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20. Abstract

The γ -aminobutyric acid (GABA) receptor-chloride channel complex and its interaction with convulsant and neurotoxic drugs was studied in mammalian brain and invertebrates by a combination of biochemical and physiological techniques. The GABA receptor complex was assayed in vitro by radioactive ligand binding studies with membrane homogenates from rat brain, insect ganglia, and crayfish muscle. We described the first binding assay for GABA receptors in the latter two tissues, using [3 H]muscimol, and the first binding assay for GABA receptor-coupled chloride channels, using [3 H]picrotoxin and [35 S]t-butyl bicyclophosphorothionate (TBPS). The GABA receptor complex in mammalian brain was shown by binding assays to interact with a variety of convulsant and depressant drugs, including picrotoxin-like cage convulsants (very toxic to mammals), barbiturates, benzodiazepine, amidine steroid convulsants, and several pesticides (avermectins, cyclodiene and polychlorinated hydrocarbon insecticides, and pyrethroids). All of these agents were studied electrophysiologically on crayfish and insect preparations, revealing that picrotoxin and cage convulsants were weak GABA antagonists, cyclodienes and avermectins potently modulated GABA responses, and the others (pyrethroids, barbiturates, benzodiazepines, and steroids) were inactive. [3 H]Muscimol and [35 S]TBPS binding in insects was specific for GABA receptor complexes. Cyclodienes and barbiturates inhibited [35 S]TBPS binding, suggesting a possible chloride channel site of action. Pyrethroids were shown to act much more potently on neuronal sodium channels by both physiological and binding studies. In summary, the mammalian brain GABA receptor-chloride channel complex is the target of many known convulsant drugs and several important environmental pesticides. Conversely, some of the many drug categories known to affect GABA neurotransmission in mammals may provide new pesticides, because of the apparent similarities in the GABA receptors across the animal kingdom.



Neuroexcitatory Drug Receptors in
Mammals and Invertebrates

Richard W. Olsen and Thomas A. Miller

November 30, 1986

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A. Statement of the Problem Studied

Ten years ago, the applicants synthesized a radioactive analog of the potent plant convulsant, picrotoxin, and demonstrated specific binding to sites in membrane homogenates of crayfish muscle and mammalian brain that had properties of receptors for picrotoxin's action of blocking the postsynaptic response to the inhibitory neurotransmitter, γ -aminobutyric acid (GABA) (1,2). The picrotoxin receptor site appeared to involve the chloride channel associated with the GABA receptor, as opposed to the GABA recognition site itself. Picrotoxin binding was inhibited potently by the cage convulsants of Casida, some of the most toxic chemical agents ever described (3), and, less potently, by depressant barbiturates (4) and other drugs (5). One of the cage convulsants, [35 S]TBPS, was made radioactive by Squires et al. (6) and provides a better binding assay for the picrotoxin-convulsant site on the GABA receptor-chloride channel complex. This complex contains receptor sites for a variety of drugs, including benzodiazepines, and can be isolated as a single protein (7). [35 S]TBPS binding in mammalian brain has been reported to be sensitive to a variety of pesticides that can have mammalian toxicity, such as avermectins (8), pyrethroids (9), and cyclodiene/chlorinated hydrocarbon insecticides (10). The other drug receptors on the GABA receptor complex, namely, the binding sites for GABA, benzodiazepines, and barbiturates, also have neuroexcitatory/convulsant ligands that act on them, such as bicuculline (GABA site), beta-carbolines (benzodiazepine site), and convulsant barbiturates (7).

The project proposed in 1982 for 1983-86 was to analyze the interactions of drugs with the GABA receptor-chloride channel complex using a combination of biochemical and physiological methods, with these aims: 1. to determine whether potent convulsant drugs and/or environmental pesticides had important actions on the mammalian brain at the level of the GABA receptor complex (as major mechanism or major side-effects); 2. to determine whether pesticides in use may act to kill their target organisms through a GABA mechanism; and 3. whether new pesticides based on GABA mechanisms might be found in the mammalian pharmacology repertoire. Limited studies on non-GABA receptors were included where appropriate.

B. Summary of the Most Important Results

1. Interactions of convulsants including pesticides with the mammalian brain GABA receptor complex. a. We described (11) potent (nanomolar) inhibition of GABA receptor binding by the amidine steroid RU5135. The effects were consistent with an antagonist action at the GABA site, although slightly different than those of the standard bicuculline. b. We described (8) interactions of the macrocyclic lactone anthelmintic/insecticide avermectin B1a with GABA receptor binding, and proposed a 3-state allosteric model of the receptor to explain drug interactions. Others, e.g. (12), had described potent effects of avermectin on mammalian brain benzodiazepine receptors. c. We studied pyrethroid insecticides and found little if any effect on mammalian [35 S]TBPS binding (despite the report by Lawrence & Casida, 9).

2. Interactions of pesticides and "GABA-drugs" with voltage-regulated nerve membrane sodium channels, monitored by the binding of [3 H]batrachotoxin (in collaboration with Dr. Bud Brown, University of Alabama). We observed insecticidal pyrethroids of the cyano-type to show a stereospecific enhancement of [3 H]BTX binding (13, and manuscript in preparation), consistent with a sodium

channel mechanism. Other pyrethroids, and other "GABA" convulsants like picrotoxin, showed a "nonspecific" interaction with [³H]BTX binding at high concentrations. This "nonspecific" overlap of drugs acting principally on GABA chloride channels or voltage-regulated sodium channels may imply some similarities in nerve cell channel structure, e.g., protein homology, or may simply reflect the sensitivity of the cell membrane environment to any and all hydrophobic molecules.

3. Glutamate receptors: we utilized the Krogsgaard-Larsen analog of L-glutamic acid, AMPA, to try and assay invertebrate receptors, without success. We did some work on rat brain binding, as published (14).

4. GABA receptor complex in invertebrates: biochemistry.

a. [³H]Muscimol binding was demonstrated and characterized as receptor-specific in crayfish muscle by our lab in 1979 (15) and in insect ganglia in the current project (16). The latter publication describes the properties of these binding sites. This binding is now stable (at -70°C) and reproducible, using thoroughly washed membrane homogenates of housefly heads, locust ganglia, and crayfish muscle. No allosteric effects of other drugs were observed. We have also obtained preliminary results on binding of [³H]muscimol to frozen-thawed sections of ganglia from locust, honeybee, flies, and cockroaches (Schouest, Miller & Olsen, unpublished) using quantitative autoradiography (17).

b. [³⁵S]TBPS binding to housefly heads and crayfish muscle membranes (18, and manuscript in preparation) has been demonstrated. The properties can be summarized briefly:

[³⁵S]TBPS binding to housefly heads and crayfish muscle membranes was readily detectable by a filtration assay, using nonradioactive cage convulsants or picrotoxinin as the blank. The binding was saturable and specific, with K_d values of 50 nM and 30 nM and B_{max} values of 200-300 and 100 fmol/mg protein in housefly and crayfish, respectively. The following properties were determined:

- Binding was linear with protein (0.6 - 2.5 mg/ml in housefly, 0.1 - 1.0 mg/ml in crayfish).
- Binding was stimulated by, but not absolutely dependent upon chloride ions in both species.
- The temperature maximum was 22°C in housefly but 0 - 10°C in crayfish.
- The pH optimum was 7.3 - 7.5 in both species.
- The binding kinetics in housefly were reasonably fast, apparently homogeneous in association ($k_{on} = 8 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$) but heterogeneous in dissociation ($k_{off} = 0.28 \text{ min}^{-1}$ and 0.042 min^{-1}). Equilibrium was reached in 60 min. In crayfish, binding was very slow (150 min).

In both species, [³⁵S]TBPS binding was inhibited by cage convulsants, picrotoxin and analogues, barbiturates, with a specificity similar to that observed for crayfish picrotoxinin binding (1) and mammalian brain [³⁵S]TBPS binding (6).

In houseflies, [³⁵S]TBPS binding was most potently inhibited by the insecticides dieldrin, aldrin, and lindane, as previously reported by Cohen & Casida for housefly binding (10) and rat brain binding by Abalis et al. (19).

Although the invertebrate [35 S]TBPS binding is not identical in properties with its vertebrate counterpart, e.g., insensitivity to GABA modulation, the similarities are more compelling (see also refs. 20,21). Physiological studies showing that these drugs that inhibit [35 S]TBPS binding can also block GABA receptor-chloride channels (22,23) suggest that the binding sites are at least partly GABA-coupled. Thus a similar GABA receptor complex as that seen in vertebrate CNS (7) is likely to exist in the invertebrates. Numerous pharmacological agents are likely to have at least qualitatively similar actions across these wide species lines, not the least important of which are the chlorinated hydrocarbon insecticides, cage convulsants, barbiturates, and benzodiazepines.

c. [3 H]Flunitrazepam binding. Following the lead of our collaborator, George Lunt (21), we have observed the binding of [3 H]flunitrazepam to housefly heads and crayfish muscle. In locust the binding is enhanced by GABA (21) but not in our hands. This is being studied further. We have also initiated a collaboration with Dr. Mogens Nielsen in Denmark to measure the target sizes of these three invertebrate receptors by the method of radiation inactivation (27).

5. GABA Receptor Complex in Invertebrates: Physiology.

Dr. Alison E. Chalmers, working with Dr. Thomas Miller at the University of California, Riverside, studied GABAergic drugs and pesticides on several invertebrate preparations, including the crayfish stretch receptor neuron, crayfish walking leg muscle, and insect nerve and muscle (Heliothis virescens).

a. Pyrethroids. Dose-dependent, picrotoxin-sensitive GABA responses were measured in crayfish muscle and stretch receptor neuron ($EC_{50} = 25 \mu M$). Deltamethrin at 0.1-10 nM did not inhibit GABA in the absence or presence of 1 μM tetrodotoxin ((to prevent sodium channel effects), despite its marked activity to depolarize resting membrane potential, increase nerve firing frequency, and eventually block action potentials (24). Pyrethroids appear to affect sodium channels, not GABA chloride channels.

b. Avermectins. Avermectin B1a at 5-50 nM caused an increase in membrane conductance in crayfish muscle that was reversed by 50 μM picrotoxin. The response to higher concentrations (> 100 nM) was irreversible, and GABA responses were blocked. In the stretch receptor neuron, 10-100 nM avermectin caused a picrotoxin-sensitive conductance increase accompanied by decreased responsivity to GABA (25). Consistent with other work (26), avermectins appear to open GABA-regulated chloride channels and perhaps also non-GABA chloride channels. This can lead to an overall depressant action (e.g., flaccid paralysis of nematodes) or excitatory action (convulsions). In some cases, GABA synapses are blocked.

c. Convulsants. The amidine steroid RU5135 and cage convulsants like ethyl bicyclopophosphate (0.1 mM) did not inhibit GABA in crayfish stretch receptor neuron. Bicuculline was also inactive, but weakly active in crayfish muscle. More potent cage convulsants like TBPS ought to inhibit GABA, but we did not have these drugs to test.

d. Cyclodienes and halogenated hydrocarbon insecticides. Dieldrin, aldrin, heptachlor, and endrin appear to have some activity in blocking GABA in crayfish stretch receptor neurons (preliminary observations).

e. Benzodiazepines. Flurazepam (50 μ M, no effects below) depolarized crayfish nerve or muscle with a dose-dependent decrease in conductance, an effect unlike GABA or picrotoxin and unlikely to involve chloride channels. Flurazepam did not affect the response to GABA, except for a modest inhibition explained by the conductance increase. The effects of flurazepam were insensitive to both picrotoxin and tetrodotoxin.

f. Barbiturates. Pentobarbital, phenobarbital, and DMBB (0.1 - 1 mM) disrupted action potentials but did not alter GABA responses in crayfish nerve and muscle, indicating that, unlike mammalian neurons, invertebrate cells are more sensitive to other barbiturate actions like effects on voltage-regulated channels than to enhancement of GABA-mediated inhibition. Barbiturates may have had some activity to reverse picrotoxin's block of GABA responses.

In summary, the GABA receptor complex appears ever more important as the target of numerous drugs, pesticides, and neurotoxicants, including some convulsants highly toxic to mammals. In both vertebrates and invertebrates, desirable or (more often) harmful effects on the nervous system result from agents that modulate the action of the inhibitory neurotransmitter GABA at the postsynaptic receptor-chloride channel site. Numerous types of chemical structures can modulate GABA function in a positive or negative manner, providing potential for useful drugs (anesthetics, anxiolytics, sedative-hypnotics, muscle relaxants, stimulants, memory-enhancers, or pesticides) but also the danger of compounds highly toxic to non-target animals and man.

Further studies on drug interactions with the GABA receptor complex in mammals and invertebrates, using physiological, anatomical, and biochemical approaches are proposed in our renewal application to USARO (1986-89). Genetic approaches will also complement biochemical comparisons of the protein subunits and sequence between mammals and invertebrates for this important brain receptor protein.

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