20030210204

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SECURITY CLASSIFICATION OF THIS PAGE

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REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY AD-A220 255	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited.
	5. MONITORING ORGANIZATION REPORT NUMBER(S) ARO 24118.3-LS
6a. NAME OF PERFORMING ORGANIZATION Univ. of California, Los Angeles 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION U. S. Army Research Office
6c. ADDRESS (City, State, and ZIP Code) 405 Hilgard Avenue Los Angeles, CA 90024	7b. ADDRESS (City, State, and ZIP Code) P. O. Box 12211 Research Triangle Park, NC 27709-2211
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U. S. Army Research Office 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAAL03-87-K-0020
8c. ADDRESS (City, State, and ZIP Code) P. O. Box 12211 Research Triangle Park, NC 27709-2211	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. / PROJECT NO. / TASK NO. / WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification)
Neuroexcitatory Drug Receptors in Mammals and Invertebrates (unclassified) Contract number DAAL 03-87-K-0020

12. PERSONAL AUTHOR(S)
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13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 1/19/87 TO 1/18/90	1990 March 16	7

16. SUPPLEMENTARY NOTATION The view, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	γ-Aminobutyric acid receptors; Chloride channels; Convulsants; Neurotoxicants; Pesticides

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

See reverse side

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20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Richard W. Olsen	22b. TELEPHONE (Include Area Code) (213) 825-5093 22c. OFFICE SYMBOL

DD FORM 1473, 84 MAR

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19. *ABSTRACT*

Previous work by the principal investigators and others has shown that the major type of synaptic receptor for the major inhibitory neurotransmitter, the GABA-A receptor/chloride channel complex, is the target of numerous drugs and toxins. The GABA-A receptor function is directly potentiated by several categories of central nervous system depressants including benzodiazepines, barbiturates, steroid anesthetics, avermectin pesticides, and possibly ethanol. GABA-A receptor function is directly blocked by the GABA antagonist bicuculline, benzodiazepine inverse agonists, convulsant barbiturates, and a series of cyclic convulsant molecules like picrotoxin. These neuroexcitatory GABA blockers include pentylenetetrazol, chlorinated hydrocarbon insecticides like dieldrin and lindane, and the synthetic cage convulsants of Casida, such as t-butyl bicyclophosphorothionate (TBPS), one of the most toxic substances to mammals ever encountered.

We demonstrated that these convulsant drugs acted potently on GABA-A receptors in mammals and invertebrates, using a combination of electrophysiology and biochemistry. However, the differences in pharmacological profiles for GABAergic drugs between different animal species appeared important to define, such as how dangerous to non-target species are the currently used pesticides that act via the nervous system, and are there any potential new pesticides among the numerous GABAergic drugs active in mammals including man?

Using radioligand assays that we developed for sites on the GABA-A receptor complex, we were able to localize for the first time GABA-A receptors in the insect nervous system. This will be useful in understanding the physiology and toxicology of GABA. We also began studies on biochemical isolation of invertebrate GABA-A receptor proteins in hopes of determining their molecular structure.

A phylogenetic comparison of the GABA-A receptors was begun, and the subunit/gene composition in several animal species investigated. We identified the codfish as having a single polypeptide/gene for GABA-A receptors, as compared to 10-15 different subunits/genes in mammals. The codfish is thus closely related to the ancestral gene from which all the mammalian genes evolved. Structural and pharmacological comparisons of the different subtypes of GABA-A receptors in various species and in various regions of human brain are underway. This will help to define the specificity of the neurotransmitter and drug binding sites in the various receptors and should lead to the development of new useful drugs.

NEUROEXCITATORY DRUG RECEPTORS IN
MAMMALS AND INVERTEBRATES

Final Report

Richard W. Olsen and Thomas A. Miller

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Availability Codes	
Dist	Avail and/or Special
A-1	

March 16, 1990

U.S. Army Research Office

Contract/Grant Number: DAAL 03-87-K-0020

Institution: University of California, Los Angeles

Approved for public release;
distribution unlimited

The view, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

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A. Statement of the Problem Studied

As described in the final report (11/30/86) for our previous contract with the same title (DAAG 29-83-K-0156), this project deals with comparative pharmacology of the $GABA_A$ inhibitory synaptic transmission in vertebrates and invertebrates, using a combination of biochemical, physiological, and anatomical approaches. We (1,2) and others (3) have described the interaction of a remarkable array of drugs with the mammalian brain $GABA_A$ receptor-chloride channel complex. These include many highly toxic convulsants and several important environmental pesticides, drugs which effect their actions through blocking of GABA receptor functions. The GABA receptor-chloride channel system is important in wide-spread regions of the mammalian nervous system and also wide-spread throughout the animal kingdom. We developed radioligand binding assays for GABA receptors ([^{3}H]muscimol; 4,5,23), and the associated chloride channels ([^{3}H]picrotoxin, 6; and [^{35}S]TBPS, 7) in invertebrates, contributed significantly to the development of such assays in vertebrates, e.g., (1,8,9), and studied numerous drugs for effects on these binding assays and on GABA receptor function assayed by tracer radiolabeled $^{36}Cl^-$ flux in mammalian brain slices (10,11) and by electrophysiological techniques in invertebrate tissues (12,13).

The objectives for the current contract period 1986-89 were: [A] to compare the GABA receptor proteins at the molecular level across animal species with the comparative pharmacological profile of drugs active on these species; and [B] to use the binding assays we had developed for the invertebrate GABA receptor chloride channel complex to: [i] describe the anatomical distribution of receptors in the insect nervous system for the first time, and [ii] to isolate the receptor proteins from invertebrate species for the first time, for comparative structure-function studies including molecular cloning.

B. Summary of the Most Important Results

1. Localization of GABA Receptors in Insect CNS

Conventional film autoradiography was used at the light microscopic level for the localization and quantification of γ-aminobutyric acid (GABA) receptors in the locust brain (*Schistocerca americana*). Localization of the receptor sites was achieved via binding with the receptor-ligand probe [^{3}H]muscimol (4). Frozen sections were cut and subsequently incubated either in 40 nM [^{3}H]muscimol or by coincubating sections with [^{3}H]muscimol and one of the following: GABA (50 μM), a receptor specific agonist (muscimol, 1 μM, or isoguvacine, 1 μM), an uptake inhibitor (nipecotic acid, 50 μM), or a noncompetitive channel modulator (avermectin B1a, 1 μM, or aldrin, 50 μM).

Through computer image enhancement and densitometric analysis of the optical density of [^{3}H]muscimol binding sites, the interaction of the above compounds with the putative GABA receptor was determined for various anatomical regions of the locust brain. By comparing the differently treated, but adjacent sections, GABA receptor distribution was quantitated and mapped (14). For this analysis, we employed an image analyzer purchased by Drs. Olsen and de Vellis under support of USARO.

Receptor sites were found distributed in the antennal lobes, central body, alpha-lobe and beta-lobe of the corpus pedunculatum, protocerebral bridge, and calyx as well as the optic lobe regions.

The GABA system is an important component of the insect central nervous system. Previous work has shown that both GABA concentrations and glutamic acid decarboxylase (GAD) specific activity exceeded by 10-fold that found in comparable vertebrate brain tissue (15).

The invertebrate tissues afford more accessible GABA sites and should be preferred for both neurochemical and receptor binding studies. Neuropharmacology combined with radiolabeled binding studies using computer-aided autoradiography will provide strong and direct evidence in substantiating that the GABA receptor in insects is a site of drug action. Through image enhancement and analysis, computer-acquired images of autoradiograms can now be quantified using standard binding procedures. Competitive binding studies can be performed on adjacent tissue sections *in situ* and receptors localized to specific anatomical regions (5).

The data indicate specific binding to GABA receptor sites. Serial sections are being analyzed by computer (16) to allow 3-dimensional reconstruction of the receptor distribution in the entire nervous system of locust and other insects. Work in progress includes similar autoradiography on [^{35}S]TBPS binding to the GABA receptor-associated chloride channels (7) in frozen unfixed tissue sections of insect ganglia.

2. Invertebrate GABA Receptor Neurochemistry

An undergraduate student from Bath University, United Kingdom, Mr. Damian Cotton, working under our collaborator Dr. George Lunt, came to Dr. Olsen's laboratory at UCLA (April-September, 1988) to work on crayfish and insect GABA receptors. Mr. Cotton found that GABA receptors are present in high density in abdominal muscles of crayfish (living outdoors) only during warm summer months. He successfully managed to solubilize with mild detergent the crayfish muscle GABA receptor complex, preparatory to its biochemical purification. We have not had anyone continuing the project recently, but plan to return to it soon. However, we may actually obtain the invertebrate receptor protein sequences more rapidly by cloning, using the vertebrate cDNA (17-19) as probes.

In addition, Mr. Cotton learned our housefly head membrane preparations and assays of [^{3}H]muscimol and [^{35}S]TBPS binding to teach them to the Lunt laboratory in Bath, preparatory to measuring their molecular weights (target size) by irradiation inactivation, in collaboration with Dr. Mogens Nielsen in Roskilde, Denmark. Unfortunately, ill health prevented Mr. Cotton from completing this project, but we (Olsen, Lunt, Nielsen) plan to continue the work soon.

Comparative biochemistry of GABA receptor protein: Lynn Deng, graduate student in Dr. Olsen's laboratory at UCLA, has been working on the purification, photoaffinity labeling, and subunit composition of the GABA/benzodiazepine receptor from codfish. In collaboration with Dr. Mogens Nielsen of Denmark, we found codfish brain [^{3}H]flunitrazepam binding protein to have a molecular weight of 58 kiloDaltons on SDS-PAGE (20). Then the protein was purified by benzodiazepine affinity chromatography as we described for rat receptor (21): a single stained band was observed on SDS-PAGE at 58 kD. Photoaffinity labeling with both [^{3}H]flunitrazepam and [^{3}H]muscimol resulted in a single radioactive peptide band corresponding to the single stained band at 58 kD (22). The purified codfish receptor was subjected to 2-dimensional gel electrophoresis and showed only 1 spot. This preparation gave a specific activity of about 1000 pmol of [^{3}H]muscimol and 500 pmol of [^{3}H]fl·nitrazepam binding per mg protein. The latter was enhanced by GABA in the assays and showed central benzodiazepine receptor specificity. Thus, the codfish receptor appears to consist of a single subunit, in contrast to mammalian species which have two subunits of different size but homologous sequence. The mammalian subunits of the GABA receptor may have evolved from a common ancestral gene, while the codfish protein still is coded for by the single ancestral gene (Deng, Nielsen and Olsen, manuscript in prepatation). It will be of interest also to examine the subunit composition of invertebrate GABA receptors.

Current studies include attempts to measure the native molecular weight of the codfish receptor, and the question of whether barbiturate, picrotoxin, and steroid receptor sites are present on the complex, as they are in mammals. We will also attempt to isolate fresh mRNA from codfish brain for the construction of a cDNA library and cloning of the gene(s) for codfish GABA receptor using mammalian cDNA probes and the polymerase chain reaction. In addition, we have prepared receptor protein (~1 mg) from 200 g of codfish brain for two approaches to molecular structure: [i] sequencing of ligand binding active sites using proteolytic fragmentation of photoaffinity labeled subunits as we have done on mammalian protein; [ii] attempt to produce a water-soluble large fragment containing the GABA binding site, presumably in the N-terminal 200 residues, using [^{3}H]muscimol photolabeled protein as starting material. Such a fragment might be suitable for crystallization and X-ray structural work, especially since the codfish protein, unlike the mammalian protein, appears to be a <u>homo</u>-oligomer. Note that <u>no</u> neurotransmitter receptor even in part has been crystallized so this active site fragment is a potential solution both to the overall structure and to accurate ligand binding site information.

<u>References/Bibliography</u>

1. Olsen, R.W. (1982) Drug interactions at the GABA receptor-ionophore complex. *Annu. Rev. Pharmacol. Toxicol.* <u>22</u>, 245-277.

2. Miller, T.A. and A.E. Chalmers (1987) Actions of GABA agonists and antagonists on invertebrate nerves and muscle. In: <u>Sites of Action for Neurotoxic Pesticides</u>, B.M. Hollingworth and M.B. Green, Editors, ACS Symposium Series, Washington, pp. 1-13.

3. Biggio, G. and E. Costa, Editors (1988) <u>Chloride Channels and Their Modulation by Neurotransmitters and Drugs</u>. *Adv. Biochem. Psychopharmacol.* <u>45</u>, 384 pp.

4. Lunt, G.G., T. Robinson, T. Miller, W.P. Knowles and R.W. Olsen (1985) The identification of GABA receptor binding sites in insect ganglia. *Neurochem. Int.* <u>7</u>, 751-754.

5. Schouest, L.P., Jr., T.A. Miller and R.W. Olsen (1988) Quantitative autoradiography of GABA receptors in locust (Schistocerca americana). *Brain Pestic. Sci.* 24, 299-309.

6. Olsen, R.W., M.K. Ticku and T.A. Miller (1978) Dihydropicrotoxinin binding to crayfish muscle sites possibly related to gamma-aminobutyric acid receptor/ionophores. *Mol. Pharmacol.* 14, 381-390.

7. Olsen, R.W., O. Szamraj and T. Miller (1989) [^{35}S]t-Butyl bicyclophosphorothionate (TBPS) binding sites in invertebrate tissues. *J. Neurochem.* 52, 1311-1318.

8. Olsen, R.W. and J.C. Venter, Editors (1986) Benzodiazepine/GABA Receptors and Chloride Channels: Structural and Functional Properties. Receptor Biochemistry and Methodology, Volume 5, Alan R. Liss, New York.

9. Williams, M. and R.W. Olsen (1988) Benzodiazepine and tissue function. In: Receptor Pharmacology and Function, M. Williams, R.A. Glennon and P.B. Timmermans, Editors, Marcel Dekker, New York, pp. 385-413.

10. Wong, R.H.F., L.M.F. Leeb-Lundberg, V.I. Teichberg and R.W. Olsen (1984) γ-Aminobutyric acid activation of $^{36}Cl^-$ flux in rat hippocampal slices and its potentiation by barbiturates. *Brain Res.* 303, 267-275.

11. Yang, J. and R.W. Olsen (1987) γ-Aminobutyric acid receptor regulated $^{36}Cl^-$ flux in mouse cortical slices. *J. Pharmacol. Exp. Ther.* 241, 677-685.

12. Chalmers, A.E., T.A. Miller and R.W. Olsen (1986) The actions of avermectin on crayfish nerve and muscle. *Eur. J. Pharmacol.* 129, 371-374.

13. Chalmers, A.E., T.A. Miller and R.W. Olsen (1987) Deltamethrin: A neurophysiological study of the sites of action. *Pesticide Biochem. Physiol.* 27, 36-41.

14. Wamsley, J.K., D.R. Gehlert and R.W. Olsen (1986) The benzodiazepine/barbiturate-sensitive convulsant/GABA receptor-chloride ionophore complex: Autoradiographic localization of individual components. In: Benzodiazepine/GABA Receptors, R.W. Olsen and J.C. Venter, Editors, Liss, New York, pp. 299-314.

15. Robinson, T.N. and R.W. Olsen (1988) GABA. In: Comparative Invertebrate Neurochemistry, G.G. Lunt and R.W. Olsen, Editors, Croom Helm, London, pp. 90-123.

16. Toga, A.W., E.M. Santori and M. Samaie (1986) Regional distribution of flunitrazepam binding constants: Visualizing Kd and Bmax by digital image analysis. *J. Neuroscience* 6, 2747-2756.

17. Levitan, E.S., P.R. Schofield, D.R. Burt, L.M. Rhee, W. Wisden, M. Köhler, N. Fujita, H.R. Rodriguez, A. Stephenson, M.G. Darlison, E.A. Barnard and P.H. Seeburg (1988) Structural and functional basis for $GABA_A$ receptor heterogeneity. *Nature* 335, 76-79.

18. Pritchett, D.B., H. Sontheimer, B.D. Shivers, S. Ymer, H. Kettenmann, P.R. Schofield and P.H. Seeburg (1989) Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature* 338, 582-585.

19. Khrestchatisky, M., A.J. MacLennan, M.-Y. Chiang, W. Xu, M.B. Jackson, N. Brecha, C. Sternini, R.W. Olsen and A.J. Tobin (1989) A novel alpha-subunit in rat brain $GABA_A$ receptors. *Neuron* 3, 745-753.

20. Deng, L., R.W. Olsen and M. Nielsen (1987) Biochemical properties of the benzodiazepine-GABA receptor protein from codfish brain. *Abstr. Soc. Neurosci.* 13, 965.

21. Stauber, G.B., R.W. Ransom, A.I. Dilber and R.W. Olsen (1987) The γ-Aminobutyric acid-benzodiazepine receptor protein from rat brain: Large-scale purification and preparation of antibodies. *Eur. J. Biochem.* 167, 125-133.

22. Deng, L., R.W. Olsen and M. Nielsen (1988) [^{3}H]Muscimol and [^{3}H]flunitrazepam photoaffinity label the same molecular weight band in codfish brain GABA/BZ receptor. *Abstr. Soc. Neurosci.* 14, 344.

23. Meiners, B.M., P. Kehoe, D.M. Shaner and R.W. Olsen (1979) γ-Aminobutyric acid receptor binding and uptake in membrane fractions of crayfish muscle. *J. Neurochem.* 32, 979-990.

C. Publications

Miller, T.A. and Chalmers, A.E. Actions of GABA agonists and antagonists on invertebrate nerves and muscle. In: Sites of Action for Neurotoxic Pesticides, B.M. Hollingworth and M.B. Green, Editors, ACS Symposium Series, Washington, pp. 1-13 (1987).

Chalmers, A.E., Miller, T.A., and Olsen, R.W. Deltamethrin: a Neurophysiological Study of the Sites of Action. *Pesticide Biochem. Physiol.*, 27, 36-41 (1987).

Lunt, G.G. and Olsen, R.W. (eds.) Comparative Invertebrate Neurochemistry. Croom Helm, London (1988).

Robinson, T.N. and Olsen, R.W. GABA, in Comparative Invertebrate Neurochemistry eds. G.G. Lunt & R.W. Olsen, Croom Helm, London, pp. 90-123 (1988).

Brown, G.B., Gaupp, J.E. and Olsen, R.W. Pyrethroid Insecticides: Stereospecific, Allosteric Interaction with the Batrachotoxin-A Benzoate Binding Site of Mammalian Voltage-Sensitive Sodium Channels. *Mol. Pharmacol.* 34, 54-59 (1988).

Williams, M. and Olsen, R.W. Benzodiazepine Receptors and Tissue Function, in Receptor Pharmacology and Function, eds. M. Williams, R.A. Glennon and P.B. Timmermans, Marcel Dekker, New York, pp. 385-413 (1988).

Schouest, L.P., Jr., Miller, T.A. and Olsen, R.W. Quantitative Autoradiography of GABA Receptors in Locust (Schistocerca americana) *Brain. Pestic. Sci.* 24, 299-309 (1988).

Olsen, R.W., Szamraj, O. and Miller, T. [^{35}S]t-Butyl Bicyclophosphorothionate (TBPS) Binding Sites in Invertebrate Tissues. *J. Neurochem.* 52, 1311-1318 (1989).

Abstracts

Deng, L., Olsen, R.W. and Nielsen, M. Biochemical properties of the benzodiazepine-GABA receptor protein from codfish brain. *Abstr. Soc. Neurosci.* 13, 965 (1987) #266.3.

Deng, L., Olsen, R.W. and Nielsen, M. Biochemical properties of the benzodiazepine-GABA receptor protein from codfish brain. *Abstr. Soc. Neurosci.* 13, 965 (1987), #266.3.

Schouest, L., Miller, T.A. and Olsen, R.W. Quantitative three-dimensional morphological analysis of locust CNS GABA receptors. *Abstr. Neurotox. '88.*

Schouest, L., Miller, T.A. and Olsen, R.W. Autoradiography of locust CNS GABA receptor. *Abstr. Soc. Neurosci.* 15, 1155 (1989) #459.17.

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