicyon*), and extinct guanaco (*Lama gracilis*), were discovered as well as remains of extant fauna such as the modern guanaco (*Lama guanicoe*), tuco-tuco (*Ctenomys*), and various birds.

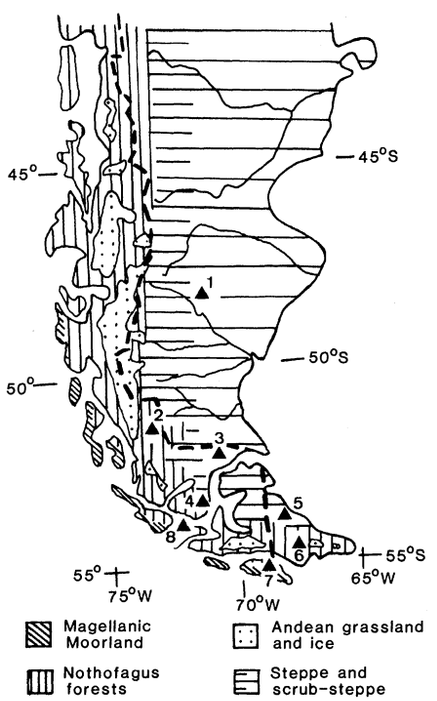

Fig. 1. Map of southernmost South America (Chile and Argentina) showing major vegetation types and site localities mentioned in text: 1, Los Toldos; 2, Mylodon Cave; 3, Fells Cave and Palli Aike shelter; 4, Puerto Hambre; 5, La Mision; 6, Lago Yehuin; 7, Isla Navarino; and 8, Isla Clarence.

Most extinct fauna were found at archeological excavations that were undertaken to determine the antiquity of man in South America. Data on cultural remains from cave and shelter sites, especially the meticulous excavation of Fells Cave (*5, 6*), proved the contemporaneity of early man and the extinct fauna. Radiocarbon dates on material associated with the oldest phase of human occupation in southern Patagonia are 12,600 ± 600 years before present (B.P.) in Los Toldos (*7*); 11,000 ± 170 and 10,720 ± 300 B.P. for Palli Aike and Fells Cave, respectively (*6, 8, 9*). Radiocarbon dates on the skin and dung of groundsloth range from 13,470 ± 180 to 10,575 ± 440 B.P. (Table 1), with most falling around 12,000 B.P.

Contemporaneity of extinct fauna and artifacts alone is insufficient to prove predation on extinct fauna (*10, 11*) or domestication by paleoindians (*12*). Furthermore, available data suggest that Patagonian paleoindians did not specialize in hunting the now extinct fauna (*13*), but primarily killed now extant taxa, preferentially guanaco, small mammals, and birds (*5, 8*). This implicates factors other than just predation pressure (or human overkill) (*14*) in the late Pleistocene faunal extinctions in southern Patagonia.

The foremost alternative proposal to human overkill is paleoenvironmental change. Past changes in vegetation in southern Patagonia and Tierra del Fuego, interpreted from dated pollen and plant macrofossils contained in sediment sections from lake, bog, and cave sites (*15–19*), strongly suggest a climatic cause of faunal extinction. Analysis of cuticle material and pollen in groundsloth dung reveal shifts in the animals' dietary habits that are similar to the vegetational changes.

Pollen and plant macrofossil records that reflect changes in vegetation and climate (Table 2) are known from (i) the steppe and desert-scrub environment of eastern Patagonia (Fells Cave), (ii) the mesic steppe environment in eastern Tierra del Fuego (La Mision) (*17, 18*), (iii) the steppe–*Nothofagus* forest transition in Patagonia (Mylodon Cave) (*20*) and Tierra del Fuego (Lago Yehuin) (*18*), and (iv) the *Nothofagus* rainforest environment in the western Magellan Strait and the Beagle Channel [Isla Clarence (*16, 18*) and Isla Navarino, Puerto Hambre (*19*)].

The steppe and desert-scrub record from Fells Cave (Table 2) shows only one environmental change during the last 11,000 years. This is a shift from mesic

Table 1. Radiocarbon dates on groundsloth dung from Mylodon Cave.

Sample	Date (B.P.)	
GX-6248	10,575 ± 400	
C-484*	10,832 ± 400	(*25*)
GX-6243*	10,880 ± 300	
GX-6246*	11,775 ± 480	
BM-1210	11,810 ± 299	(*8*)
GX-6247*	11,905 ± 335	
GX-6244*	12,020 ± 460	
A-2445	12,270 ± 350	
BM-1210B	12,308 ± 288	(*8*)
A-2447*	12,240 ± 150	
GX-6245*	12,285 ± 480	
BM-1209	12,496 ± 148	(*8*)
BM-1375	12,552 ± 128	(*8*)
A-2448*	12,870 ± 100	
A-2446*	13,470 ± 180	

*Analyzed for cuticle and pollen.