FY 2000 ANNUAL REPORT

OF

INTRAMURAL RESEARCH PROGRAM ACTIVITIES

OF THE

NATIONAL INSTITUTE ON ALCOHOL ABUSE AND ALCOHOLISM

US DEPARTMENT OF HEALTH AND HUMAN SERVICES

NATIONAL INSTITUTES OF HEALTH

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FY 2000 REPORTING COMPONENTS

DIVISION OF INTRAMURAL CLINICAL & BIOLOGICAL RESEARCH

LABORATORY OF CLINICAL STUDIES

Unit of Clinical & Biochemical Pharmacology - Paolo B. DePetrillo, M.D., Chief
Section of Brain Electrophysiology & Imaging - Daniel W. Hommer, M.D., Chief
Section of Clinical Science - David T. George, M.D., Acting Chief
Section of Neurochemistry & Neuroendocrinology – Daniel W. Hommer, M.D., Acting Chief

LABORATORY OF MEMBRANE BIOCHEMISTRY & BIOPHYSICS

Office of the Chief - Norman Salem, Jr., Ph.D., Chief
Unit of Metabolic Control - Richard Veech, M.D., Ph.D., Chief
Unit of Molecular Biology - Byoung-Joon Song, Ph.D., Chief
Section of Fluorescence Studies - Burton J. Litman, Ph.D., Chief
Section of Mass Spectrometry - Hee-Yong Kim, Ph.D., Chief
Section of Nuclear Magnetic Resonance - Klaus Gawrisch, Ph.D., Acting Chief

LABORATORY OF MOLECULAR & CELLULAR NEUROBIOLOGY

Section on Molecular Neuroscience - Forrest F. Weight, M.D., Acting Chief
Section on Pharmacology - Forrest F. Weight, M.D., Acting Chief
Unit on Cellular Neuropharmacology - Robert Peoples, Ph.D., Chief
Section on Physiology - Forrest F. Weight, M.D., Chief

LABORATORY OF NEUROGENETICS

Section of Human Neurogenetics - David Goldman, M.D., Chief
Section of Molecular Neurobiology - David Goldman, M.D., Acting Chief
Section of Population Genetics & Linkage - Jeffrey Long, Ph.D., Chief

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LABORATORY OF MEMBRANE BIOCHEMISTRY & BIOPHYSICS

2-DG	2-deoxyglucose
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine or serotonin
AA	arachidonic acid
AAP	acetaminophen
ADH	alcohol dehydrogenase
ADHD	Attention Deficit Hyperactivity Disorder
ALDH2	aldehyde dehydrogenase
ASPD	antisocial personality disorder
ATP	adenosine 5'-triphosphate
AWS	alcohol withdrawal syndrome
BAC	blood alcohol concentration
BOLD	blood oxygen level dependent
CNS	central nervous system
COMT	catechol-O-methyltransferase
CRH	corticotropin-releasing hormone
CSF	cerebrospinal fluid
DHA	docosahexaenoic acid
DPH	diphenylhexatriene
DRG	dorsal root ganglion
ECF	Executive cognitive functions
EEG	electroencephalogram
EFA	essential fatty acids
Emax	maximal response
ENT	entorhinal cortex
EPA	eicosapentaenoic acid
ERK	extracellular-signal regulated protein kinase
ERP	event-related brain electrical potentials
Et	ethanol
fMRI	functional magnetic resonance imaging
FRET	Fluorescence Energy Transfer
FUR	Furosemide
GABA	gamma-aminobutyric acid
GABAA	gamma-aminobutyric acid type A
Gcc	glucocorticoid
GC/MS	gas chromatography/mass spectrometry
HIPP	hippocampal
HNE	4-hydroxynonenal
HPA	hypothalamic-pituitary-adrenal
HRV	heart rate variability
HVA	homovanillic acid
IBI	inter-beat interval
ICV	intracranial volume
IED	Intermittent Explosive Disorder
IV	intravenous
JNK	c-Jun N-terminal protein kinase
LC	liquid crystalline
LD	linkage disequilibrium
LDH	lactate dehydrogenase
LHPA	Limbic-Hypothalamic-Pituitary-Adrenal
LS	long-sleep
LVA	low voltage alpha
MAP	mitogen activated protein
Mcc	mineralocorticoid
MDA	malondialdehyde
MESSI	MultiplE Simultaneous Stable Isotopes
MHPG	3-methoxy-4-hydroxyphenyl glycol
MII	meta-rhodopsin II
MR	mother-reared
MRI	magnetic resonance imaging
MTP	methyltryptophan
n-3	omega-3
nACh	nicotinic acetylcholine
NER	nucleotide excision repair
NIDDM	noninsulin-dependent diabetes mellitus
NMDA	N-methyl-D-aspartate
NMR	nuclear magnetic resonance
OCD	obsessive-compulsive disorder
PC	phosphatidylcholines
PDE	phosphodiesterase
PET	positron emission tomography
PKA	protein kinase A
PKC	protein kinase C
PL	phospholipids
PNS	parasympathetic nervous system
PR	peer-reared
PS	phosphatidylserine
PTSD	post traumatic stress disorder
RP	Retinitis Pigmentosa
SAD	seasonal affective disorder
sMRI	structural magnetic resonance imaging
SNPs	single nucleotide polymorphisms
SPR	surrogate-peer-reared
SS	short-sleep
SSCP	single-strand conformational polymorphism
SVZa	anterior subventricular zone
TBW	total body water
TCA	tricarboxcyclic acid
TDO2	tryptophan 2,3-dioxygenase
TDT	transmission/disequilibrium test
TEA	tetraethylammonium
TPH	tryptophan hydroxylase
TPQ	Tridimensional Personality Questionnaire
Vd	volume of total body water
XP	xeroderma pigmentosum

FY 2000 ANNUAL REPORT SUMMARIES

(1 OCTOBER 1999 - 30 SEPTEMBER 2000)

LABORATORY OF CLINICAL STUDIES

DANIEL W. HOMMER, M.D., ACTING CHIEF

DIVISION OF
INTRAMURAL CLINICAL & BIOLOGICAL RESEARCH
NATIONAL INSTITUTE ON ALCOHOL ABUSE & ALCOHOLISM
NATIONAL INSTITUTES OF HEALTH

SYNOPSIS
LABORATORY OF CLINICAL STUDIES
1 OCTOBER 1999 - 30 SEPTEMBER 2000

INTRODUCTION

During FY 2000, the 11-bed ward of the laboratory continued to be one of the busiest inpatient services in the Clinical Center. Long-term, projects were expanded in the area of brain imaging, to develop new serotonin receptor ligands, to use functional MRI to explore the functional neuroanatomy of reward and punishment and to develop techniques for automated measurement of neuroanatomically defined brain structures. The unique brain imaging resources and expertise at the NIH Clinical Center make these efforts particularly important and rewarding.

SECTION OF BRAIN ELECTROPHYSIOLOGY AND IMAGING

Investigators in the Section of Brain Electrophysiology and Imaging conduct sophisticated electrophysiological, neuropsychological and brain imaging studies on alcoholics, individuals at risk and carefully matched controls. The lack of an acceptable method for determining statistical significance of differences in brain images derived from functional magnetic resonance imaging (fMRI) or positron emission tomography (PET) studies has been a major problem. Over the past several years, we have made significant progress in applying rigorous statistical methods, based on a Gaussian random field model, to the analysis of image data. In addition, other methods have been developed for determining the statistical significance of differences observed in arbitrary regions of group average images. These include methods of spatial frequency decomposition as well as wavelet analysis. We have compared the relative merits and shortcomings of these methods with Gaussian random field based techniques. Gaussian random field based techniques are the most conservative statistically and give the most precise spatial localization; they are particularly suited to analysis of PET data. We have developed an approach to extend Gaussian random field based techniques to the analysis of fMRI time series data. This technique is particularly useful in fMRI studies of drug effects or emotions where it is difficult to collect temporally independent scans of functional activity.

Development of advanced image analysis and co registration for PET, CT, structural and fMRI has continued. Methods have been developed to achieve 3-D registration of PET images with structural MRI, as have techniques for the automated detection of midsagittal lines or planes. Using these techniques, we can now identify structures as small as the head of the caudate nucleus or nucleus accumbens on co registered PET and MRI scans.

Using these analysis techniques, we have been able to demonstrate significant differences in glucose metabolism in the brains of normal controls and individuals that have developed anti-social behavior following a serious closed head injury. Analysis was performed using both absolute-pixel glucose uptake values and means-adjusted pixel values obtained by subtracting the overall mean brain glucose uptake from each pixel glucose uptake. Both mean-adjusted and absolute glucose uptake produced similar results. Head injured subjects had significantly lower glucose uptake in the posterior orbital cortex bilaterally, in the right caudate, in the right dorsal thalamus and in mesial superior frontal cortex. Glucose uptake in the caudate, thalamus and superior frontal cortex was significantly correlated with ratings of aggressive behavior developed after the injury. In addition, we have co registered these PET images with subjects' structural MRI (sMRI) to show that the regional reduction in glucose uptake corresponds nearly perfectly to the regions of tissue loss in the caudate and thalamus.

Over the past two years we have developed fMRI techniques to measure changes in cerebral blood flow/blood volume that occur during operant tasks which are under either appetive or aversive control. In healthy normal subjects, we have found a differential effect of reward or punishment in the human brain. Both reward and punishment increases activity in the mesial frontal cortex (BA 24 and 32), the dorsal thalamus and the medial caudate nucleus. However, only expectation of reward activates the nucleus accumbens. The magnitude of activation appears to be a function of the magnitude of the expected reward or punishment. These studies are being extended to recovered alcoholics in order to determine if alcoholics show a differential sensitivity to reward and punishment compared to non-alcoholic controls.

Fully automated segmentation techniques have been developed for use on T-1 weighted MRI. These techniques allow for the automated labeling of cerebrospinal fluid (CSF) and white and gray matter regions in sMRI data. We have applied these techniques and found evidence for reduction in the volume of the hippocampus among alcoholics. In addition, we have found both gender and laterality differences in this structure, among both controls and alcoholics. We have also demonstrated significantly greater reductions in brain gray matter volume among alcoholic women compared to alcoholic men. Techniques to expand this type of analysis to other brain regions are now being developed. An automated method of measuring the volume of the medial prefrontal cortex is now being tested for reliability and validity. Also measure of the cerebellum in alcoholics and controls is nearly completed in over 150 subjects.

UNIT ON CLINICAL AND BIOCHEMICAL PHARMACOLOGY

We are investigating the mechanisms of alcohol-related neurotoxicity, as well as the effects of alcohol on the autonomic nervous system. The activity of a class of calcium-activated proteases called calpains, which regulate neuronal cellular signaling by degrading proteins, is strongly altered by alcohol exposure at concentrations that are clinically relevant. The activity of these proteases has been implicated in mediating the toxic effects of agents that activate NMDA receptors, which are thought to be overactive during alcohol withdrawal in mammals. We have developed cytotoxicity assays that are being used to screen compounds that may have neuroprotective effects after a variety of cellular insults. We found that a series of agents that interfere with calcium activation of these proteases protects neural cells in culture from l-glutamate related cell death, and have shown that l-glutamate is acting through NMDA, and not AMPA receptors. We have determined that calpain activation is a necessary step in the activation of a series of enzymes called caspases, which participate in the cycle of programmed cell death called apoptosis.

We have shown that calpains are regulated by intracellular glutathione, which functions as an anti-oxidant, and is known to decrease in organ systems of animals exposed to alcohol. Taken together, these studies allow us to test compounds of interest shown to be neuroprotective in cell culture models for their ability to protect against excitotoxic and alcohol-related neuronal loss in animal models.

In human studies, we have shown that alcohol has a long-term effect on cardiac rhythm regulation that does not normalize for up to 4 weeks after the onset of alcohol abstinence. Working with primates, in a collaborative study, we have shown that one exposure to doses of alcohol reaching maximum blood concentrations of 200 mg% interacts with stress to produce changes in CSF 5-HIAA during subsequent administrations of the drug, and possibly as long as 24 weeks later. These studies suggest that alcohol may be playing a role in cardiac rhythm dysregulation, and that the effects may be relatively long lasting. We are examining if a drug known to antagonize NMDA receptors may have similar effects to determine which neuroreceptors are responsible for the alcohol-induced alteration in cardiac rhythms.

This laboratory also serves a supportive role as an analytical laboratory within LCS. We are currently running assays for CSF and plasma monoamines and their metabolites, as well as l-tryptophan, as part of collaborative work. We are in the process of developing a fluorescent amino acid assay (AA) to quantify CSF AA for several of our collaborators.

SECTION OF CLINICAL SCIENCE

The major research objectives in the Section of Clinical Science are to utilize pharmacological challenge paradigms, PET studies, and CSF metabolite determinations to understand the etiology of alcoholism and violent behavior; to describe and understand the interaction between alcohol and acts of domestic violence; to characterize the concept of "losing control" as it relates to acts of domestic violence and alcoholic drinking; to introduce new pharmacological interventions to treat perpetrators of domestic violence; to investigate the effect of glucoprivic stress on hypothalamic-pituitary-adrenal (HPA) axis in patients with alcoholism; and, to explore the effect of protracted alcohol withdrawal on the HPA axis function.

The majority of our research this year has been directed toward understanding the psychological and biological features of individuals who initiate acts of domestic violence. Domestic violence is a major problem in the United States; it is estimated that approximately 30% of all women will be assaulted by their spouse/significant other at some time in their lives. Of special interest, 70% of the perpetrators

have an alcohol problem. To date, the majority of studies have focused on psychosocial issues; minimal emphasis has been given to biological factors that could contribute to the violence.

We have studied approximately 50 perpetrators of domestic violence and 100 non-violent controls, both with and without alcoholism. Based on our clinical evaluations of perpetrators and the results from our lactate infusions, we formulated the hypothesis that some perpetrators have an abnormality in their ability to inhibit fear-induced aggression. Using the animal literature we have developed a model outlining the neuropathways that potentially could be involved in the mediation of fear-induced aggression. Our recently analyzed PET data shows that, compared to non-violent controls, perpetrators have significantly decreased glucose metabolism in the hypothalamus. These results are consistent with our proposed model of the functional neuroanatomy of fear-induced aggression.

Approximately 50% of our perpetrators were exposed to violence growing-up. This led to the hypothesis that exposure to violence could alter the central noradrenergic function of some perpetrators. Previous studies have shown that subjects with post traumatic stress disorder (PTSD) have disturbances in norepinephrine metabolism that predispose them to over-react to stimuli which they perceived as threatening. To explore this possibility, we employed the alpha-2 adrenergic antagonist, yohimbine, to study adrenergic receptor function in perpetrators and non-violent controls. The study has been completed and the results are being analyzed.

Previous studies on aggression have shown a negative correlation between impulsive acts of aggression and the CSF metabolite of serotonin (5HT), 5-hydroxyindoleacetic acid (5HIAA). To test the hypothesis, i.e., 5HT has a role in the modulation of fear-induced aggression, we measured CSF 5HIAA in perpetrators and non-violent controls. Pair-wise comparisons revealed perpetrators without alcoholism had lower concentrations of CSF 5HIAA than perpetrators with alcoholism, non-violent alcoholics and healthy comparison subjects. These results suggest that 5HT may have a role in modulating pathways that mediate fear-induced aggression. The fact that perpetrators with alcoholism had higher 5HIAA concentrations than perpetrators without alcoholism suggests that there may be a prolonged effect of alcohol withdrawal. This possibility is supported by studies showing that CSF 5HIAA can remain elevated for weeks following alcohol cessation. We also examined testosterone in alcoholic and non-alcoholic perpetrators. Testosterone was significantly higher in the alcoholic perpetrators than in the non-alcoholic perpetrators and normal controls. In addition, testosterone was significantly lower in non-alcoholic perpetrators compared to the normal controls.

SECTION ON NEUROCHEMISTRY AND NEUROENDOCRINOLOGY

The Section on Neurochemistry and Neuroendocrinology has continued research on biochemical concomitants of violent behavior in alcoholics and on variables associated with increased vulnerability of developing alcoholism-related behavior. The major focus of this research has remained the serotonergic system. Interesting insights have been gained into regulation of serotonergic neuronal networks, developmental and genetic influences on serotonin functions and serotonergic regulation of metabolism and excessive alcohol consumption.

(INTENTIONAL BLANK)

Burgdorf J, Knutson B, Panksepp J: Anticipation of rewarding electrical brain stimulation evokes ultrasonic vocalization in rats. Behavioral Neuroscience 2000;114(2):320-7

Castellanos FX, Marvasti FF, Ducharme JL, Walter JM, Israel ME, Krain A, Pavlovsky C, Hommer DW: Executive function oculomotor tasks in girls with ADHD. Journal of the American Academy of Child and Adolescent Psychiatry 2000;39(5):644-50

DePetrillo PB, Bennett AJ, Speers D, Suomi SJ, Shoaf SE, Karimullah K, Higley JD: Ondansetron modulates pharmacodynamic effects of ketamine on electrocardiographic signals in rhesus monkeys. European Journal of Pharmacology 2000;391(1-2):113-9

DePetrillo PB, Yang QF, Rackoff J, SanMiguel A, Karimullah K: Surface fractal computation and its application to immunofluorescent histochemical studies of calpain and calpastatin in PC12 cells. Journal Neuroscience Methods 2000;103(2):191-7

Fahlke C, Lorenz JG, Long J, Champoux M, Suomi SJ, Higley JD: Rearing experiences and stress-induced plasma cortisol as early risk factors for excessive alcohol consumption in nonhuman primates. Alcoholism-Clinical & Experimental Research 2000;24(5):644-50

George DT, Hibbeln JR, Ragan PW, Umhau JC, Phillips MJ, Doty L, Hommer D, Rawlings RR: Lactate-induced rage and panic in a select group of subjects who perpetrate acts of domestic violence. Biological Psychiatry 2000;47(9):804-12

Habash T, Eskay RL, Kuenzel WJ, et al: Interactions of glucocorticoids, NPY and hypothalamic serotonin. Nutr Neurosci 2000;3(3):183-92

Habib KE, Weld KP, Rice KC, Pushkas J, Champoux M, Listwak S, Webster EL, Atkinson AJ, Schulkin J, Contoreggi C, Chrousos GP, McCann SM, Suomi SJ, Higley JD, Gold PW: Oral administration of a corticotropin-releasing hormone receptor antagonist significantly attenuates behavioral, neuroendocrine, and autonomic responses to stress in primates. Proceedings of the National Academy of Sciences USA 2000;97(11):6079-84

Heinz A, Weingartner H, George D, Hommer D, Wolkowitz OM, Linnoila M: Severity of depression in abstinent alcoholics is associated with monoamine metabolites and dehydroepiandrosterone-sulfate concentrations. Psychiatry Research 1999;89(2):97-106

Hommer DW: Functional imaging of craving. Alcohol Research & Health 1999;23(3):187-96

Knutson B, Westdorp A, Kaiser E, Hommer D: fMRI visualization of brain activity during a monetary incentive delay task. Neuroimage 2000;12(1):20-7

Kunos G, Jarai Z, Batkai S, et al: Endocannabinoids as cardiovascular modulators. Chem Phys Lipids 2000;108(1-2):159-68

Mehlman PT, Westergaard GC, Hoos BJ, Sallee FR, Marsh S, Suomi SJ, Linnoila M, Higley JD: CSF5-HIAA and nighttime activity in free-ranging primates. Neuropsychopharmacology 2000;22(2):210-8

Nicolson R, Lenane M, Singaracharlu S, Malaspina D, Giedd JN, Hamburger SD, Gochman P, Bedwell J, Thaker GK, Fernandez T, Wudarsky M, Hommer DW, Rapoport JL: Premorbid speech and language impairments in childhood-onset schizophrenia: Association with risk factors. American Journal of Psychiatry 2000;157(5):794-800

Shen JN, Wenger N, Glaspy J, Hays RD, Albert PS, Choi C, Shekelle PG: Electroacupuncture for control of myeloablative chemotherapy-induced emesis - A randomized controlled trial. Journal American Medical Association 2000;284(21):2755-61

Shoaf SE: Pharmacokinetics of intravenous alcohol: Two compartment, dual Michaelis-Menten elimination. Alcoholism-Clinical and Experimental Research 2000;24(4):424-5

Shoaf SE, Carson RE, Hommer D, Williams WA, Higley JD, Schmall B, Herscovitch P, Eckelman WC, Linnoila M: The suitability of [C-11]-alpha-methyl-L-tryptophan as a tracer for serotonin synthesis: Studies with dual administration of [C-11] and [C-14] labeled tracer. Journal of Cerebral Blood Flow and Metabolism 2000;20(2):244-52

Torrey EF, Miller J, Rawlings R, Yolken RH: Seasonal birth patterns of neurological disorders. Neuroepidemiology 2000;19(4):177-85

Westergaard GC, Chavanne TJ, Lussier ID, Suomi SJ, Higley JD: Hormonal correlates of hand preference in free-ranging primates. Neuropsychopharmacol 2000;23(5):502-7

Zajicek KB, Price CS, Shoaf SE, Mehlman PT, Suomi SJ, Linnoila M, Higley JD: Seasonal variation in CSF5-HIAA concentrations in male rhesus macaques. Neuropsychopharmacology 2000;22(3):240-50

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00002-08

Title: EYE MOVEMENTS IN ALCOHOLISM AND INDIVIDUALS AT RISK FOR ALCOHOLISM

Staff Years: 2.2

Principal Investigator: DW Hommer, MD (BEI, LCS, NIAAA)

Other Lab Personnel: EZ Davis
M Israel, BS

NIH Collaborators: NIAAA, LCS (LC Doty, MSW)
NIAAA, LCS (VM Moore, SW)
NIMH, CHP (F Castellanos, MD)
NIMH, CHP (JL Rapoport, MD)

Extramural Collaborators: None

Sample Type: Human Subjects

Keywords: behavioral research, neurosciences, electrophysiology/EEG, impulsive behavior, risk-taking

SUMMARY: The study of human eye movements provides an extremely useful approach to the examination of a variety of cognitive functions. It is obvious that the latency and goal of saccadic eye movements are related to attention. What is not so obvious is that other aspects of cognition such as short-term memory, preparatory set and inhibition of context inappropriate responses can also be assessed using eye movement techniques. Short-term memory, preparatory set and inhibition of context inappropriate responses constitute core functions of the prefrontal cortex, the brain region most involved in the control of higher order cognitive processes. We have used a number of different tasks to elicit saccades, including Go/No Go and delayed response tasks. These tasks allow us to independently assess core functions of the prefrontal cortex by measuring the accuracy and latency of memory guided saccades, as well as the frequency of context inappropriate saccades that should be inhibited. Using these tasks we have demonstrated that schizophrenics are impaired in the three core aspects of prefrontal cortex function while children with Attention Deficit Hyperactivity Disorder (ADHD) are impaired in only their ability to inhibit context inappropriate saccades. Similarly, adult alcoholics have difficulty inhibiting context inappropriate saccades. The smooth pursuit eye movements of alcoholics are completely normal. Over the past year, we have added several new computer-based cognitive tasks designed to assess risk-taking and impulsive behavior. We expect that performance during these tasks will distinguish alcoholics from non-alcoholics as well as correlate with performance during eye movement tasks that assess inhibition of inappropriate saccades. In addition, we expect that impulsivity and risk-taking will be associated with smaller gray matter volume in the mesial and orbital frontal cortices.

RESEARCH HIGHLIGHTS: We have found that the tendency to make saccadic eye movements in anticipation of expected target motion is higher in alcoholics than non-alcoholics. This tendency reflects a failure to inhibit responses that are, in general, high probability responses, but are inappropriate for the current behavioral context.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: The failure to inhibit responses that are inappropriate for the current behavioral context is thought to be a characteristic of frontal lobe function. Our methods of eye movement testing may provide a rapid and objective method for detecting frontal lobe dysfunction. Studies are currently underway to determine if failure to inhibit behaviorally inappropriate saccades is associated with structural changes in the frontal lobes as measured by MRI.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00061-09

Title: CEREBRAL STRUCTURAL AND METABOLIC CORRELATES OF AGGRESSIVE OR ADDICTIVE BEHAVIOR

Staff Years: 7.3

Principal Investigator: DW Hommer, MD (BEI, LCS, NIAAA)

Other Lab Personnel:
JM Bjork, PhD
M Israel, BS
M Kerich, MS
L Lu, BS
K Moon, BS
H C Nguyen, BS
S Porges
D E Rio, PhD
J Shen, MD
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NIH Collaborators:
NIAAA, LCS (LC Doty, MSW)
NIAAA, LCS (AJ Granger)
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NIAAA, LCS, PS (ED Singley, BS)
NIAAA, LCS, CS (MJ Phillips, BS)
NIAAA, LCS, CN (CL Jones, BS)
NIAAA, LCS, CN (RR Rawlings, MS)
NIAAA, LCS, CABC (DM Hill, MSW)
CC, PET (P Herscovitch, MD)

Extramural Collaborators: None

Sample Type: Human Subjects

Keywords: neurosciences, imaging, PET, MRI

SUMMARY: This research is designed to determine neuroanatomical and neurochemical correlates in addictive and aggressive-impulsive behavior in human subjects. The principal focus of these studies is the measurement and correlation of regional cerebral glucose metabolic activity, using positron emission tomography (PET) and brain volumes using magnetic resonance imaging (MRI), cerebrospinal fluid metabolites and measures of impulsive-aggressive behavior and excessive alcohol consumption. We collected full, volumetric T-1 weighted MR images using a 1.5 T scanner to measure intracranial volumes in 140 alcoholics (89 males and 51 females) and 104 healthy, non-alcoholic comparison subjects (53 males and 51 females). An automated segmentation program was used to divide the intracranial contents into CSF, gray and white matter (Human Brain Mapping 1997;5:194-205). When brain volume is measured, we are measuring the combined effect of two processes: growth and degeneration. Growth determines maximum brain size achieved during life. Maximal brain growth can be estimated by intracranial volume (ICV) and since ICV remains constant throughout life, brain degeneration can be measured by the ratio of cerebral volume or gray matter or white matter volume to the remainder of the intracranial contents. Alcoholics show greater brain degeneration than non-alcoholics. Women appear to be more affected than men. Alcoholics, by their mid to late twenties, also show significantly greater brain shrinkage than controls. In addition, alcoholics have smaller intracranial volumes than controls but this difference is small and barely reaches statistical significance, and brain degeneration accounts for a greater amount of the difference in brain volume than brain growth does. Neither estimated lifetime alcohol consumption nor number of years of heavy alcohol use predict brain degeneration among alcoholics. Similarly, presence or absence of comorbid psychiatric disorder or other substance abuse does not affect brain shrinkage among alcoholics.

RESEARCH HIGHLIGHTS: We found that alcoholics, particularly women alcoholics, have smaller brains than non-alcoholics. In addition, we found that alcoholics also have smaller intracranial volumes than non-alcoholics. Since intracranial volume is a function of growth, and not affected by the brain degeneration normally seen with aging, our results suggest that alcoholics may have mildly reduced brain sizes even before they begin drinking heavily.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: Risk factors that predict alcohol use problems at present are primarily related to family history and social behavior. Intracranial volume may represent a purer biological risk factor that could be used to help identify individuals at risk for alcohol dependence.

Reporting Period:	10/01/1999 – 09/30/2000
Project Number:	Z01 AA00065-09
Title:	SEMIAUTOMATED METHODS OF SEGMENTATION OF BRAIN IMAGES
Staff Years:	0.58
Principal Investigator:	DE Rio, PhD (BEI, LCS, NIAAA)
Other Lab Personnel:	DW Hommer, MD
NIH Collaborators:	None
Extramural Collaborators:	None
Sample Type:	Interview, Questionaires, or Surveys Only
Keywords:	neurosciences, imaging, PET, MRI

SUMMARY: The establishment of associations between structure and function among various areas of the brain is an important step in the identification of neurologic mechanisms in both normal and disease states. The pursuit of this goal requires the geometrical co-registration of digital images in two types of major applications: (1) data fusion, where brain images from the same subject are acquired by different modalities; (2) data comparison, for the detection of significant differences in images acquired by the same modality between different subject groups. The first application requires identification and delineation ("segmentation") of distinct areas and landmarks in the brain. A procedure based on dynamic clustering and region growing algorithms has been developed that segments T1-weighted MR images into regions of CSF, gray and white brain matter. This technique is currently being applied to automatic/semi-automatic identification of the following specific brain structures: cerebellum [good automated detection allowing operator editing] and caudate [limited success at automated detection]. This technique has been found to be of particular value in clinical research projects that use morphometric measures in addition to other physiological and sociological measures. Associated with the above developments, a technique based on a previously investigated algorithm has been further developed for automatic detection of axis of symmetry (intrahemispheric fissure) in PET and MR images. This algorithm is based on the correlation of similar but possible phased signals in the Fourier domain between values of corresponding spatial points. Using continuity conditions, the technique has been extended to 3-D. This technique allows us to first identify the fissure in brain images and then, using previously developed algorithms, to orient the brain images of all subjects in the same vertical manner. This is helpful in obtaining consistent volumetric measures of brain structures that have traditionally been viewed as 2-D cross-sections. In addition, brain image visual presentation for clinical review editing purposes and landmark orientation for multimodality registration is made more consistent. For the second (within-modality) application, the gray-level information itself can be employed for image registration without the need for segmentation. A multiscale registration procedure has been implemented that determines parameters of a general 3-D affine transformation (translation, rotation about an arbitrary center, anisotropic scaling and skewing) between volumes to be registered that minimize the average squared gray-level difference between corresponding voxels. Successful registration of PET images achieving homogeneous registration variance across the entire brain section has been achieved for both within and between subject analyses. This technique is now being used routinely in many clinical investigations. This method is also being used for the co-registration of long time series (e.g., 199 time points) of volumes acquired in fMRI studies.

RESEARCH HIGHLIGHTS: We have developed semi-automated methods to measure the volume of the mesial surface of the frontal lobes as well as the orbital surface. These techniques are being applied to alcoholics and controls. We have also found evidence for a difference in intra-cranial volume between alcoholics and controls, suggesting that a portion of the difference in brain volume between alcoholics and controls may exist prior to the development of alcoholism.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: The ability to do quantitative human neuroanatomy has been limited by the requirement that brain structures be outlined by hand on each

slice of an MRI scan. This requirement has resulted in very slow progress because outlining by hand is an extremely slow and time-consuming method. The methods we have developed are at least 100 times faster than outlining individual brain structures by hand and make it practical to compare the regional brain volumes of large numbers of alcoholics and controls.

Reporting Period:	10/01/1999 – 09/30/2000
Project Number:	Z01 AA00077-07
Title:	IMPAIRED CNS SEROTONIN ACTIVITY: TOLERANCE, NEUROIMAGING, AND NEUROANATOMY
Staff Years:	1.7
Principal Investigator:	JD Higley, PhD (NN, LCS, NIAAA)
Other Lab Personnel:	R Andrews, BS AJ Bennett, PhD T Graham, BS S Lindell, BS JG Pushkas, BS
NIH Collaborators:	NIAAA, AMB, ULAS (AL Chedester, DVM) NIAAA, LCS, UCBP (PB DePetrillo, MD) NIAAA, LCS, UPS (ED Singley, BS) NIAAA, LCS, BEI (DW Hommer, MD) NIDA, OCD (C Contoreggi, MD) NIMH (J Gorey) NIMH, CNE (PW Gold, MD) NIMH, CNE (K Habib, MD) NIMH, CBDB (D Jones, PhD) NICHD (M Bastian, BS) NICHD (M Champoux, PhD) NICHD (SB Higley) NICHD (R Hommer, BS) NICHD (C Shannon, BS) NICHD, LCE (H Rupp, BS) NICHD, LCE (SJ Suomi, PhD)
Extramural Collaborators:	University of Michigan, Ann Arbor (J Lopez, MD)
Sample Type:	Neither Human Subjects nor Human Tissues
Keywords:	social behavior, violence, tolerance, neurosciences, neuroanatomy, genetics (nonhuman), alcohol and alcoholism, neuroimaging, serotonin

SUMMARY: During the past year, we continued to investigate brain mechanisms of low cerebrospinal fluid (CSF) concentrations of the serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA) and its relationship to alcohol consumption and tolerance.

Biochemistry of Alcohol Use and Preference--We examined the relationship between the HPA axis and CNS monoamine response to initial alcohol exposure and relationship to subsequent alcohol consumption. Results showed that concentrations of plasma cortisol and ACTH and CSF MHPG, HVA and 5-HIAA were increased following an intravenous alcohol infusion. Nevertheless, individual differences in CSF monoamine and plasma hormone concentrations were positively correlated between the baseline and intravenous alcohol conditions, showing that individual differences in neurotransmitter levels were stable and trait-like even after alcohol intake. Baseline 5-HIAA, MHPG, HVA and ACTH were negatively correlated and cortisol was positively correlated with future alcohol consumption. In addition, alcohol-induced MHPG and HVA were negatively correlated and ACTH was positively correlated with future voluntary alcohol consumption. These findings suggest that CNS monoamine and hormonal functioning may be important factors predicting and possibly mediating alcohol consumption.

Pharmacokinetics—In monkeys, blood alcohol concentration (BAC) is highly related to volume of total body water (TBW), therefore, developing a measure of TBW is important for assessing dosage and calculating an individual subject's response to a particular dose of ethanol. Blood samples were obtained 5, 10, and 60 minutes following an alcohol infusion and analyzed for BAC (g/dl). The resulting dose and

BAC data were used to compute the Vd (L/kg) and the rate of metabolism of ethanol, expressed as g/kg/min. The Vd was taken to be the volume of total body water. Non-linear modeling software (NLREG) was used to compare and contrast a series of equations modeling the relationship between Vd (L) and Weight (kg), gender, age (months) and metabolic rate. This equation has practical applications for researchers using rhesus macaques to study alcohol kinetics and dynamics. We found that by knowing the weight and gender of an animal, its body water content can be calculated, which in turn can be used to calculate a dose of ethanol needed to produce a specific peak BAC.

Psychopharmacology Studies (Serotonin and NMDA)--In collaboration with Dr. Paolo DePetrillo, Dr. Bennett began a series of studies to more closely examine the underlying physiology and mechanisms of alcohol-related temperament. These psychopharmacological studies employ cardiac signal complexity as a measure of rhesus monkeys' response to drugs that act on serotonergic and NMDA receptors. The first of these studies showed that heart rate variability increased with ketamine administration and naturally occurring interindividual differences in CSF 5-HIAA concentrations were inversely correlated with heart rate variability.

Effects of Diet on CNS Serotonin Functioning--Our collaboration with Drs. Salem and Hibbeln (LMBB) investigating the role of essential fatty acids (EFA) in CNS serotonin functioning continued. Subjects that were fed a diet high in EFA as infants were shown in adolescence to possess high heart rate variability, suggesting long-lasting effects of diets rich in EFA. As a corollary of this investigation, in a laboratory-wide assessment of cholesterol and EFA, we found that plasma eicosapentaenoic acid, low-density lipoprotein cholesterol and arachidonic acid were positively correlated with CSF 5-HIAA concentrations, suggesting a modest effect of diet on CNS serotonin functioning.

Neuroimaging:

- In collaboration with Dr. Hommer (LCS), we performed MRI scans on 31 rhesus infants to examine the effect of rearing condition on brain volume. Consistent with other findings showing the importance of maternal care on the CNS, we found that after co-varying for age and weight, mother-reared infants had larger brain volumes than did peer-reared infants. Paralleling studies in humans, we also found that male infants had significantly larger brain volumes than did females.

- A high alcohol tolerance and a genetic disposition for increased serotonin transporters predispose humans to alcoholism. In collaboration with Dr. Andreas Heinz (Germany), we used SPECT neuroimaging to measure serotonin transporter availability in rhesus monkeys with varied CSF 5-HIAA concentrations and correlated that with future alcohol consumption. The availability of brainstem serotonin transporters correlated with alcohol intake in a free access paradigm, accounting for 50% of the interindividual variance in alcohol consumption. Only serotonin transporter availability statistically predicted alcohol intake in a multiple regression analysis using serotonin transporter availability, tolerance to the acute effects of alcohol intake and CSF 5-HIAA concentrations as independent variables. These observations provide clues to the neurobiological substrates of human alcoholism.

- SPECT scans were performed to assess CNS dopamine function. Type-2 dopamine (D2) receptors were measured using clozapine and a novel derivative of clozapine that may be a safer and more effective neuroleptic than clozapine. The radioligand, iodobenzamide, a potent D2 receptor antagonist, tagged with iodine-123, was used in these studies. Preliminary analysis of the data indicates evidence of D2 receptor occupancy by both clozapine and the novel derivative of clozapine. We are in the process of comparing the binding of the radioligand in subjects with low and high CSF 5-HIAA and in those that were reared with and without their parents.

Neuroanatomy--To assess the brain differences of subjects with high and low CSF 5-HIAA concentrations, we continued our collaboration with Drs. Stanley Watson and Juan Lopez (University of Michigan). These studies used classic neuroanatomical techniques to assess correlates of low CSF 5-HIAA concentrations. Studies this past year investigated the interaction between the Limbic-Hypothalamic-Pituitary-Adrenal (LHPA) "stress axis" and the serotonin (5-HT) system in the brains of parentally-deprived, peer-reared (PR), mother-reared (MR) and surrogate-peer-reared (SPR) monkeys. We quantified glucocorticoid (Gcc) and mineralocorticoid (Mcc) receptors, as well as 5-HT1a and 5-HT2a receptor messages in the hippocampus and prefrontal cortex using *in situ* hybridization. Our results showed that:

- unlike rats, in primate prefrontal cortex Mcc mRNA is abundantly present. This constitutes anatomical evidence suggesting that Mcc receptors may have a more expanded role in modulating brain function in primates than in rodents;
- Mcc and Gcc are co-localized with 5-HT2a and 5-HT1a in monkey prefrontal cortex and hippocampus;
- Mcc gene expression is significantly decreased in the hippocampus of both PR and SPR monkeys (interestingly, these findings are in the same direction as those found in the hippocampus of suicide victims);
- compared to both MR and PR monkeys, 5-HT1a gene expression is significantly increased in the hippocampus of SPR monkeys and SPR monkeys also have a tendency for higher levels of 5-HT2a but, surprisingly, we did not find any differences in Gcc levels in the hippocampus between any of the groups; and,
- in the outer cortical layers of the prefrontal cortex, PR animals had significantly higher levels of 5-HT1a mRNA than MR animals and both PR and SPR animals had a strong tendency for higher Gcc mRNA levels in this region, although the increase did not achieve statistical significance.

SIGNIFICANCE TO BIOMED RESEARCH AND TO THE PROGRAM: Male Type 2 alcoholics show a variety of behavioral deficits that may be in part a function of impaired CNS serotonin functioning. Much of our results this year focused on females with low CSF 5-HIAA concentrations, showing that they exhibit behaviors that may be related to impaired impulse control that impacts on their maternal behaviors. Our work with other species suggests that these findings may have wide generalizability.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00079-07

Title: PSYCHOBIOLOGY OF ANTISOCIAL BEHAVIOR AND HEALTH

Staff Years: 2.41

Principal Investigator: JD Higley, PhD (NN, LCS, NIAAA)

Other Lab Personnel:
AJ Bennett, PhD
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NIH Collaborators:
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NIAAA, LNG, SPGL (JG Lorenz, PhD)
NIAAA, LCS, UPS (ED Singley, BS)
NICHD (M Champoux, PhD)
NICHD (R Hommer, BS)
NICHD (C Shannon, BS)
NICHD, LCE (SJ Suomi, PhD)

Extramural Collaborators: Yerkes Regional Primate Center, Emory University (M Dario)

Sample Type: Neither Human Subjects nor Human Tissues

Keywords: behavioral/social research, violence, neurosciences, sleep, sexually transmitted diseases, suicide, risk-taking behavior, gene mapping (nonhuman)

SUMMARY: **Longitudinal Studies of Type 2 Personality In Nonhuman Primates: Follow-up of Middle-Aged Males --** As an investigation of Type 2 personality in nonhuman primates, in 1989 we initiated a study of 104 rhesus juvenile males with varying CSF 5-HIAA concentrations. Our first samples were taken when the subjects were yearlings, still living with their mothers. The subjects have all since migrated from their natal family troop, typically living in a new troop for a period and then moving to other troops. Ten years later, in the first study to longitudinally follow male rhesus from childhood through the middle-aged period of life, interesting data have emerged suggesting that the varying life history behavior patterns of individual males are predicted by differences in CNS serotonin function.

- **Stability of Individual Differences in CSF 5-HIAA concentrations** -- Individual differences in CSF 5-HIAA concentrations obtained from subjects while they were juveniles were positively correlated with individual differences in CSF 5-HIAA concentrations obtained eight to ten years later. Differences in CNS serotonin functioning were predictive of life-long behavior patterns.

- **Emigration** -- Analysis of the data revealed a curvilinear relationship between CSF 5-HIAA concentration and the age that males leave their family group. Examination of the data show that prior to reaching sexual maturity, there is a positive correlation between CSF 5-HIAA concentration and age at emigration for animals. On the other hand, for males that migrate after reaching sexual maturity, there was a negative correlation between CSF 5-HIAA concentration and age at emigration.

- **Social Dominance** -- Males from families high in natal dominance (as measured by high or low maternal social dominance rank within the troop) were able to delay their eventual migration until they were fully mature and better able to integrate themselves into a new troop. Family social dominance status was positively predictive of the eventual rank of the male when it left its natal troop and joined a new troop (i.e., males from high ranking families became high in social dominance rank in their new troop). In addition, juvenile CNS serotonin was associated with social dominance rank in both the natal troop and the migratory troop. Low CSF 5-HIAA concentrations

were correlated with high family natal dominance rank. Low CSF 5-HIAA concentrations as a juvenile predicted the acquisition of high social dominance rank in the new troop that the subjects joined years later.

- **Aggression** -- Replicating what we previously discovered in juveniles and adolescents, subjects with low CSF 5-HIAA concentrations as infants and juveniles were more likely, as adults, to engage in violent behavior that resulted in wounds and scars. During the mating season, these same adults (with low CSF 5-HIAA concentrations) were less likely to find sexual partners and to inseminate females.

- **Mortality** -- Further analysis showed that 47 of the original 104 study subjects were alive. Among the 57 animals that were dead, there was a positive correlation between age at emigration and age in months at death. Also, CSF 5-HIAA concentrations were lower in animals that died than in animals that survived.

Studies of Females with Low CSF 5-HIAA concentrations in Feral Environments -- To date our naturalistic studies of the phenotypic expression of low concentrations of CSF 5-HIAA have focused mainly on males. To assess the phenotype in females, three years ago, we began to gather a sample of young female macaques with varying levels of CSF 5-HIAA. This year, sufficient data were collected to report on these females. Consistent with laboratory studies, there was an age graded change in CSF 5-HIAA concentrations with younger females exhibiting higher CSF 5-HIAA concentrations. Replicating what was found in males, we found that independent of age differences, there was a positive interindividual correlation between infant and adolescent CSF 5-HIAA concentrations, with an average correlation between the yearling and adolescent sample of about $r = 0.45$. This finding suggests that the interindividual differences in CSF 5-HIAA concentrations are trait-like, showing continuity across time and situation. Initial analyses show that among these females, low CSF 5-HIAA concentrations places them at risk for a number of behavioral problems and for antisocial behavior. Females with low CSF 5-HIAA concentrations seldom engaged in affiliative social interactions. They were more likely to act in a bully-like fashion by "kidnapping" other females' infants. CSF 5-HIAA concentrations of the first 7 females that died were more likely to be lower than that of the living females. This finding replicates earlier data from males showing premature mortality in males with low CSF 5-HIAA concentrations.

Impaired CNS Hemispheric Specialization -- A number of studies have shown that alcoholics and alcohol abusers are more likely to show abnormalities of laterality. In the rhesus species, there is a bias to left-handedness. For adult subjects not showing this typical left bias, we found they had low CSF 5-HIAA concentrations and high cortisol as juveniles and adolescents, suggesting that the atypical right-hand preference was negatively correlated with low CNS serotonin functioning and stress reactivity. In a subsequent study, we examined the development of hemispheric specialization by comparing familial influences on hand preference in two closely related macaque species: rhesus macaques (Macaca mulatta) and pigtailed macaques (Macaca nemestrina). In the rhesus macaque, we found a positive correlation in the direction of hand preference (right vs left) between mothers and juvenile offspring, but in the pigtailed macaque, a negative correlation was found. Such findings indicate that maternal influences on offspring hand preference vary between closely related primate species and lead us to question the generalizability of universal single-factor theories used to explain intergenerational transmission of hand preference in humans.

SIGNIFICANCE TO BIOMED RESEARCH AND TO THE PROGRAM: Male Type 2 alcoholics show a variety of behavioral deficits that may be in part a function of impaired CNS serotonin functioning. Much of our results this year focused on females with low CSF 5-HIAA concentrations, showing that they exhibit behaviors that may be related to impaired impulse control that impacts on their maternal behaviors. Our work with other species suggests that these findings may have wide generalizability.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00081-07

Title: FUNCTIONAL MAGNETIC RESONANCE IMAGING OF EMOTION AS RELATED TO ALCOHOLISM

Staff Years: 7.3

Principal Investigator: DW Hommer, MD (BEI, LCS, NIAAA)

Other Lab Personnel:
JM Bjork, PhD
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NIH Collaborators:
NIAAA, LCS (CR Copeland, BS)
NIAAA, LCS (AJ Granger)
NIAAA, LCS, PS (ED Singley, BS)
NIAAA, LCS, CS (MJ Phillips, BS)
NIAAA, LCS, CN (D Parma-Dotson)
NIAAA, LCS, CN (RR Rawlings, MS)
NIAAA, LCS, CABC (DM Hill, MSW)

Extramural Collaborators: None

Sample Type: Human Subjects

Keywords: neurosciences, alcoholism, emotion, affect, MRI

SUMMARY: Much of the recent work in the neurobiology of emotion has divided emotion into two categories: positive and negative emotion. Positive emotions involving high levels of arousal often occur in the context of the anticipation of reward, while negative emotions involving high levels of arousal often occur in the context of the anticipation of punishment. Thus, we have sought to characterize neural activity associated with the anticipation of rewards and punishments. Comparative studies have implicated a ventral forebrain dopaminergic path in the anticipation of reward. We aimed primarily to replicate our work from FY 1999, which verified activation of these areas during monetarily rewarded approach and avoidance behavior in humans. In addition, we examined whether these ventral forebrain areas were activated prior to motor behavior, specifically, during anticipation of reward and punishment, as opposed to during responses for reward and punishment. Twelve healthy right-handed females (age 20-40) participated in 10-minute approach and active avoidance tasks in counterbalanced order. During the approach task, subjects saw a cue indicating that they could either win money or not, waited a variable delay, and then pressed a button in response presentation of a target. If subjects responded before the target following the reward cue disappeared, they won $1.00, whereas their response to the neutral target did not affect their total. During the active avoidance task, subjects were given $20.00 and responded to targets that followed either a punishment cue or a neutral cue. If they failed to respond before the disappearance of the target following the punishment cue, they lost $1.00, whereas their response to the neutral target again did not affect their total. 200 T2*-weighted gradient echoplanar MR volumes depicting BOLD-contrast were acquired using a 1.5 Tesla GE Signa System. The volume consisted of 10 slices spanning the corpus callosum (voxel size; 3.8 X 3.8 X 7.0 mm, TR: 3000 ms). After correcting for in-plane motion, individual voxel activations were correlated with an ideal waveform corresponding to the expected activation timecourse using AFNI. The ideal waveform consisted of the task On-Off waveform convoluted with the hemodynamic response function. Significant voxels ($r > 0.30$, $p < 0.0001$) were highlighted on the functional images. Similar to results found in our

previous male sample, group analysis indicated that anticipation of reward activated striatal areas (caudate and putamen) and mesial forebrain areas (anterior cingulate, mesial prefrontal cortex and thalamic regions). Anticipation of punishment also activated these regions relative to anticipation of no monetary outcome. However, anticipation of reward but not punishment produced activation in the left nucleus accumbens and regions of the medial forebrain bundle near the ventral tegmental area. These results extend our previous findings with men and suggest that projections from the ventral tegmental area, including the nucleus accumbens, may play a selective role in the anticipation of reward.

RESEARCH HIGHLIGHTS: We have used functional Magnetic Resonance Imaging (fMRI) to identify the brain regions that are specifically involved in the anticipation of reward and punishment. Anticipation of reward activates the rostral pole of the nucleus accumbens, while in contrast; anticipation of punishment activates a more posterior location in the medial aspect of the ventral caudate nucleus.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: It is possible that sensitivity to reward and punishment may differ between individuals who are at high risk for development of alcoholism or other substance abuse. The ability of fMRI to provide a precise brain localization for anticipation of reward or punishment will provide a new method for understanding the differences in brain function that predispose individuals to substance abuse.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00082-07

Title: STATISTICAL ANALYSIS OF IMAGE FEATURES

Staff Years: 1

Principal Investigator: DE Rio, (BEI, LCS, NIAAA)

Other Lab Personnel: BD Knutson, PhD
DW Hommer, MD
J Shen, MD

NIH Collaborators: NIAAA, LCS, CN (RR Rawlings, MS)

Extramural Collaborators: None

Sample Type: Human Subjects

Keywords: neurosciences, imaging, PET, MRI

SUMMARY: The aim of this project is the development of statistical methods that either take into account interpixel correlation or apply global image transform methods that permit an analysis of uncorrelated image components. Of typical interest is the investigation of differences between either images from individual subjects acquired under different experimental conditions or between average images of subjects from different diagnostic groups. Three different statistical methods have been developed, based on the Fourier transform, the wavelet transform, and the theory of Gaussian random fields in the spatial domain. In the Fourier domain, the statistics at different wave numbers are uncorrelated and inference tests can be performed unencumbered by spatial correlations. This method provides for rigorous statistical tests with well-known properties and interpretations, but results in spatially uniform image blurring and may yield relatively poor spatial localization. For the wavelet-transform based analysis, a mathematically rigorous theory has been established that applies parametric statistical tests on wavelet coefficients and results in estimates of local image differences by inverse wavelet transform of only significant coefficients. This method provides for good spatial localization and the implementation of locally adaptive image smoothing, but there has not been much experience accumulated for the interpretation of test outcomes and estimates of image differences. Gaussian random field analysis has good spatial localization properties and permits the investigation of correlations with external variables (e.g., age), but it results in spatially uniform image blurring and does not provide for statistically reconstructed estimates of images differences (either across group or conditions). The three methods have been applied to the analysis of PET images from normal and alcoholic subjects and have identified significant differences in the same general brain regions. Gaussian random field analysis was able to demonstrate, in PET images from alcoholics, a significant negative correlation of glucose utilization in the prefrontal cortex with age. Current research on these topics includes the development of a 1-D Gaussian random field method to analyze fMRI time series data. This methodology can be used to analyze fMRI data acquired from experiments designed to incorporate a long (that is long enough, as determined experimentally, to estimate the variance associated with the acquired data) baseline condition and transition to another activated state. It uses the long baseline data to estimate the variance measure associated with the temporal data from a voxel within the image and sets a statistically rigorous threshold for activation in spite of the known temporal correlation in the data. This analysis technique is being validated with simulated and experimental data. Furthermore, this analysis technique is being incorporated into numerous experiments, including one designed to look at the blood flow changes in the brain associated with alcoholic intake in normal subjects. This presents an ideal demonstration of this analytic technique to basically establish a response curve for alcohol intake. Finally, statistical analysis in the temporal domain, based on traditional time series analysis in the Fourier domain, have been developed and gave similar results, in terms of localization of the signal in fMRI blood flow studies, to other less rigorous and generalizable techniques. This analytic methodology has the potential to: (1) localize fMRI activation changes, (2) estimate or reconstruct the activated signal without the associated noise, (3) estimate the hemodynamic response function locally without prior assumptions as to its structure and (4) detect multiple responses to multiple input stimuli. Currently this technique is being used to study both simple finger tap data as well as more complex experimental designs.

RESEARCH HIGHLIGHTS: A major problem with the analysis of fMRI data involving data collected over time is that this data is correlated in time. That is, the signal value at time t +1 is similar to that at time t. To correctly analyze these data, methods must be developed that take this correlation into account. Previous attempts to analyze this data have depended on ad hoc assumptions and incorrect statistical methods. We have extended several established statistical methods to correctly analyze this data depending on the experimental setup.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: Methods have been developed to analyze fMRI data acquired over time using established statistical methods that do not depend on contrived or ill-suited statistics. This will enable us to better reproduce results and will have an impact on the development and analysis of many experiments in both this program, and biomedicine in general, since many questions being asked are dependent on reliable and correct analysis of fMRI data.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00092-05

Title: MONITORING OF HEART RATE VARIABILITY DURING ALCOHOL WITHDRAWAL SYNDROME

Staff Years: 0.8

Principal Investigator: PB DePetrillo, MD (UCBP, LCS, NIAAA)

Other Lab Personnel: K Karimullah

NIH Collaborators: NIAAA, LCS, CS (DT George, MD)
NIAAA, LCS, BEI (DW Hommer, MD)

Extramural Collaborators: None

Sample Type: Human Subjects

Keywords: heart, alcohol withdrawal, ethnicity, gender, signal dynamics

SUMMARY: The purpose of the project was to examine measures of heart rate variability (HRV) in male and female alcoholics admitted for drinking cessation. Our question was whether abstention from alcohol use for a period of 4 to 6 weeks would result in normalization of the cardiac signal complexity measure back to baseline, as defined by healthy comparison subjects or if the decreased signal complexity remained stable for a period of 4 to 6 weeks. Our outcome variable of interest was the Hurst exponent of the interbeat interval time series obtained from the EKG, which is a good measure of HRV. The Hurst exponent is a non-linear measure of the autocorrelation of the time series, and in our previous work was found to be increased in alcoholic subjects when obtained 48 hours after cessation of alcohol use with no symptoms of alcohol withdrawal syndrome present. We have determined that in alcoholic subjects, measures of signal complexity do not return to baseline at the end of 5 weeks, but reach an asymptote, which is higher than controls. We also found that there were gender and age differences in this measure. We modeled this response over time and found a good fit to a sigmoid response construct. Work by others has found that subjects with decreased measures of autocorrelation, as found in this study in alcoholic subjects, have a greater risk of sudden death. Therefore we hypothesize that altered autonomic control of heart rate in alcoholics may also be a risk factor for sudden death, as has been determined from several large epidemiologic studies. We are terminating this project as we are moving to nonhuman primates and rodent models to answer several mechanistic questions resulting from this work.

RESEARCH HIGHLIGHTS: We found that chronic excessive alcohol use results in alterations in cardiac rhythm that are present both during alcohol withdrawal and up to 4 weeks after the last use of alcohol. These alterations are known to be associated with sudden death from cardiac arrhythmias, which is found to occur in alcoholics at a higher rate than non-alcoholics.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: This work raises the possibility that long-term chronic excessive alcohol use might have lasting toxic effects not only on heart muscle itself, but also on the neural regulation of heart rate.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00093-05

Title: INTERACTION OF ETHYL ALCOHOL WITH CELLULAR CYSTEINE PROTEASES

Staff Years: 1.4

Principal Investigator: PB DePetrillo, MD (UCBP, LCS, NIAAA)

Other Lab Personnel: H Arora
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JA Rackoff, BS
A San Miguel, BS

NIH Collaborators: None

Extramural Collaborators: None

Sample Type: Neither Human Subjects nor Human Tissues

Keywords: toxicology, neurosciences, molecular genetics

SUMMARY: Certain types of cellular cysteine proteases may play an important role in modulating the activity of G-protein coupled receptors. Cytoplasmic and nuclear inhibitors closely regulate the activity of this class of enzymes. The effects of ethyl alcohol exposure on cysteine protease activity have been studied in cell culture utilizing a PC12 cell line. We have previously established that exposure of PC12 cells to ethyl alcohol for 96 hours results in a decrease in calcium-stimulated protease activity. We developed assays to measure GSH, GSSG and NO and we have determined that both GSH and GSSG are important modulators of calpain activity. GSH is an uncompetitive inhibitor while GSSG is a competitive inhibitor of calpains, with inhibition constants well within range of concentrations of GSH and GSSG found in cells. We have also determined that nitric oxide strongly inhibits protease activity. These results may relate to the previous reports that both ischemia and oxidative stress increase calpain activity in several mammalian cell types, and both processes are known to decrease GSH and GSSG. We are now examining how alcohol exposure alters GSH, GSSG and NO as possible mechanisms for alcohol-induced alterations in protease activity.

RESEARCH HIGHLIGHTS: We have determined that several novel inhibitors present in nerve cells regulate a family of proteins present in nerve cells which, when activated, result in cell injury and death. We know that alcohol alters the level of these inhibitors.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: This work will enhance our understanding of the mechanisms by which alcohol alters nerve cell function and how it may injure or kill nerve cells. An appreciation of these mechanisms may lead to development of strategies to prevent or reverse alcohol-related nerve cell toxicity.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00094-05

Title: CHARACTERIZATION OF PERPETRATORS OF DOMESTIC VIOLENCE

Staff Years: 6.7

Principal Investigator: DT George, MD (CS, LCS, NIAAA)

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NIH Collaborators: NIAAA, LCS, UPS (ED Singley, BS)
NIAAA, LCS, CABC (DM Hill, SW)

Extramural Collaborators: None

Sample Type: Interviews, Questionnaires, or Surveys only

Keywords: behavioral research, violence, neurosciences, spouse abuse

SUMMARY: Domestic violence is a problem of major proportions in the United States. The majority of studies pertaining to the etiology of domestic violence have focused on psychosocial parameters and given little emphasis to biological factors. In order to explore the possibility that the aggression displayed by perpetrators is related to fear, we administered the panicogenic agent, sodium lactate, to a select group of perpetrators. Results of this study showed that perpetrators evidenced more lactate-induced fear, panic and rage than non-violent controls. These results lead us to conclude that some perpetrators of domestic violence have an exaggerated fear-related rage response to perceived threats. Our current research is directed toward understanding what abnormality(s) may be present in perpetrators, which could contribute to these exaggerated fear-related responses. To do this, we are conducting a number of research studies. We performed detailed psychiatric evaluations of perpetrators and controls. Preliminary results indicate that perpetrators have a higher incidence of anxiety-related disorders than expected. We obtained cerebral spinal fluid (CSF) from perpetrators with (ALC-PERP) and without (PERP), a diagnosis of alcoholism, and healthy comparison subjects. Compared to controls, ALC-PERP had significantly higher CSF testosterone concentrations and PERP had significantly lower CSF concentrations of 5-hydroxyindoleacetic acid (5-HIAA). These findings lead to the conclusions that there may be biological differences between perpetrators with and without alcoholism, and that both low serotonin and high testosterone may have a role in facilitating the fear-related aggression displayed by perpetrators. Additional CSF neurotransmitters are currently being assayed. We performed FDG PET scans on ALC-PERP, non-violent alcoholics and healthy comparison subjects. Preliminary results show that ALC-PERP have lower glucose metabolism in the right hypothalamus compared to non-violent alcoholics and healthy comparison subjects. To study autonomic regulation in perpetrators, we performed an orthostatic challenge. The strong association between respiratory sinus arrhythmia and heart rate present in controls was not present in the perpetrators. This suggests that perpetrators may have a disturbance in their autonomic function. To study noradrenergic function in perpetrators, we administered the alpha-2-antagonist, yohimbine, to perpetrators and controls. We are in now in the process of having the plasma, which was obtained during the challenge, assayed for various noradrenergic parameters.

Formerly titled: *Yohimbine Challenge to Study Noradrenergic Function in Individuals*

RESEARCH HIGHLIGHTS: Results of our research indicate that some perpetrators of domestic violence have an exaggerated fear-related behavioral response to perceived danger.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: The results of our research could potentially lead to new treatments for domestic violence.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00203-01

Title: *IN VITRO* AND *IN VIVO* MODELS OF CYTOPROTECTION BY INHIBITION OF CALPAIN PROTEASES

Staff Years: 1

Principal Investigator: PB DePetrillo, MD (CBP, LCS, NIAAA)

Other Lab Personnel: W Wan
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NIH Collaborators: None

Extramural Collaborators: None

Sample Type: Neither Human Subjects nor Human Tissues

Keywords: alcoholism, neurotoxicity, calpains, therapeutics

SUMMARY: NMDA receptor up regulation occurring after prolonged alcohol administration in rodent models has been implicated in neural cytotoxicity via an increase in calcium-flux resulting from l-glutamate activation of these calcium channels. Calpains are a class of calcium-activated cysteine proteases that are known to mediate calcium-mediated injury in neural cells. The focus of this project is to a) examine the mechanism of l-glutamate mediated cell injury using a PC12 cell model; b) determine whether inhibition of calpain activity would result in significant cytoprotection; c) test a variety of calpain inhibitors as cytoprotectants in a l-glutamate model of cytotoxicity; and d) extend these observations to a rodent model of neural cell injury. It was previously shown in this laboratory that ritonavir, an HIV protease inhibitor, is also a competitive inhibitor of calpain activity, with a Ki of ~10 microM, well within the range found during clinical dosing of the drug in humans when used as an anti-retroviral. Using fura-2, it was found that l-glutamate significantly increased intracellular concentrations of free calcium. The increase in intracellular free calcium induced by l-glutamate was also shown to result in a significant increase in calpain protease activity. Calpain activation is followed by degradation of a variety of cytoskeletal components, and in this model it was found that l-glutamate exposure resulted in significant degradation of actin, tau, and NF68, which was blocked by MK801, calpain inhibitor I and ritonavir. In cytotoxicity tests, it was found that MK801, calpain inhibitor I and ritonavir also inhibited l-glutamate mediated cell death. Neither a caspase inhibitor (Z-DEVD-FMK) nor DNQX (AMPA inhibitor) had any protectant effects, nor did they prevent l-glutamate induced breakdown of cytoskeletal proteins. We conclude from the initial phases of this work that l-glutamate mediated cytotoxicity in PC12 cells is a calpain-dependent process. In future work, the neuroprotective effects of these agents will be examined in a rodent model. If this is successful, this would raise the possibility that ritonavir or its analogues might be useful as neuroprotective agents when cellular injury is thought to occur via a calcium-mediated process.

RESEARCH HIGHLIGHTS: We discovered that ritonavir, an HIV protease inhibitor, is also an inhibitor of calpain proteases.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: Compounds that inhibit calpain have been shown to be effective in limiting cell damage after strokes or heart attacks. Our findings provide the basis for further work and suggest that ritonavir and drugs related to ritonavir should be investigated for the treatment of strokes or heart attacks. Since alcohol withdrawal may exert neurotoxic effects through similar mechanisms, it is also possible that this drug or related drugs may be helpful in limiting the damaging effects of alcohol on the brain.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00226-03

Title: MODULATION OF CALPASTATIN-CALPAIN INTERACTIONS BY ETHANOL

Staff Years: 1.8

Principal Investigator: PB DePetrillo, MD (CBP, LCS, NIAAA)

Other Lab Personnel: JA Rackoff, BS
A San Miguel, BS

NIH Collaborators: None

Extramural Collaborators: None

Sample Type: Neither Human Subjects nor Human Tissues

Keywords: alcoholism, neurotoxicity, PC12, post-translational modifications, protein-protein interactions, calpain, calpastatin, proteases

SUMMARY: Exposure of PC12 cells to ethanol results in large changes in calcium-stimulated protease activities. Since these proteases are critical modulators of neurotransmitter release as well as cellular toxicity and death, we are exploring the hypothesis that ethanol-mediated neural toxicity may result from calcium-activated protease dysregulation. Calpastatin, an inhibitor of calpain, is an acidic, hydrophobic protein, which interacts with the hydrophobic active site(s) of mu- and m-calpains. A series of post-translational modifications of calpastatin have been described which alter the binding affinity to calpains, among them, PKC-mediated phosphorylations. Using a PC12 model, we are examining the effects of ethanol exposure and withdrawal on protein-protein interactions. Because of the hydrophobic nature of calpastatin-calpain interactions, we examined the possibility that ethanol might modify protease-calpastatin (inhibitor) complex stability. We found that exposure of PC12 cells to ethanol results in an increase in the molecular weight of calpain and calpastatin-containing protein complexes, and that this is associated with a change in protease activity. We have extended these observations by use of immunocytochemical techniques. We developed a method to quantitate alterations in spatial organization of the immunoreactive proteins of interest and applied it successfully to fluorescent immunohistochemical images. This method allows us to not only quantitate signal magnitude *in situ*, but also to determine if the spatial signal is altered after exposure to alcohol. We found that signal magnitudes associated with mu-calpain and calpastatin are altered by alcohol exposure, but that in the case of mu-calpain, the texture of the signal is also altered. This finding suggests that *in vivo*, exposure of PC12 cells to alcohol may alter calpain epitope recognition by either a protein-protein interaction or by a post-translational modification. We are now in the process of extending these findings and developing the capability of filming the time course and translocation of the proteins of interest in live cells immediately after exposure to alcohol. This will increase our understanding of the mechanisms involved in the regulation of calpain activity by alcohol.

RESEARCH HIGHLIGHTS: We have found that when nerve cells are exposed to alcohol, alcohol probably disrupts the interaction between calpain proteins and one of their endogenous inhibitors. We also developed a method to visualize how the proteins are altered after alcohol exposure in microscopic images of nerve cells.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: This work has contributed to the methodology of cell imaging, allowing researchers to objectively quantify proteins within cells where before such comparisons were made subjectively. The finding that alcohol alters the interaction between calpains and calpastatin contributes to our knowledge of how the drug may exert toxic effects in nerve cells.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00277-12

Title: MODELS OF CNS SEROTONIN FUNCTIONING: ALCOHOL CONSUMPTION AND IMPAIRED IMPULSES

Staff Years: 3.6

Principal Investigator: JD Higley, PhD (NN, LCS, NIAAA)

Other Lab Personnel:
R Andrews, BS
AJ Bennett, PhD
M Gerald, PhD
T Graham, BS
S Lindell, BS
JG Pushkas, BS
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NIH Collaborators:
NIAAA, LNG, HN (L Akhtar, MS)
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NIAAA, LMBB (N Salem Jr, PhD)
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NIMH, CNE (PW Gold, MD)
NICHD (M Bastian, BS)
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NICHD (SB Higley)
NICHD (R Hommer, BS)
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NICHD (C Shannon, BS)
NICHD, LCE (H Rupp, BS)
NICHD, LCE (SJ Suomi, PhD)

Extramural Collaborators: U Michigan Medical School, Ann Arbor (G Flory, BS)

Sample Type: Neither Human Subjects nor Human Tissues

Keywords: behavioral/social research, violence, neurosciences, sleep, sexually transmitted diseases, suicide, risk-taking behavior, gene mapping (nonhuman)

SUMMARY: During the past year, our research included studies that investigated neurobiology and behaviors that are correlated with Type-1 and 2-like excessive alcohol consumption.

Type 1 and 2 Phenotype: Early Experiences:

- **Social Dominance** -- In a series of studies this year we focused on the acquisition of social competence by measuring the effects of early experiences on the development of social dominance. As infants, subjects were reared in one of three conditions: (1) with their mothers in families (MR), (2) without adults but constant access to age-mates in peer-groups (PR), and (3) without adults and with only limited social contact by other age-mates in what is known as a surrogate-peer-reared group (SPR). The results showed that MR subjects had higher baseline CSF 5-HIAA concentrations than PR or SPR subjects, with no difference between PR and SPR subjects in CSF 5-HIAA concentrations. Continued assessment of these subjects showed that at age 4 and as young adults, rearing condition produced a graded difference in social dominance rank. At age 4 and as adults, MR and PR subjects were higher in social dominance rank than SPR subjects, and the PR subjects were intermediate between the MR and SPR groups. Studies of how monkeys acquire social dominance also showed that high-ranking monkeys exhibit

accelerated physical development. Finally, consistent with impaired social competence in subjects at risk for alcoholism, we found that monkeys low in social dominance rank were likely to consume alcohol to excess.

- **Cognition** -- Among humans, alcoholics and children of alcoholics show evidence of cognitive deficits. To investigate the role of early experiences in patterns of learning and cognition, as well as to provide baseline assessments prior to alcohol exposure, studies of learning and cognition continued. Dr. Allyson Bennett used an automated, computerized joystick video system to measure monkeys' learning and cognitive abilities. Alcohol-naive PR monkeys (subjects at risk for excessive alcohol consumption) showed delayed acquisition of tasks that varied in difficulty. PR animals' perseveration in simple motor responses interfered with their acquisition of complex tasks. Deficits in gross motor ability were not evident, however. Preliminary data analysis indicated that PR monkeys' performance might be more vulnerable to disruptions and distractors. Finally, comparison of monkeys' performance on multiple problem-solving tasks showed consistency in the amount of time spent working to solve the tasks, but not in performance outcome. These data provide evidence of the deleterious effects of early parental deprivation on learning in subjects at risk for high alcohol consumption.

Child Abuse and Neurobiological Functioning -- In a study with Dr. Dario Maestripieri, Emory University, we investigated females with a long-term history of abusing their infants. Interindividual differences in CSF concentrations of 5-HIAA and MHPG during pregnancy were positively correlated with postpartum differences. Abusive mothers, who were typically abused as infants, had significantly higher CSF levels of corticotrophin releasing hormone (CRH), 5-HIAA and MHPG than controls. Among the abusive mothers, the females who refused to care for an unrelated infant had higher levels of 5-HIAA and MHPG than the females who adopted an infant. Across both abusers and controls, higher CRH, 5-HIAA and MHPG levels were associated with child abuse behaviors including higher frequency of maternal aggression, infant rejection and infrequent infant contact by mothers and other females. One possible explanation for this positive correlation with CSF 5-HIAA concentrations is that aggressive mothers' abusive behavior is not a result of low serotonin-mediated, impulse deficits; instead, it may be a result of high anxiety, similar to humans who exhibit posttraumatic stress disorder. These findings do concur with those of previous primate and human studies in suggesting that individuals who were exposed to early stressful experiences exhibit dysregulation of the monoamine systems and hyperactivity of extrahypothalamic CRH neurons. These data suggest that neurobiological alterations associated with infant abuse may play an important role in the occurrence of maladaptive behavior in adulthood, including the perpetuation of infant abuse across generations.

Type 1 and 2 Phenotype: Genetic Contributions Genotypic Variation -- One of the most powerful advantages of the nonhuman primate model is the capacity to closely control and investigate the role of genes and environment. In last year's report we indicated that there was an association between length variation in the serotonin transporter gene regulatory region (5-HTTLPR) CSF 5-HIAA concentrations and competitive aggression. Two other projects involving analysis of other candidate serotonergic genes are underway. In collaboration with Dr. Peter Lesch, University of Wuerzburg, our subjects were genotyped for a MAO-A polymorphism that is probably homologous to the genotype associated with panic disorders in humans. Dr. Bennett's genotypic analysis of monkeys' acute response to a stressor produced preliminary evidence for an association with the MAO-A genotype.

Reproductive History Patterns of Subjects With Low Serotonin -- In last year's report, we noted that males with low CSF 5-HIAA concentrations are more likely to die prematurely, early in life, and during the mating season they are less likely to be sought as sexual partners and to inseminate females than subjects with high CSF 5-HIAA concentrations. Given the potential loss of mating opportunities associated with diminished serotonergic functioning, it is puzzling that this trait has been maintained in the gene pool. Given these behaviors, in a recent study headed by our postdoctoral fellow, Dr. Melissa Gerald, we examined whether rhesus macaque males with low CSF 5-HIAA produce fewer offspring. Our study showed that laboratory males with low CSF 5-HIAA concentrations sired fewer offspring than males with high 5-HIAA. However, amongst males with low CSF 5-HIAA concentrations, males that successfully sired offspring were younger than those that failed to sire offspring. By contrast, among the males with high CSF 5-HIAA, those that successfully sired offspring were more likely to be older than their counterparts that failed to sire offspring. One interpretation of the age difference in sires that produce offspring may be that there is a differential reproductive life history strategy for males with low and high CSF 5-HIAA phenotypes.

Psychopharmacology Studies Corticotrophin Releasing Hormone -- Given our discovery that the strongest predictor of alcohol consumption is plasma cortisol, we began a series of studies investigating CRH. In collaboration with NIMH (Drs. Phil Gold & Kamal Habib) we found that out of several hormones measured in the CSF only CRH acutely correlated with objective measures of arousal in adult rhesus macaques. Antalarmin, a CRH type-1 receptor antagonist was administered which reduced arousal and anxiety-like behaviors. In a second series of studies, we found that during social separation, stress CRH responses were higher in parentally deprived peer-only reared infants than in mother-reared subjects. Independent of raising, repeated separations of an infant macaque from its attachment figure produced progressively greater increases in CSF CRH concentrations with each sequential separation. Two months after being placed in a new social group, juvenile monkeys demonstrated a significant elevation of CSF CRH, but not ACTH or cortisol. Taken together, these data indicate the involvement of CRH in the regulation of stress and the response of the HPA system to stress.

Formerly titled: *Models of Impaired CNS Serotonin, Alcohol Consumption and Excessive Aggression*

SIGNIFICANCE TO BIOMED RESEARCH AND TO THE PROGRAM: Male Type 2 alcoholics show a variety of behavioral deficits that may be in part a function of impaired CNS serotonin functioning. Much of our results this year focused on females with low CSF 5-HIAA concentrations, showing that they exhibit behaviors that may be related to impaired impulse control that impacts on their maternal behaviors. Our work with other species suggests that these findings may have wide generalizability.

Reporting Period:	10/01/1999 – 09/30/2000
Project Number:	Z01 AA00287-10
Title:	STRESS AXIS ACTIVATION, ETHANOL AND SITE-SPECIFIC CNS NEURODEGENERATION
Staff Years:	1
Principal Investigator:	RL Eskay, PhD (NN, LCS, NIAAA)
Other Lab Personnel:	None
NIH Collaborators:	NIMH, LCMR (AL Hampson, PhD) NIMH, SMN (CR Hamelink, PhD)
Extramural Collaborators:	University of Maryland (T Castonguay, PhD)
Sample Type:	Neither Human Subjects nor Human Tissues
Keywords:	mental health, stress axis, neuroscience, CNS, neurodegeneration, neuroprotection, glucocorticoids, ethanol, neuroendocrine

SUMMARY: Acute consumption of moderate to large amounts of ethanol (Et) activates the hypothalamic-pituitary-adrenal axis (HPAA) and chronic, excessive Et intake can lead to permanent brain damage. Activation of the HPAA, or hypercortisolism, accompanies both short- and long-term consumption of Et and the Et withdrawal syndrome. Since a relative state of elevated glucocorticoids (chronic continuous or chronic intermittent) can lead to neural changes and even cell death, particularly in the hippocampus, the progressive loss of cognitive capacity in many alcoholics may be due, in part, to hypercortisolemia and subsequent irreversible neural damage in the hippocampus and other areas of the central nervous system (CNS). Using an intragastric cannulated rodent model and short-term (4 days) intermittent or binge-type Et administration, we demonstrated site-specific CNS neurodegeneration in the dentate gyrus of the hippocampus, the entorhinal cortex and the piriform cortex. The observed Et-induced neurodegeneration was functionally validated as noted by the decline in learning and memory capacity in the Et-treated animals in the hippocampal-dependent Morris Water Maze test. Ongoing efforts to define the mechanism of Et's cytotoxicity continue. Surprisingly, the coadministration of glutamate receptor subtype antagonists or calcium uptake blocking drugs with Et are not neuroprotective, which argues against a glutamate-receptor dependent excitotoxic basis for the neurodegeneration. However, evidence for a glutamate-dependent, non-receptor or metabolic mechanism of excitotoxicity exists. Furthermore, elevated glucocorticoids exacerbate the Et-induced neurodegeneration presumably through excitotoxic mechanisms. To date the most potent cytoprotective agent in the binge-type rodent model has been shown to be Furosemide (FUR), an anion transport inhibitor. However, our finding that LY-644,711, bumetanide and SITS, which are drugs with mechanisms of action similar to FUR, are not neuroprotective would argue against a primary ionic, edema-based mechanism of neurotoxicity. Finally, with the knowledge that certain cannabinoids are neuroprotective, we coadministered cannabidiol with Et and found a significant reduction of neurodegeneration. Since *in vitro* studies have demonstrated that cannabidiol blocked glutamate-NMDA, -AMPA or -kainate receptor-mediated toxicity, it would appear that the cannabidiol site of action is downstream of receptor activation and perhaps has a generalized metabolic or antioxidant mechanism of neuroprotection. To confirm that the protection with cannabidiol was due to its antioxidant properties, two other common antioxidants, Vitamin E and butylated hydroxytoluene (BHT), were tested. Vitamin E, a well-known antioxidant, and BHT, an antioxidant commonly used as a food preservative, both protected to a similar degree as cannabidiol in our binge-type animal model of alcoholism. The neuroprotection afforded by diverse antioxidant compounds in our binge-type model of alcoholism suggests that oxidative stress and the generation of reactive oxygen species are partially responsible for the brain damage associated with excessive Et consumption.

RESEARCH HIGHLIGHTS: Excessive alcohol consumption, particularly binge-type intake, leads to site-specific brain damage and reduced cognitive (learning and memory) abilities. Administration of compounds with antioxidant properties, along with alcohol, significantly reduces alcohol-induced brain damage in an animal model of binge-type alcoholism.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: Our findings demonstrate for the first time that brain permeable antioxidants such as Vitamin E and cannabidiol are potent neuroprotectants against alcohol-induced brain damage in an animal model intended to mimic the consumption pattern of a binge drinker. Since a binge-drinking pattern of alcohol consumption is thought to be the most damaging patter in humans, it is envisioned that the treatment of active alcoholics with antioxidants should lessen alcohol-related oxidative stress and brain damage.

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FY 2000 ANNUAL REPORT SUMMARIES

(1 OCTOBER 1999 - 30 SEPTEMBER 2000)

LABORATORY OF MEMBRANE BIOCHEMISTRY & BIOPHYSICS

NORMAN SALEM, JR., Ph.D., CHIEF

DIVISION OF
INTRAMURAL CLINICAL & BIOLOGICAL RESEARCH
NATIONAL INSTITUTE ON ALCOHOL ABUSE & ALCOHOLISM

SECTION OF NUTRITIONAL NEUROSCIENCE

The first *in vivo* studies of the essential fatty acid (EFA) metabolism in alcoholics and smokers have been performed within the Section of Nutritional Neuroscience. In addition, our research with infants and adults report some of the first basic studies in the normal case. Highly sensitive and selective methodology has been developed within our research group allowing for the safe and non-invasive assessment of EFA metabolism *in vivo*. This approach takes advantage of the stable isotopically labeled EFAs, deuterated linoleic and linolenic acids, along with negative, chemical ionization gas chromatography/ mass spectrometry to simultaneously measure the precursor and product fatty acids. In this most recent reporting period, this methodology has advanced with new capabilities to simultaneously measure different components of the EFA pathway.

Our metabolic studies were among the first clinical studies performed in human adults and showed that a diet low in n-3 fats leads to an increase in formation and transport of long chain polyunsaturates to the plasma compartment. Smokers also have somewhat increased activity in this regard. Smoking alcoholics have a further marked increase in the deuterium enrichment of plasma DHA when deuterated alpha-linolenate is orally ingested. Both smoking and alcohol are peroxidative challenges that are hypothesized to lead to increased EFA degradation. The apparently greater level of formation/transport of the fatty acids may, in part, compensate for these losses. When the intensity of the alcohol challenge is too great with respect to its frequency and dosage, metabolism cannot keep pace with the increased catabolism and tissue levels fall, consistent with our findings in many species. This fall in long chain polyunsaturate levels then has adverse consequences for organ function and may lead, in particular, to liver and brain pathology. Dietary impact of these fats is variable in the alcoholic population and may be protective for alcohol-induced organ pathology; this may help to explain why some alcoholics develop liver disease or organic brain syndrome and others do not.

In human infants in the first week of life, our results indicate a general capacity to perform 18-carbon EFA metabolism to 20 and 22-carbon end products for both the n-3 and n-6 pathways. There was a surprising inverse relationship between gestational age and metabolic capacity that was strongest for docosahexaenoic acid (DHA). This is consistent with the rapid formation of neural membranes for which DHA is an important structural constituent during the brain growth spurt in the third trimester of human development. The rate of conversion in these infants is slow and appears inadequate to support the amount of DHA required for optimal brain and other organ development. We concluded, therefore, that infant formula must have preformed DHA added to support optimal nervous system growth and function; current formulas in North America are devoid of this nutrient, although they contain small amounts of the DHA precursor, alpha-linolenic acid.

Adult studies of EFA metabolism have been extended to the case of neurological disease states, for the first time. In collaboration with Dr. William Connor and colleagues, patients with Retinitis Pigmentosa (RP) have been given deuterated 18-carbon EFAs and their plasma metabolites quantified over an extended period. The amount and enrichment of isotope-labeled n-3 fatty acid metabolites, particularly DHA, was increased in Usher's II type patients but decreased in non-Usher's RP relative to normal controls. The increased metabolism observed in Ushers RP may be surprising in view of the decreased levels of DHA observed in circulation and, presumably, in the retina. However, as was the case in alcoholics, increased fatty acid metabolism may be coupled to increased catabolism related to the disease process. However, the opposite response in the non-Usher's patients indicated a quite distinct etiology of this illness.

An intractable and long-standing problem in the field of EFA biology has been the relative efficacy of interconversion of 18- and 20-carbon precursors to longer chain, more unsaturated products. This problem can be addressed by dosing an animal with multiple stable isotope labels within a given pathway. These isotopes must be chosen so that they are distinguishable isotopomers when metabolized. We have been able to simultaneously give oral doses of two n-3 precursors and two n-6 precursors simultaneously and measure the conversion of each isotope independently to AA or DHA. This novel

technique has been named MultiplE Simultaneous Stable Isotopes (MESSI). This technique will be applied to human infants and, possibly, to human adults in the future.

In concert with these studies of metabolic capacities of infants for AA and DHA formation *in vivo*, a series of studies was performed to examine the consequences of DHA depletion in the rat nervous system. Diets with varying amounts of n-3 and n-6 precursors and DHA were formulated and fed to rats over three generations so that by F2, the brains and retinas had lowered levels of DHA. Brain function was assessed by behavioral means using olfactory-based discrimination and spatial tasks. The n-3 adequate groups performed better in retaining a spatial memory in water maze tasks than did the n-3 inadequate animals. Animals with low brain or olfactory bulb levels of DHA made significantly more mistakes in acquiring an olfactory discrimination task. When the animals were over-trained, n-3 adequate animals appeared to learn a set as they acquired a strategy that enabled them to make zero or one error after an information trial. These studies provided clear evidence that lack of an adequate n-3 fatty acid dietary source in early development and persisting through adulthood results in a low brain DHA level and this has impact on memory- and learning-related brain functions.

In order to assess the behavioral underpinnings of the differences in olfactory learning and spatial memory, control experiments were performed. There were little or no differences in measures of general activity or in the plus maze either in the normal state or after restraint stress. There was no difference in the ability of rats to respond to a diluted odor even when masked with a higher level of a second odor, indicating that there was no loss in olfactory sensory ability. Also, a progressive-ratio licking task, in which the water-deprived animals worked for a water reward until extinction, showed no difference between dietary groups, suggesting that motivation was not altered by n-3 deficiency.

Once it was established that inadequate dietary n-3 sources during early development led to low brain DHA and behavioral deficits, it was of interest to understand the reversibility of this functional loss upon DHA repletion. Thus, F3 deficient rats were replete at various times in development--at birth, at weaning and at young adulthood. Repletion at birth or at weaning led to full recovery of brain DHA by 9 weeks of age and recovery of function in spatial maze tasks. However, adult recovery depended upon the duration of the repletion diet; partial recovery was observed after 2 weeks of repletion and nearly complete recovery after six weeks. A good correlation of brain DHA was obtained with spatial task performance and an inverse correlation with DPAn-6. One implication of this study is that human infants who received an n-3 deficient formula during rapid brain development may recover brain DHA as well as the more optimal brain function associated with its high membrane concentration.

In a closely related line of inquiry, an attempt was made to pioneer the quantification of alterations in brain morphology in n-3 deficient animals with low brain DHA. A loss of neuronal area was observed in the CA1 and CA2/3 regions of the hippocampus of n-3 deficient rats. There were no differences in cell number, density or layer volumes in the hippocampus between dietary groups. This finding has now been extended to the piriform cortex and the subfornical organ suggesting that there may be a general effect of low DHA on neuronal size throughout the brain.

In a further development in the area of nutritional neuroscience, a method invented by Hoshiba was adapted for the use in the study of EFAs during rat development. This system, termed "automatic feeding", makes possible the complete control of any nutrient during the entire life cycle of a rat when suitable diets are constructed. Artificial rat milks appropriate for use with this apparatus and with extremely low levels of n-3 fatty acids have been developed. It is now possible with this approach to induce a brain DHA loss of over 70% in a rat pup born to an n-3 adequate dam within 4 weeks. This system may thus allow intensive scrutiny of dietary EFA contributions to brain function and also allow novel metabolic experiments.

UNIT OF MOLECULAR BIOLOGY

During FY2000, the staff of the Unit of Molecular Biology continued to work on two main research projects: (1) the functional importance of ethanol-inducible cytochrome P450 2E1 (CYP2E1) and (2) the preparation and characterization of transgenic mice carrying the human aldehyde dehydrogenase 2 variant (*hALDH2-2*) and knock-out mice deficient in the mouse *ALDH2* gene. During this period, we investigated the signaling mechanism for apoptosis caused by various CYP2E1 substrates such as ethanol, acetaminophen (AAP, Tylenol®), 4-hydoxynonenal, carbon tetrachloride, and arachidonic acid. Our results demonstrated that AAP and other CYP2E1 substrates caused time and dose-dependent

apoptosis of C6 glioma and PC12 cells, as evidenced by DNA fragmentation and staining with fluorescent dyes. AAP and 4-hydoxynonenal, a cytotoxic lipid aldehyde, selectively activated c-Jun *N*-terminal protein kinase activity (JNK) without altering the activities of other mitogen activated protein (MAP) kinases or Akt kinase. YH439, a transcriptional inhibitor of the *CYP2E1* gene, decreased the levels of CYP2E1 and JNK activities with subsequent reduction in apoptosis, indicating the important role of CYP2E1-dependent metabolism of CYP2E1 substrates before initiating apoptosis. The critical role of the selective JNK activation in apoptosis was established by transfecting cDNA for the dominant negative mutant of JNK or SEK1, an upstream kinase of JNK, which significantly blocked the apoptosis. Similar results were observed in animal models. Because of the selective activation of JNK by CYP2E1 substrates, we are trying to identify a phosphatase, which specifically dephosphorylates phopho-p38 MAP kinase, which is often activated by many other apoptotic stimuli including cytotoxic cytokines, hydrogen peroxide, UV and X-ray irradiation

As part of an ongoing study to examine the physiological roles of ALDH2 and elevated levels of acetaldehyde generated from ethanol metabolism, an attempt has been made to prepare an *ALDH2* knockout mouse using the gene-by-gene disruption technique. During the year, we were able to produce chimera mice, which contain our DNA construct specifically designed to delete the mouse *ALDH2* gene. We are currently identifying the homozygous knockout mice using restriction analyses and Southern blot analyses. It is our hope that transgenic mice carrying the *hALDH2-2* variant or *ALDH2* gene knockout mice can be used as an animal model to study the role of ALDH2 in alcohol preference, acetaldehyde accumulation, metabolism of neurotransmitters, and acetaldehyde-mediated tissue damage following long-term alcohol abuse.

UNIT OF METABOLISM

Collaboration with the Department of Biochemistry at the University of Barcelona, last year, led this year to a publication discussing the implications of thiamine deficiency in cancer. Participation in the Institute of Medicine's review of fluid therapy in trauma, led to the adoption of our recommendations for new fluid formulation in its report. The Office of Naval Research is currently implementing our fluid therapy recommendations. Work performed in collaboration with the Department of Neurology of Tottori University and the Department of Biochemistry of Oxford University led to the publications of a paper suggesting that dietary treatment with ketone bodies may be useful in two major neurodegenerative diseases, Alzheimer's and Parkinson's disease. A meeting held this year under the auspices of the Office of Rare Diseases, NIH, suggested further that ketone body therapy may be useful in Leprechaunism and other forms of insulin resistance, Freidriech's ataxia and drug resistant epilepsy. The reception of the Unit's work on control strength analysis and its applicability to neurodegenerative disease led to its being awarded funding from a private foundation to carry on this work for the succeeding two years.

SECTION OF FLUORESCENCE STUDIES

Our studies are targeted at determining the role of both membrane acyl chain composition and ethanol on the function of G protein-coupled signaling systems. Receptors for many neurotransmitters, sight, taste, and smell are among the members of this superfamily of receptors. Previous studies in our section demonstrated that the formation of metarhodopsin II, which binds and activates the G protein, was most favored in bilayers containing DHA-phospholipids (PL). In addition, these bilayers showed the greatest resistance to the inhibitory effects of added cholesterol and were most sensitive to the enhancing effects of ethanol. Our recent studies have shown that ethanol interacts with both the protein and phospholipid membrane components. Physiological levels of non-ionic osmolytes increase the potency of ethanol by about 2.5-fold, demonstrating the importance of simulating *in vivo* conditions, while carrying out *in vitro* experiments. In other experiments, we have found that less unsaturated acyl chain phosphatidylcholines (PCs) result in a lag-time between the formation of metarhodopsin II and its interaction with G protein, relative to DHA containing PC bilayers. Integrated visual signaling, as measured by the effector enzyme activity, the cGMP-specific phosphodiesterase (PDE) was also found to be optimized in DHA containing PC bilayers. These studies show that the level of function in the visual pathway greatly enhanced in DHA bilayers. In other studies, we developed a set of acyl chain-specific fluorescent probes to investigate the presence of lateral domains in DHA containing bilayers. These measurements demonstrate the presence of a rhodopsin-phospholipid domain, highly enriched in di22:6 PC, suggesting another mechanism whereby DHA-containing bilayers may modulate the efficiency of membrane

signaling pathways. These findings support our hypothesis that acyl chain packing properties are strong modulators of receptor and signaling pathway function.

Dr. Drake C. Mitchell was appointed to a tenure-track position in this Section in June, 2000. He is undertaking a study of the effect of acyl chain composition and ethanol on the GABA(A) receptor. The first phase of this project involves purification of the GABA(A) receptor from bovine brain. An agarose affinity resin is being synthesized, which will be used to purify the GABA(A) receptors from detergent-solubilized membranes. Conditions for optimal solubilization and reconstitution are under investigation. Single-turnover activity in real time will be measured using a stopped-flow fluorescence spectrometer. The second phase of this project will involve the purification and reconstitution of specific isoforms of human GABA(A) receptor expressed in insect (Sf-9) cells.

We have found that inadequacies of dietary n-3 fatty acids leads to reduced membrane DHA content and both cognitive and visual deficiencies. These deficiencies manifest as reduced capabilities, rather than complete loss of function. Our studies of acyl chain packing and receptor and visual pathway activity are predictive of these observations in that reduced levels of signaling are associated with membranes containing lower amounts of unsaturated acyl chains.

SECTION OF MASS SPECTROMETRY

It has been proposed that an important mechanism underlying many of the effects of ethanol is its capacity to alter metabolism of polyunsaturates. The principal objective of our research is to elucidate biologic and metabolic functions of polyunsaturated fatty acids, docosahexaenoic acid (DHA) and arachidonic acid (AA) in the nervous system with particular reference to their modulation by ethanol. We previously found that DHA positively affects the survival of neuronal cells, at least in part, through the accumulation of phosphatidylserine (PS). Both alcohol and n-3 fatty acid deficiency lowered the accumulation of DHA in PS, resulting in the reduction of membrane PS concentration. During this period, we investigated mechanisms underlying the observed protective effect by examining the signaling pathways leading to cell death and survival.

We established another model where DHA enrichment prevented the apoptotic cell death in Neuro 2A cells. In addition to serum starvation, the treatment of cells with staurosporine, a general protein kinase inhibitor, induced apoptotic cell death and DHA enrichment significantly inhibited this effect. When the signaling mechanism affected by DHA enrichment was examined using these two model systems, we found that the PI-3 kinase pathway must be operative in order for DHA to be protective, while other kinases such as MEK, MAPK or PKA did not have any influence. Concurrently, the Akt phosphorylation inhibited by serum starvation or staurosporine treatment was partially restored in DHA treated cells, suggesting that DHA enrichment may affect the Akt activation, possibly through facilitating translocation of Akt to membrane and/or activation of Akt phosphorylating enzymes such as PDKs. Thus, it is suggested that enrichment of PS caused by a high DHA status in cell membranes may support neuronal survival under adverse conditions where Akt activation is greatly compromised. The modification of neuronal membranes by inadequate DHA supply or by ethanol may have significant implications in neuronal dysfunction.

During this period, we also continued to investigate the role of melatonin in polyunsaturated lipid metabolism. We found evidence that melatonin may be a negative endogenous regulator of $cPLA_2$ which plays a pivotal role in selective release of AA. In culture, melatonin decreased the release of AA and expression of $cPLA_2$ at both protein and mRNA levels. *In vivo*, endogenous non-esterified AA and $cPLA_2$ mRNA levels in the rat pineal gland showed an off-phase diurnal pattern in relation to melatonin levels. Intravenous administration of melatonin or isoproterenol, which has been shown to elevate melatonin production, also decreased the levels of non-esterified AA, $cPLA_2$ protein and $cPLA_2$ mRNA significantly. The data strongly suggests that melatonin may be an endogenous negative modulator of polyunsaturate metabolism. In a continuing effort to determine trace levels of neurosteroids from biological fluids using a GC/MS/NCI technique, we were able to detect species differences in $GABA_A$ active neurosteroids present CSF or plasma.

Section of Nuclear Magnetic Resonance

The effect of membrane composition on ethanol partitioning into biological membranes composed of protein and lipid was assessed by NMR methods and headspace gas chromatography. A large number of model membranes with different composition but also total brain and liver membranes have been investigated at the physiological ethanol concentration of 20 mM. The degree of ethanol partitioning depended strongly on the interface area, but was independent of membrane hydrophobic volume. The interface concentration of ethanol is modulated by ethanol affinity of polar groups in the interface. Based on this data, we suggest a layer model of ethanol partitioning. Membrane-bound ethanol almost exclusively resides within the membrane-water interface region, with very little or no ethanol in the hydrophobic core of the bilayer. From 5 to 15% of total ethanol in tissues is bound to proteins and lipids.

The decrease of ethanol concentration in plasma and the increase in thin interface layers surrounding proteins and lipids must be accounted for in studies of ethanol metabolism. The data suggest existence of differences in ethanol content between various tissues that is poorly correlated with tissue water content. We propose that the presence of ethanol at hydrophobic/hydropilic interfaces influences protein structure, ligand binding, and protein-lipid interaction.

We investigated conformation and flexibility of docosahexaenoic acid (DHA) chains in lipid membranes by magic angle spinning NMR approaches. Order parameters of all 22-carbon segments were measured. The excellent chemical shift resolution of DHA resonance enabled us to partially assign order parameters to specific chain segments. Furthermore, we measured NOESY cross-relaxation rates between the well-resolved DHA proton resonance using novel experimental approaches developed in the laboratory. In comparison to saturated chains, DHA order parameters are very low, reflecting both a change in bond geometry and an increase in angular fluctuation amplitudes for some bond angles. Docosahexaenoic acid is surprisingly flexible with rapid structural transitions between large numbers of conformations. The high flexibility of DHA is the consequence of lower potential barriers for rotations around the C-C bonds between methylene and vinyl groups compared to C-C bonds in saturated chains. This enables these chains to adapt to looped conformations that have shorter length and larger area per molecule.

NMR experiments on reconstituted membranes containing rhodopsin suggest that lipids with polyunsaturated DHA chains preferentially locate near the receptor. Within the limits of experimental sensitivity, we found no evidence for existence of DHA that is tightly bound to protein. The energy required for elastic deformation of DHA-containing membranes has been probed by application of osmotic stress.

Perhaps adaptability to the structure of imbedded membrane protein is a crucial biophysical property of DHA-rich membranes that enables structural transition during receptor activation without paying a high energetic penalty.

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BIBLIOGRAPHY
LABORATORY OF MEMBRANE BIOCHEMISTRY & BIOPHYSICS
1 OCTOBER 1999 – 30 SEPTEMBER 2000

Cascante M, Centelles JJ, Veech RL, Lee WNP, Boros LG: Role of thiamin (vitamin B-1) and transketolase in tumor cell proliferation. Nutrition and Cancer 2000;36:150-4

Contreras MA, Greiner RS, Chang MCJ, et al: Nutritional deprivation of alpha-linolenic acid decreases but does not abolish turnover and availability of unacylate docosahexaenoic acid and docosahexaenoyl-CoA in rat brain. J Neurochem 2000;75(6):2392-2400

Cott J, Hibbeln JR: Lack of seasonal mood changes in the Icelandic population: results of a cross-sectional study. Am J Psychiatry (letter, in press).

Hamilton J, Greiner R, Salem N Jr, Kim HY: N-3 fatty acid deficiency decreases phosphatidylserine accumulation selectively in neuronal tissues. Lipids 2000;35:863-9

Hibbeln JR: Homicide mortality rates and seafood consumption: a cross-national analysis. World Rev Nutr Diet (in press)

Hibbeln JR: Membrane disturbances in depression: A critical role for polyunsaturated fatty acids? In: Peet M, et al (eds), Phospholipid Spectrum Disorder in Psychiatry. England:Marius Press. 1999, pp 195-210

Hibbeln J, Salem N Jr: Omega-3 fatty acids and psychiatric disorders. In: Mostofsky D et al (eds), Fatty Acids: From Neuronal Membrane to Physiological and Behavioral Function. NJ:Humana (in press)

Huster D, Arnold K, Gawrisch K: Binding of Ca^{2+} ions to polyunsaturated phospholipids – consequences for lateral lipid organization. Biophysical Journal 2000;73:1954-66

Huster D, Dietrich U, Gutberlet T, Gawrisch K, Arnold K: Lipid matrix properties in cationic membranes interacting with anionic polyelectrolytes: A solid-state NMR approach. Langmuir 2000;16(24):9225-32

Huster D, Gawrisch K: New insights into biomembrane structure from two-dimensional nuclear Overhauser enhancement spectroscopy. In: Katsaras J, Gutberlet T (eds), Lipid Bilayers: Structure and Interactions. Berlin:Springer. 2000, pp 109-26

Jeffrey BG, Weisinger HS, Neuringer H, Mitchell DC: The Role of Docosahexaenoic Acid in Retinal Function. Lipids (in press)

Jeong KS, Park SJ, Lee CS, Kim TW, Kim SH, Ryu SY, Williams BH , Veech RL, Lee, YS: Effects of cyclocreatine in rat hepatocarcinogenisis model. Anti Canc Res 2000;20:1627-34

Jeong KS, Soh Y, Jeng J, Felder M, Hardwick JP, Song BJ: Cytochrome P450 2E1 (CYP2E1) dependent production of a 37 kDa acetaldehyde-protein adduct. Archives of Biochemistry and Biophysics 2000;384(1):81-7

Jeong KS, Song BJ, Keck-Waggoner CL, Huh CG, Popescu NC: Assignment of the mitochondrial aldehyde dehydrogenase-2 (ALDH2) gene to mouse chromosome 5 by fluorescence in situ hybridization. Cytogenetics Cell Genetics 2000;89:54-5

Karanian JK, Salem N Jr: Essential Fatty acids and the cardiovascular system. In: Watson RR, Myers A (eds), Alcohol and Heart Disease. (in press)

Kashiwaya Y, Takeshima T, Mori N, Nakashima K, Clarke K, Veech RL: D-beta-Hydroxybutyrate protects neurons in models of Alzheimer's and Parkinson's disease. Proc Natl Acad Sci USA, 2000;97:5440-4

Kim HY, Hamilton J: Accumulation of docosahexaenoic acid (22:6n-3) in phosphatidylserine is selectively inhibited by chronic ethanol exposure in C-6 glioma cells. Lipids 2000;35:187-95

Kim HY, Akbar M, Kim K: Inhibition of neuronal apoptosis by polyunsaturated fatty acids. J Mol Neurosci (in press)

Kim HY, Akbar M, Lau A and Edsall L: Inhibition of neuronal apoptosis by docosahexaenoic acid (22:6n-3): Role of phosphatidylserine in antiapoptotic effect. J Biol Chem 2000;275(45):35215-23

Kim YS, Zhang H, Kim HY: Profiling neurosteroids in CSF and plasma by gas chromatography/electron capture negative chemical ionization mass spectrometry. Anal Biochem 2000;277:187-95

Koenig BW, Mitchell DC, Litman BJ, Grzesiek S, Bax A: Measurement of dipolar couplings in a transducin peptide fragment weakly bound to oriented photo-activated rhodopsin. J Biomolecular NMR 2000;16:121-5

Li B, Zhang H, Akbar M, Kim HY: Negative regulation of cytosolic phospholipase A(2) by melatonin in the rat pineal gland. Biochem J 2000;351:709-16

Litman BJ, Niu SL, Polozova A, Mitchell DC: The role of docosahexaenoic containing phospholipids in modulating G protein-coupled signaling pathways: visual transduction. J Molecular Neuroscience (in press)

Mitchell DC, Litman BJ: Effect of ethanol and osmotic stress on receptor conformation: reduced water activity amplifies ethanol's effect on metarhodopsin II formation. J Biol Chem 2000;275:5355-60

Moriguchi T, Greiner RS, N Salem Jr: Behavioral deficits associated with dietary induction of decreased brain docosahexaenoic acid concentration. J Neurochem 2000;75(6):2563-73

Pawlosky R and Salem N Jr: Recent advances in the supply of docosahexaenoate to the nervous system. In: Mostofsky DI, Yehuda S, Salem N Jr (eds), Fatty Acids: From Neuronal Membrane to Physiological and Behavioral Function. New Jersey:Humana Press (in press)

Polozova A, Litman BJ: Cholesterol dependent recruitment of di22:6-PC by a G protein-coupled receptor into lateral domains. Biophys J 2000;79(5):2632-43

Salem N Jr., Moriguchi T, Greiner RS, McBride K, Ahmad A, Catalan JN, Slotnick B: Alterations in brain function after loss of docosahexaenoate due to dietary restriction of n-3 fatty acids. J Molecular Neurosci (in press)

Simopoulos AP, Leaf A, Salem N Jr: Workshop on essentiality of and recommended dietary intakes for omega-6 and omega-3 fatty acids. Prostag Leukotr Ess 2000;63(3):119-21

Soh Y, Jeong KS, Lee IJ, Kim YC, Song BJ: Selective activation of c-Jun N-terminal kinase pathway during apoptosis of PC12 cells by 4-hydroxynonenal. Molecular Pharmacology 2000;58:535-41

Song, BJ, Soh Y, Bae MA, Pie J, Wan J, Jeong KS: Apoptosis of PC12 cells by 4-hydroxynonenal is mediated through selective activation of the c-Jun N-terminal protein kinase pathway. In: Weiner H, Maser R, Hurley T, Lindahl R (eds), The Enzymology and Molecular Biology of Carbonyl Metabolism 10. Ireland:Elsevier (in press)

Song, BJ, Soh Y, Jeong KS: Stress biology research exemplified by alcohol mediated tissue and cell damage. Proceedings of Korean Journal of Microbiology 1999; pp 1-13

Tanskanen A, Hibbeln JR, Tuoileheto J, Uutela A, Haukkala A, Viinamaki H, Lehtonen J, Vartainen E: Infrequent fish consumption is associated with depressive symptoms at the population level. Psych Services (in press)

Yau WM, Gawrisch K: Lateral lipid diffusion dominates NOESY cross-relaxation in membranes. J Am Chem Soc 2000;22:3971-2

Reporting Period: 10/01/1999 - 09/30/2000

Project Number: Z01 AA00003-08

Title: NMR INVESTIGATIONS OF CELL MEMBRANE STRUCTURE
Staff Years: 6.33

Principal Investigator: K Gawrisch, PhD (NMR, LMBB, NIAAA)

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NIH Collaborators: None

Extramural Collaborators: University of Leipzig, Germany (K Arnold, PhD)

Sample Type: Neither Human Subjects nor Human Tissues

Keywords: cognitive deficits/central nervous system, NMR spectroscopy, cell membrane, polyunsaturation, docosahexaenoic acid, alcohol

SUMMARY: The objectives of this project are to: (1) study structure and dynamics of membranes composed of lipids with polyunsaturated fatty acids such as docosahexaenoic acid (DHA) 22:6n-3, (2) study lipid-protein interactions related to lipid polyunsaturation and alcoholism and (3) investigate the interaction of alcohol with proteins and lipids in biological membranes.

The membranes of brain synaptosomes and retinal rod outer segments contain 30-50 mol% of the six-fold unsaturated DHA as lipid hydrocarbon chains. One possible role of DHA is to alter membrane mechanical properties important for activity of receptor proteins. There is controversy as to the nature of the perturbation that DHA chains induce on membrane hydrocarbon order. The six methylene-interrupted cis double bonds within DHA 22 carbon unit reduced the number of degrees of freedom for structural transitions, which led to the suggestion that these chains have a specific rigid conformation such as angle-iron or helical. However, direct measurements of DHA chain order parameters reveal a different picture. Using a magic angle spinning NMR experiment which re-couples 13C-1H dipolar interactions, assigned DHA order parameters were obtained. A unique membrane probe, perdeuterated DHA, was synthesized and incorporated into the lipid matrix. Six distinct order parameters and their corresponding signal intensities were measured. Furthermore, the dimensions of the DHA chain unit cell were determined by x-ray diffraction. In comparison to saturated chains, DHA order parameters were low, reflecting both a change in bond geometry and an increase in angular fluctuation amplitudes. This is contrary to the widespread belief that high concentrations of double bonds result in less flexible hydrocarbon chains. The results suggest that DHA chains in membranes adapt to looped and extended conformations in rapid succession, providing increased flexibility to receptor-rich neural membranes.

We developed quantitative methods for interpretation of NMR NOESY cross-relaxation rates between lipid resonances. In addition to providing information on lipid structure, these rates are sensitive to the dynamics of membrane reorganization in the correlation time range form pico- to microseconds. The comparison of experimental rates and rates from molecular dynamic calculations suggests that distance variation between protons caused by lateral diffusion of lipid molecules is the primary mechanism of cross-relaxation in lipids. The analysis quantifies the high degree of molecular disorder in biological membranes, showing a finite probability of close approach between even the most distant segments of neighboring lipid molecules (e.g., the methyl groups in the choline headgroup and the terminal methyl groups of the fatty acid chains). Intermolecular cross-relaxation rates are an ideal tool to study lateral lipid organization in the liquid-crystalline phase of lipids. Inhomogeneous lipid distribution and

preferences in the interaction of lipid species as well as preferences in the location of substances that incorporate into membranes can be detected.

There is evidence that a high content of DHA in retinal membranes modulates physical properties of membranes, creating an environment that is optimal for function of rhodopsin, the primary visual receptor, and a member of the G-protein coupled receptor family. We investigated this hypothesis by solid-state NMR methods. Using 2H-labeled lipids, we compared lipid order parameters in the absence and the presence of the protein. Rhodopsin was reconstituted into fully hydrated, solid-supported, oriented multi-bilayer samples. With this novel approach we obtained, for the first time, highly resolved spectra from deuterated acyl chains in membranes containing a reconstituted integral membrane protein under physiological conditions. Oriented samples also improve NMR sensitivity, enabling use of milligram-size samples. The presence of rhodopsin induced differential changes in order parameters along acyl chains suggesting existence of membrane curvature stress. We also compared rhodopsin effects on the deuterium order parameter profile of saturated chain in mono- and polyunsaturated lipid systems. Protein incorporation decreased the order parameters of polyunsaturated PC, while not affecting that of the monounsaturated PC. The data suggest that rhodopsin preferentially interacts with polyunsaturated lipids resulting in lateral phase separation within the lipid matrix.

We obtained direct evidence by NMR that ethanol interacts preferentially with the lipid-water interface of membranes. Ethanol's interactions are driven by both the opportunity for hydrogen bonding and hydrophobic interactions. We quantitated ethanol binding to membranes composed of lipids and proteins at the physiological ethanol concentration of 20 mM by headspace gas chromatography. This method is ideally suited for partitioning studies because it is non-perturbing. Under physiological conditions, nearly10% of total ethanol is bound to the interfaces of lipids and proteins. NMR measurements indicate that free and bound ethanol molecules are in rapid exchange and that ethanol passes through membranes at rates that are only slightly lower than permeation rates of water. Interfacial binding of ethanol raises effective ethanol concentrations at surfaces but lowers concentrations in the electrolyte solutions of living organisms. The interface location of ethanol lowers interfacial energy of lipids and proteins. In lipid membranes, this results in an increase of area per lipid molecule and a disordering of lipid hydrocarbon chains. Ethanol-induced chain disordering is smaller in polyunsaturated bilayers, most likely because polyunsaturated hydrocarbon chains already occupy a larger area per molecule and are therefore less sensitive to ethanol-induced disordering. The ethanol molecules at the lipid-water interface block pathways for water diffusion through lipid bilayers as seen in decreased rates of water permeation.

RESEARCH HIGHLIGHTS: The interface concentration of ethanol is modulated by ethanol affinity of polar groups in the interface. Our data suggests a layer model of ethanol partitioning. Membrane-bound ethanol almost exclusively resides within the membrane-water interface region, with very little or no ethanol in the hydrophobic core of the bilayer. Five to 15% of total ethanol in tissues is bound to proteins and lipids. The decrease of ethanol concentration in plasma and the concentration increase in thin interface layers surrounding proteins and lipids must be accounted for in studies of ethanol action on neural signaling and on ethanol metabolism. The data suggest existence of differences in ethanol content between various tissues that is poorly correlated with tissue water content. We propose that the presence of ethanol at hydrophobic/hydrophilic interfaces influences protein structure, ligand binding and protein-lipid interaction.

NMR experiments on reconstituted membranes containing rhodopsin suggest that lipids with polyunsaturated DHA chains preferentially locate near the receptor. Within the limits of experimental sensitivity, we found no evidence for existence of DHA that is tightly bound to protein. The energy required for elastic deformation of DHA-containing membranes has been probed by application of osmotic stress. Adaptability to the structure of imbedded membrane protein is a crucial biophysical property of DHA-rich membranes that enables structural transition during receptor activation without paying a high energetic penalty.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: Ethanol interacts with lipids and proteins to influence neural receptor signaling. The function of membrane receptor molecules, like rhodopsin, is substantially modulated by membrane lipid composition, in particular by the degree of hydrocarbon chain unsaturation. We are studying molecular interactions with resolution at the level of atoms to uncover the physico-chemical mechanism of these sensitivities.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00036-14

Title: REGULATION AND BIOLOGICAL ROLE OF ETHANOL INDUCIBLE CYTOCHROME P450 2E1 (CYP2E1)

Staff Years: 2.58

Principal Investigator: B Song, PhD (MB, LMBB, NIAAA)

Other Lab Personnel: MA Bae, PhD
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NIH Collaborators: None

Extramural Collaborators: None

Sample Type: Neither Human Subjects nor Human Tissues

Keywords: hepatology, cell and molecular biology, alcohol, metabolism, apoptosis, tissue damage, oxidative stress

SUMMARY: It is well established that ethanol-inducible cytochrome P450 2E1 (CYP2E1) is induced by alcohol drinking in humans and animal models. In the past, we have cloned the genes for human and rat CYP2E1 and demonstrated multiple regulatory mechanisms: induction via transcription after birth, mRNA stabilization in ketotic states, activation of mRNA translation and protein stabilization by CYP2E1 substrates, suppression via transcription by YH439, mRNA degradation and protein degradation by carbon tetrachloride. Increased CYP2E1 after chronic alcohol consumption or certain pathological states leads to elevated production of acetaldehyde, reactive oxygen species, free radical metabolites and lipid peroxides while reducing cellular antioxidants such as glutathione. Therefore, cells or tissues with increased CYP2E1 become more susceptible to damage or cell death. In fact, others have already demonstrated that hepatoma HepG2 cells or PC12 cells transfected with the CYP2E1 cDNA are far more sensitive to cell death than the non-transfected counterparts after exposure to various CYP2E1 substrates such as ethanol, acetaminophen (AAP), carbon tetrachloride and arachidonic acid. Despite numerous studies on cell death by CYP2E1 substrates, the signaling mechanisms by which these CYP2E1 substrates exert their toxicological effects are unknown. Therefore, we continued to examine the signaling mechanisms during apoptosis caused by various CYP2E1 substrates. We have been particularly interested in the potential role of mitogen activated protein (MAP) kinases involved in the early signal transduction pathway during apoptosis and the enzymes involved in the cell survival pathway. These enzymes include: c-Jun N-terminal protein kinase (JNK), p38 MAP kinase, extracellular-signal regulated protein kinase (ERK), phophatidylinositol 3-kinase, Akt protein serine/threonine kinase and their upstream kinases or downstream target proteins including various caspases and bax/bcl-2 proteins involved in cell apoptosis/survival processes. Our initial hypotheses were: CYP2E1 substrates and their metabolites would activate the JNK and p38 MAP kinase while suppressing the enzymes involved in the cell survival pathway. In addition, inhibitors of CYP2E1 and other enzymes elevated during apoptosis effectively prevent cell death caused by CYP2E1 substrates. Our results showed that treatment of target cells (C6 glioma and PC12 cells) with AAP or other CYP2E1 substrates caused time- and concentration-dependent apoptosis of these cells as evidenced by DNA fragmentation and fluorescent staining of the apoptotic cells. In these cells, JNK activity was selectively and transiently activated after treatment with AAP or 4-hydoxynonenal (HNE), a cytotoxic lipid aldehyde. Unexpectedly, these toxic compounds did not activate p38 MAP kinase or affect the ERK activity. The selective and transient activation of the JNK in target cells was critical for their apoptosis since blockade of the JNK pathway by transfecting the cDNA of a dominant negative mutant of JNK or SEK-1 significantly blocked the apoptosis. In addition, our results showed that 10 uM YH439, a transcriptional inhibitor of the CYP2E1 gene, not only reduced the CYP2E1 and JNK activities but also suppressed the apoptosis caused by CYP2E1 substrates, indicating the critical role of CYP2E1-dependent metabolism during apoptosis. The non-involvement of p38 MAP kinase in the apoptosis was further confirmed by the use of its selective inhibitor, SB203580. Our results, therefore, are in contrast with other apoptotic stimuli such as hydrogen peroxide, UV and x-ray irradiations and pro-inflammatory cytokines including tumor

necrosis factor alpha and interleukin 1-beta, all of which can activate P38 MAP kinase along with the JNK. Our results from *in vitro* cultured cells were replicated in *in vivo* models where AAP and carbon tetrachloride selectively and transiently activated the JNK and its upstream kinases, SEK-1 and MEK. Because of the selective activation of JNK by CYP2E1 substrates, we are investigating the potential activation of a phospho-protein phosphatase, which specifically dephosphorylates phopho-P38 MAP kinase, after treatment with CYP2E1 substrates.

RESEARCH HIGHLIGHTS: During the year, we studied the molecular signaling mechanisms to determine how lipid peroxides and other potentially toxic substances including alcohol can cause cell or tissue injury. Our data indicate that JNK is selectively activated during cell injury caused by the toxic chemicals. By studying the signaling mechanisms for the tissue injury, we can develop potential methods to prevent or treat tissue injury caused by the toxic compounds. In fact, we are testing the benefits of various food components or natural substances that are considered safeguards against chemical induced tissue or cell injury.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: We are investigating the signaling mechanism to determine how alcohol and other potentially toxic substances can cause cell or tissue injury. We hope that our biochemical and molecular studies will lead to the development of potential therapeutic agents or preventive measures against organ damage caused by long-term alcohol consumption or other toxic compounds. We also hope that our findings will be used to prevent alcohol-related medical disorders.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00072-09

Title: FLUORESCENCE STUDIES OF BIOPHYSICAL PROPERTIES OF POLYUNSATURATED PHOSPHOLIPIDS

Staff Years: 2.4

Principal Investigator: BJ Litman, PhD (FS, LMBB, NIAAA)

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KG Hines, BS
DC Mitchell, PhD
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SB Shelton

NIH Collaborators: NIDDK, LCP, MBS (IW Levin, PhD)

Extramural Collaborators: None

Sample Type: Neither Human Subjects nor Human Tissues

Keywords: neurosciences, nutritional disorders

SUMMARY: Neuronal and retinal tissues are high in phospholipids containing one or two long chain polyunsaturated acyl chains. These studies are directed towards determining a molecular basis for the modulation of G protein-coupled receptor signaling by phospholipids containing polyunsaturated acyl chains, in particular 22:6n-3. Fluorescence Energy Transfer (FRET) was used to determine the degree of lateral domain formation in bilayers containing di16:0 and di22:6 PCs plus cholesterol. Lateral domain formation, which was dependent on the presence of rhodopsin, was detected. Polyunsaturated acyl chains result in the highest level of formation of the active conformation of the G protein-coupled receptor, rhodopsin. The role of lateral domain formation in bilayers containing 22:6n-3 lipids is being further investigated. Other experiments indicate that ethanol may disrupt the lateral domains, suggesting a potential mechanism by which ethanol can modulate membrane function. Both acyl chain free volume and curvature stress have been invoked as bilayer properties, which modulate membrane protein function. Experiments are currently being carried out to determine the relationship between these two properties. These studies are important in understanding the role of 22:6n-3 acyl chains in domain formation, the mechanism of alcohol action and the modulation of membrane protein function.

RESEARCH HIGHLIGHTS: Current studies on mixed phospholipid systems indicate that highly unsaturated omega-3 fatty acid chains tend to form lateral domains in the presence of cholesterol and the receptor, rhodopsin. The domains around rhodopsin are highly enriched in phospholipids containing the omega-3 fatty acid chains.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: Neurotransmitter signaling occurs through the interaction of several proteins in the surface of membranes. The localization of these proteins in a limited domain would greatly enhance the efficiency of the signaling pathway. The finding that highly unsaturated phospholipids, of the type found in high levels in the nervous, form lateral domains, suggests that such domains may exist in the cell membranes of the nervous system, enhancing neurotransmitter signaling efficiency. This finding may offer some insight into the visual and learning deficits observed in animals and infants with omega-3 deficiency.

Reporting Period: 1 October 1999 to 30 September 2000

Project Number: Z01 AA00080-07

Title: INFLUENCE OF PROTEIN/LIPID INTERACTIONS ON SIGNAL TRANSDUCTION

Staff Years: 3.7

Principal Investigator: BJ Litman, PhD (FS, LMBB, NIAAA)

Other Lab Personnel: LL Chatwood
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DC Mitchell, PhD
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SB Shelton

NIH Collaborators: NIDDK (I Levin, PhD)

Extramural Collaborators: None

Sample Type: Neither Human Subjects nor Human Tissues

Keywords: neurosciences, nutritional disorders, signal transduction

SUMMARY: G protein-coupled receptors are ubiquitous components of signal transduction pathways, including many neurotransmitter systems. This project is designed to assess the role of polyunsaturated phospholipids in modulating G protein-coupled signal transduction and to elucidate the mechanism of action of ethanol in these systems. The visual transduction pathway of the retinal rod photoreceptor is being used as a model system. System properties we are studying include: (1) the kinetics and extent of formation of metarhodopsin II (MII), the G protein activating form of rhodopsin; (2) MII-G protein complex formation; (3) the rate of G protein activation; (4) cGMP phosphodiesterase (PDE) activation; and (5) the GTPase activity of the G protein. Both functional measures in the transduction pathway and acyl chain packing properties of the phospholipid bilayer are being investigated. Current studies demonstrate that the kinetics and extent of formation of the MII-G protein complex are dependent on both acyl chain formation and cholesterol content. In particular the kinetic coupling of MII to G protein is slowed by 50% in 18:0,18:1PC relative to18:0,22:6PC. The addition of cholesterol doubles the lag time in complex formation in 18:0,18:1PC, whereas the lag time is essentially unchanged upon the addition of cholesterol to 18:0,22:6PC. In other experiments, it is shown that the PDE activity, a measure of the integrated visual pathway function, is also dependent upon acyl chain composition. Here again, 22:6n-3 phospholipids yield the highest levels of activity. The role of lateral domain formation in signaling systems was investigated using acyl chain specific fluorescence probes and fluorescence energy transfer techniques. These studies demonstrate that rhodopsin prefers an environment enriched in polyunsaturated acyl chain lipids relative to a more saturated phospholipid environment. Taken together, these studies indicate that 22:6n-3 containing phospholipids enhance the efficiency of a G protein-coupled signaling system and appear to impart unique structural properties to bilayers in the form of lateral domains.

RESEARCH HIGHLIGHTS: We have determined that alcohol and water compete for binding sites on the surface of both proteins and lipids in membranes. The binding of alcohol induces a change in structure of the membrane receptor, rhodopsin, resulting in an increase in the activity of this receptor. This effect is a combination of the direct binding of alcohol to both the receptor and the lipid in the membrane.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: These findings uniquely demonstrate that alcohol can modulate the activity of a membrane receptor through a mixed mode of action, effecting both the lipid and protein in the membrane. Many receptors involved in the signaling pathways of neurotransmitters in the nervous system are members of the same superfamily of receptors as rhodopsin. Therefore, it is highly likely that their activity is modulated by alcohol in a manner similar to what has been observed for rhodopsin.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00089-06

Title: MEASUREMENTS AND METABOLISM OF NEUROSTEROIDS IN THE CENTRAL NERVOUS SYSTEM

Staff Years: 1

Principal Investigator: HY Kim, PhD (MS, LMBB, NIAAA)

Other Lab Personnel: K Kevala, MS

NIH Collaborators: NIMH, CPB (D Rubinow, MD)
NINDS (M Rogawski, MD, PhD)

Extramural Collaborators: None

Sample Type: Human Tissues, Fluids, Cells, etc.

Keywords: mental health research, neurosciences, anxiety disorders, alcohol withdrawal, mass spectrometry, cerebrospinal fluid

SUMMARY: The principal objective of this study is to determine the effect of ethanol on the metabolism of neurosteroids in the central nervous system (CNS). Neurosteroids such as 5a-pregnane-3a-ol-20-1 (5a,3a-THP or Allopregnanolone) and 5a-pregnane-3a,21-diol-20-1 (THDOC) have been shown to modulate the GABA/benzodiazepine binding sites and to exert anxiolytic and hypnotic effects. Modulation of these neurosteroids in the CNS by ethanol may be one of the underlying mechanisms for stress observed in human alcoholics, especially during withdrawal.

The GC/MS-NCI technique that we previously established for the measurements of trace levels of neurosteroids in human cerebrospinal fluids (CSF) has been improved to include the analysis of progesterone and dihydroprogesterone and extended to the analysis of human and rat plasma samples. We were able to quantitate pregnenolone, 5a,3a-, 5b,3a-, 5a,3b-THP, androsterone and dihydrotestosterone, dihydroprogesterone and progesterone in those samples. Using this technique, we participated in NINDS investigators' development of a pseudopregnancy model of catamenial epilepsy. Investigations on the effect of progesterone metabolism on postpartum depression are now in progress in collaboration with NIMH.

RESEARCH HIGHLIGHTS: Docosahexaenoic acid positively modulates the enrichment of phosphatidylserine in neuronal membranes. Deficiency of docosahexaenoic acid or long-term ethanol treatment prevents the enrichment of phosphatidylserine in neuronal membranes. High levels of docosahexaenoic acid prevent neuronal cell death in adverse conditions.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: Long-term ethanol exposure may adversely affect neuronal survival, contributing to the neuropathological effects of ethanol.

Reporting Period:	10/01/1999 – 09/30/2000
Project Number:	Z01 AA00090-06
Title:	ROLE OF ALDH2--TRANSGENIC MICE CARRYING ASIAN ALDH2-2 VARIANT ALLELE
Staff Years:	1
Principal Investigator:	BJ Song, PhD (MB, LMBB, NIAAA)
Other Lab Personnel:	K Jeong, DVM, PhD Y Lee, PhD
NIH Collaborators:	NCI (FJ Gonzalez, PhD) NCI, LNMR (T Wang, PhD)
Extramural Collaborators:	None
Sample Type:	Neither Human Subjects nor Human Tissues
Keywords:	alcoholism, acetaldehyde, alcohol metabolism, health and behavior, animal models of organ damage, drinking patterns & causes, ethnicity, molecular genetics, transgenic mice, knock-out mice

SUMMARY: Organ damage caused by chronic alcohol consumption can be initiated, in part, by the accumulation of cytotoxic acetaldehyde in the target tissues, since highly reactive acetaldehyde, produced from ethanol metabolism, interacts with the free amino group of various cellular macromolecules including DNA and proteins, usually altering their physiological functions while initiating autoimmune responses. Accumulation of acetaldehyde can be achieved through inhibition of the major aldehyde-metabolizing enzyme, the mitochondrial aldehyde dehydrogenase 2 (ALDH2), by either chemical inhibitors or genetic mutation (G to A nucleotide substitution) with a subsequent change in Glu487Lys in the ALDH2 protein. Individuals with the genetic variation (ALDH2-2) possess reduced ALDH2 activity through dominant inactivation of the enzyme and show aversive reactions and facial flushing, due to acetaldehyde accumulation following alcohol consumption, as observed in many East Asian people. Although chemical inhibitors are frequently used in clinics, they cause many problems due to non-selective interactions with other enzymes and proteins, short duration of action due to rapid metabolism and numerous side effects. Because of the problems associated with the chemical inhibitors of ALDH2, we have taken genetic approaches using molecular biology techniques. We hypothesized that transgenic mice carrying the dominantly negative human ALDH2-2 (hALDH2-2) transgene or knock-out mice deficient in mouse ALDH2 gene would have reduced ALDH2 activity with elevated levels of acetaldehyde upon alcohol treatment, compared to their wild-type controls. Our results showed that the recombinant human ALDH2-2 variant protein interacted with the mouse ALDH2 protein and dominantly inhibited the activity of the mouse enzyme. In addition, we produced transgenic mice carrying the hALDH2-2 using a pcDNA3 vector containing the full-length cDNA for the hALDH2-2 coding region and the mitochondrial leader sequence under the control of cytomegalovirus promoter with a polyadenylation signal and transcription termination sequences from bovine growth hormone. Expression of the hALDH2-2 transgene in the liver and brain of transgenic mice was detected by RT-PCR of mRNA, enzyme assays and immunoblot analyses using polyclonal antibodies directed against ALDH2. To our surprise, hALDH2-2 protein was expressed at low levels in the liver and brain of transgenic mice. Subsequently, mouse ALDH2 enzyme activity was slightly inhibited in these animals. Although there was no significant difference in liver or brain pathology in mice treated with 20% ethanol (4 g/kg/day) for 2 weeks, hepatic acetaldehyde levels in transgenic mice were increased 50% at 2 h after ethanol injection ($p<0.03$) relative to control mice while hepatic alcohol concentrations were unchanged. In addition, female transgenic mice, but not males, drank less alcohol than did control mice ($p<0.03$). These transgenic mice carrying the hALDH2-2 variant can be used as a model to study the role of ALDH in alcohol preference, acetaldehyde accumulation, metabolism of neurotransmitters and acetaldehyde-mediated tissue damage after long-term alcohol treatment. Furthermore, to have clear results for the physiological roles of ALDH2, we have been trying to prepare knockout mice deficient in mouse ALDH2 using gene-by-gene disruption technique. During the past year, we were able to produce chimeric mice, which contain our DNA

construct specifically designed to delete the mouse ALDH2 gene. We are now identifying the homozygous mice without the mouse ALDH2 gene using restriction analyses and Southern blot analyses.

RESEARCH HIGHLIGHTS: Mitochondrial aldehyde dehydrogenase 2 (ALDH2) is the major enzyme (protein) involved in the metabolism of acetaldehyde produced from alcohol consumption. During the year, we characterized the properties of transgenic mice carrying the inactive human ALDH2-2 variant gene. Because of the dominant inactivation of mouse ALDH2 activity by the human ALDH2-2 variant, we observed 50% more accumulation of acetaldehyde in the livers of transgenic mice than the background mice without the human gene. In addition, we observed reduced voluntary alcohol drinking in female transgenic mice, possibly due to accumulation of acetaldehyde, an aversive compound for alcohol consumption. We also prepared mice deficient in mouse ALDH2 using gene-by-gene disruption technique.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: We have prepared transgenic mice and mice deficient in mouse ALDH2 gene. In these mice, we would expect to observe more liver and brain damage after long-term alcohol treatment, due to accumulation of toxic acetaldehyde. These mice would be a good animal model to study the alcohol-mediated tissue damage, drinking behavior and the role of this enzyme (protein) in the metabolism of endogenous substances such as neurotransmitters and lipid aldehydes. We hope that our biochemical and molecular studies will lead to the development of potential therapeutic agents or preventive measures against organ damage caused by long-term alcohol consumption or other toxic compounds We also hope that our findings will contribute to the prevention of alcohol-related medical disorders.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00099-05

Title: ION GRADIENTS AND METABOLIC ENERGY IN ANIMAL TISSUE

Staff Years: 2.1

Principal Investigator: RL Veech, MD, PhD (UMC, LMBB, NIAAA)

Other Lab Personnel: C Crutchfield
MT King, MS

NIH Collaborators: None

Extramural Collaborators: Oxford University (K Clarke, PhD)

Sample Type: Neither Human Subjects nor Human Tissues

Keywords: metabolism, ion gradients

SUMMARY: The energy of the gradients of the nine major inorganic ions in working perfuse heart are in near equilibrium with each other, the electrical potential between extra- and intracellular phase and the DG of ATP hydrolysis (Masuda T et al, J Biol Chem 1990;265:20321-34). The metabolism of ethanol increases the resting electrical potential of hepatocytes from 28 to 40 mV (Veech RL et al, Alcoholism Clin Expt Stud 1994;18:1040-56). We have completed a study of the effects of ethanol-induced change in hepatic voltage upon the gradients of all the amino acids between portal vein blood and liver and the gradients of the 9 major inorganic ions between intracellular and extracellular space in rat liver *in vivo*. To our knowledge, this is the first such study. Our work is also the first to show that the end product of hepatic ethanol metabolism, acetate, completely blocks the gut uptake of l-glutamine. l-Glutamine had been thought to be essential not only for its function as a building block for proteins, but also for its action as a bacterial barrier. The implication of the ability of acetate to inhibit the gut utilization of l-glutamine remains to be determined.

Previously, we showed that merely changing the substrate available altered the DG of ATP hydrolysis in heart (Kashiwaya Y et al, Am J Cardiol 1997;80:50-64A). Since injuries of any sort induce a stereotypic change in cellular ionic distributions wherein the cell gains Na+, loses K+ and swells, these stereotypic changes of injury can possibly be reversed by simple changes in the compositions of fluids administered to victims of injuries or burns. As a result of these studies and our suggestions to a panel convened by the Academy of Medicine, a recommendation has been made that investigation of the feasibility of making new resuscitation fluids be initiated (see: Fluid Resuscitation, *State of the Science for Treating Combat Casualties and Civilian Injuries*, National Academy Press, 1999). The goal is to improve the standard treatment of hemorrhage and burns, which has not changed over the past 50 years. We are collaborating in this effort.

RESEARCH HIGHLIGHTS: This research led the Institute of Medicine to issue a call for changes to be made in the compositions of fluids used to treat trauma and burns (See Fluid Resuscitation: *State of the Science of Treating Combat Casualties and Civilian Injuries,* National Academy Press, 1999). Subsequently the Office of Naval Research has instituted a multimillion-dollar program to create and test new parenteral fluids for use in trauma, burns and hemorrhage. The research has shown that standard fluid administration after hemorrhage leads to apoptosis in lung, the beginning of fatal multiple organ failure, while the new fluids stop this process.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: Ion gradients are central to the life of cells. The extent and direction of these gradients are determined by the metabolic energy of the cell. This energy and hence the ion gradients are changed by the parenteral fluids used in a side variety of medical and surgical therapies. This therapy can be improved by understanding the control of metabolic energy and fluid gradients.

Reporting Period: 06/18/2000 - 09/30/2000

Project Number: Z01 AA00105-01

Title: EFFECTS OF BILAYER COMPOSITION AND ETHANOL ON THE GABA(A) RECEPTOR

Staff Years: 0.25

Principal Investigator: DC Mitchell, PhD

Other Lab Personnel: None

NIH Collaborators: NIDDK, LCP, BNMR (A Bax, PhD)

Extramural Collaborators: University of Miami School of Medicine (GJ Turner, PhD)

Sample Type: Neither Human Subjects nor Human Tissues

Keywords: GABA(A) receptor, ethanol, receptor purification, lipid-protein interaction, signal transduction, neurotransmitter

SUMMARY: The first goal of this project is to use purified GABA(A) receptors reconstituted in liposomes to determine the membrane composition required for optimal receptor function. The second goal is to use the receptor-liposome system to investigate the action of acute ethanol on GABA(A) receptors, with and without postulated cofactors. GABA(A) receptors are among the most sensitive neuronal signaling systems to ethanol and clearly play a role in the neural adaptation that underlies ethanol dependence. GABA(A) receptor agonists mimic many behavioral effects of ethanol and behavioral effects of acute ethanol are sharply reduced by GABA(A) receptor antagonists. Changes in GABA(A) receptor function are associated with ethanol tolerance and dependence. The mechanism whereby acute ethanol exposure potentiates GABA(A) receptor activity remains controversial. Current proposals in the literature range from direct binding of ethanol to involvement of cofactors such as neurosteroids or protein kinase C.

Reconstitution studies have demonstrated that GABA(A) receptor activity is acutely sensitive to the composition of the membrane, although the specific requirements for optimal function have not been delineated. The first phase of this project will involve purification of GABA(A) receptor from bovine brain. We have begun to synthesize an agarose affinity resin that will be used to purify GABA(A) receptors from detergent-solubilized membranes. Conditions for optimal solubilization and reconstitution are under investigation. A stopped-flow fluorescence spectrometer will be used to measure single-turnover activity in real time. The second phase of this project will involve the purification and reconstitution of specific isoforms of human GABA(A) receptor expressed in insect (Sf-9) cells.

RESEARCH HIGHLIGHTS: This project has been underway for less than two months, thus we are in the process of optimizing the conditions required for purifying intact, functional GABA receptor from bovine brain.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: The goal of this project is use purified GABA(A) receptor to directly test molecular mechanisms for the effect of ethanol on this receptor, and to determine the membrane composition required for optimal function. The mechanism whereby acute ethanol exposure potentiates GABA(A) receptor activity remains controversial. Current proposals in the literature range from direct binding of ethanol to involvement of cofactors such as neurosteroids or protein kinase C. Detailed examination of single-turnover, real time activity of GABA(A) receptor under the controlled conditions afforded by a receptor-liposome system will enable a thorough analysis of ethanol-receptor interactions.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00110-03

Title: Metabolic Control Analysis

Staff Years: 0.9

Principal Investigator: RL Veech, MD, PhD (UMC, LMBB, NIAAA)

Other Lab Personnel: None

NIH Collaborators: None

Extramural Collaborators: Oxford University (K Clarke, PhD)

Sample Type: Neither Human Subjects nor Human Tissues

Keywords: metabolism, flux control, neurological disease, nutrition

SUMMARY: It is widely believed that the steps in the major metabolic pathways are known and that the control of flux through these pathways occurs at a very limited number of "rate limiting" steps. This concept has lead to the design of drugs to alter the kinetics of these "rate-limiting enzymes". It has also led to attempts to alter metabolic pathways by altering the amounts of rate limiting enzymes using the techniques of molecular biology. To the dismay of many, such interventions often fail to alter the rates of the pathways under study. These failures have led to an increased awareness that "control" of pathway flux is distributed among many enzymes of a metabolic pathway and can vary from enzyme to enzyme depending upon conditions. Metabolic control theory predicts distribution of control among many enzymes of a pathway (Veech RL and Fell DA: Cell Biochem Function 1996;14:229-36). However, actual demonstration and testing of such theories was technically difficult. We were the first laboratory to make the required measurements of flux, kinetic and thermodynamic constants of each step, and the levels of all substrates and products required to make such a formal analysis of flux control in a major metabolic pathway (Kashiwaya Y et al: J Biol Chem 1994;269:25502-14). We went on to show that ketone bodies could act in heart to overcome insulin resistance in heart (Kashiwaya J et al: Am J Cardiol 1997;80:50-64A). Since Dr. Kashiwaya left this laboratory, I have continued to collaborate with him and he, with others at the Department of Neurology of Tottori University in Yonago, Japan, have applied these insights from our previous work to investigate the effects of ketone bodies on two neuronal culture models of the two most common degenerative neurological diseases. Alzheimer's disease was modeled by adding amyloid beta 1-42 to embryonic rat hippocampal neuronal cultures and Parkinson's disease was modeled by adding MPP+ to mesencephalic neuronal cultures. In both case, ketone bodies protected neurons from death induced by these very different toxins. The ability of ketone bodies to protect neurons under these conditions offers the possibility of therapy for these very common diseases as well as other diseases resulting from failures in either glycolysis or mitochondrial energy generation. Our findings have been published (Kashiwaya Y et al: Proc Natl Acad Sci (USA) 2000;97:5440-4). Discussions of the uses of ketone bodies in the treatment of neurological diseases including refractory epilepsy and insulin resistance, such as Leprechaunism, was held in a Rare Disease Meeting at NIH on May 3, 2000. Work on metabolic control was done in collaboration with the Dept of Biochemistry, University of Barcelona and the Harbor UCLA Research Institute and was directed this year toward the hexosemonophosphate pathway and the role of thiamine in certain cancers (Cascante M et al: Nutrition & Cancer 2000;l36:150-4).

RESEARCH HIGHLIGHTS: We have shown (Kashiwaya Y et al: PNAS 2000;97:5440-4), in primary neuronal cultures, that supplementation with ketone bodies at the physiological level protect hippocampal neurons from death caused by amyloid b1-42 and the death of mesencephalic dominergic neurons exposed to MPP+. This strongly suggests that elevation of blood ketone bodies would provide effective therapy in Alzheimer's and in Parkinson's disease.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: Alzheimer's disease affects 2.5% of the population at age 65, 14% at 75 and 44% at 85. About 20% of the cases are attributable to defects in 1 of 5 genes. There is currently no effective therapy. Parkinson's disease has only minor genetic weighting in its etiology. Dietarily-induced mild ketosis may be an effective therapy in both forms of

neurodegeneration. In addition, this should be effective therapy in insulin-resistance including the extreme form of Leprechaunism, in Friedreich's ataxia, in refractory epilepsy and other orphan diseases such as LaFora body dementia.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00115-01

Title: ESSENTIAL FATTY ACIDS IN PSYCHIATRIC DISORDERS

Staff Years: 3

Principal Investigator: JR Hibbeln, MD (LMBB, NIAAA)

Other Lab Personnel: KM Makino, BA
D McMakin, BA

NIH Collaborators: NIAAA, LCS, NN (JD Higley, PhD)
NIAAA, LCS, BEI (DW Hommer, MD)
NIMH (W Fenton, MD)
NIDDK, DDB, LD (DW Herion, MD)
NICHD, LCE (M Champoux, PhD)
NICHD, BMSB (MA Klebanoff, MD)

Extramural Collaborators: The Stanley Foundation (M Knable, DO)

Sample Type: Human Subjects

Keywords: neurosciences, docosahexaenoic acid, omega-3 fatty acids, depression, violence, infant development

SUMMARY: The central question addressed in our studies is whether a low nutritional status of essential fatty acids (EFA) increases the predisposition to psychiatric disorders, including depression and schizophrenia, or to pathological behaviors such as homicide and suicide. Some EFA, in particular docosahexaenoic acid (DHA), are selectively concentrated in the brain, but these nutrients are ultimately available only from dietary sources, e.g., seafood, that is rich in omega-3 fatty acid. In an effort to elucidate EFA mechanism of action, we examined these questions from population comparisons in epidemiological studies, clinical interventional studies in adults with psychiatric disorders and observational studies of the relationship of EFA status to the function of key neurotransmitter systems. In addition to the status of adults, nutritional inadequacies in early development may contribute to an increased predisposition toward psychiatric disorders or abnormal behaviors. Thus, two developmental outcome studies were conducted. One was a four-decade follow-up study examining the maternal EFA status at time of birth and the lifetime risk of developing schizophrenia. The second, a nonhuman primate intervention study that examined the effects of supplementing infant formulas with DHA and arachidonic acid (AA) on the short- and long-term consequences to neurological development. The cross-national ecological studies that have compared the rates of seafood consumption to prevalence rates of psychiatric disorders indicate that there may be a specific relationship to prevalence rates of affective and impulsive disorders, but not to schizophrenia.

In a prior study, we reported that seafood consumption predicted lower prevalence rates of major depression ($r = -0.84$, $p<0.0005$) over a nearly 60-fold range across countries. Consistent with this report we found that bipolar spectrum disorders (manic depressive disorders) have a well-defined relationship to seafood consumption with an apparent threshold of about 75 lbs/person/y. We conducted each of these studies using data from the gold standard of psychiatric epidemiological studies, the Epidemiological Catchment Area Study. Two studies within countries yielded similar results. A study of 1,767 subjects within Northern Finland found that those who consumed fish twice a week or more were at lower risk of reporting depressive symptoms (odds ratio 0.63) and suicidal thinking (odds ratio 0.57), compared to infrequent fish consumers. Also, subjects who consumed vegetable oils were more than twice as likely to report being depressed compared to those with no consumption. This is significant because vegetable oils are high in omega-6 fatty acids that compete with the effects of omega-3 fatty acids. Similar results were found among a sample of 200 subjects that represented 80% of the elderly people in two counties in Iowa. Low plasma concentrations of DHA alone significantly predicted more severe sleep complaints and reports of anxiety as well as depression among women.

A series of three studies done by our group indicated that there is no relationship between EFA status and schizophrenia. 1) Across 14 countries, there was no significant relationship between prevalence rates of schizophrenia and seafood consumption using Epidemiological Catchment Area data. 2) These cross-national data are consistent with the results of a double blind, placebo-controlled, multi-center trial, conducted in collaboration with the Stanley Foundation, among 74 chronic schizophrenics. No clinical improvements were found when comparing consumption of supplements of 3 g/d of EPA to 3 g/d of mineral oil, for 4 months. 3) We examined the EFA composition of maternal plasma drawn on the day of birth to compare 51 control mothers to mothers of 27 children that developed psychosis over the next four decades. In contrast to the predicted hypothesis, the mothers of children who developed psychosis were not deficient in any EFA. These data suggest that there could be a specific relationship of seafood consumption and omega-3 status to prevalence rates of affective and impulsive disorders, but not to the prevalence of schizophrenia.

Three studies indicate that inadequate omega-3 fatty acid consumption may contribute to an increased risk of mortality from suicide and homicide. The findings were: that across 31 countries, greater seafood consumption predicted a lower risk of death due to suicide; that among suicide attempters, low concentrations of the omega-3 fatty acid, eicosapentaenoic acid (EPA), alone was robustly correlated with greater psychopathology scores; and that in a cross-national analysis of 26 countries, higher homicide mortality rates correlated with lower rates of seafood consumption ($r= -0.63$, $p<0.0005$).

One of the most replicated findings in biological psychiatry is that suicide is associated with low concentrations of a marker of central serotonin concentrations called CSF 5-HIAA. Regulating central serotonin concentrations is also the cornerstone of pharmacological therapy for major depression. Thus, we found that low plasma concentrations of omega-3 fatty acids predicted low concentrations of CSF 5-HIAA in healthy control subjects and late onset alcoholics, higher concentrations of plasma DHA predicted higher concentrations of CSF 5-HIAA. We replicated this finding among 104 adult rhesus monkeys that also showed that higher concentrations of the omega-3 fatty acids in plasma predicted higher concentrations of CSF 5-HIAA. Among these animals, higher EPA and DHA concentrations in plasma predicted more functional dominance behaviors. These findings suggest that omega-3 fatty acids may modulate impulsive behaviors through regulation of the serotonergic nervous system. The basic mechanisms by which EFA status might regulate serotonergic function merits further exploration.

We have begun studies to determine if these disorders are, in part, a long-term consequence of a nutritional deficiency during early development. In prior experiments, it has been established that separating rhesus infants from their mothers at birth and raising them in a nursery imparts a lifetime predisposition toward abnormal and aggressive behaviors. We noted that while in the nursery the infants are fed formulas that are virtually devoid of DHA and AA, which is similar to human infant formulas in this country. Thus, we compared two groups of infant rhesus monkeys that were removed from their mothers at birth and were raised in a stringently controlled nursery for the first six months of life. One group received standard infant formula while the other received formulas supplemented with AA (0.8%) and DHA (0.8%), a composition similar to the milk of rhesus monkey mothers. DHA/AA fed infants had profoundly improved motor development and visual orientation scores in as little as seven days. The heart rate variability, a measure of CNS function, remained improved in adolescence up to 3.5 years after the dietary intervention had stopped, indicating an enduring developmental effect. CSF 5-HIAA was decreased in the DHA/AA group, but only during the six months of formula feeding. We cannot determine directly whether the supplementation raised or lowered the brain concentrations of serotonin among these infants. However, the behavioral and physiological improvements, noted above, were consistent with improved serotonergic function.

RESEARCH HIGHLIGHTS: Seafood contains fats that the human body cannot make. These fats are important for optimal brain function. In countries where large amounts of seafood is consumed there are lower rates of major depression, postpartum depression and deaths due to homicide. We will give recovering alcoholics some of these omega-3 fats or a placebo and test to see if they become less depressed and/or less violent. Alcoholics are important to study because many are often depressed and/or violent and because alcohol decreases the amount of omega-3 fats in brain.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: This research is significant because the amounts of omega-3 fats in our diets has decreased markedly over the last century and is very different form the diets we consumed during evolution. If treatment with these fats does, in fact, reduce violence or depression, we can make better choices on what fats to include in modern diets.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00235-18

Title: NUTRITIONAL EFFECTS ON ESSENTIAL FATTY ACID COMPOSITION

Staff Years: 7.21

Principal Investigator: N Salem Jr, PhD (NS, LMBB, NIAAA)

Other Lab Personnel:
A Ahmad, PhD
ST Chaklos
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S Lim, PhD
JD Loewke
K McBride, BA
T Moriguchi, PhD
M Murthy, PhD
J Nicklay-Catalan, PhD
H Weisinger, PhD

NIH Collaborators: None

Extramural Collaborators: USUHS, Bethesda (J Curtis, MD)

Sample Type: Human Subjects

Keywords: neurosciences, cirrhosis, liver disease, fatty acids, nutritional disorders, prevention strategies, fetal alcohol exposure, docosahexaenoic acid, arachidonic acid

SUMMARY: Past studies have indicated that alcohol abuse leads to a loss of docosahexaenoate (DHA), the major polyunsaturate in the nervous system. Nutritional inadequacies, particularly during early development, may also lead to such losses in this essential fatty acid (EFA). In following up this work, it is important to establish what losses in physiological functions are caused by the loss of DHA in various organ systems. In collaboration with several investigators at Wayne State University, the relationships of alcohol intake during pregnancy is related to the mother's and newborn infant's EFA and vitamin status. Dietary information is collected from the mothers in order to ascertain whether alcohol affects food selection or has a more direct metabolic effect in mediating potential losses in blood stream essential fats. Initial work on 225 women at their first prenatal care visit indicates that alcohol intake inversely correlates with plasma vitamin E content. A novel application to the field of EFA biology was made with the introduction of olfactory-based learning and memory-related tasks for brain function assessment. This modality was used since Slotnick has reported that rats are capable of high level learning of olfactory-based tasks of a nature usually only ascribed to non-human primates or higher mammals. Our principal findings are that there is poorer performance in the acquisition of olfactory set learning in rats where brain and olfactory bulb DHA was lowered through dietary insufficiency. That is, after the rats had acquired the task, they were over-trained in order to determine whether they could achieve the learning set, i.e., make zero or only one mistake in the first twenty trials after an information trial in a two-odor discrimination task. Rats given a safflower oil-based diet for two generations were significantly poorer in this regard than rats to which diets contains the oils alpha-linolenate and DHA were added. Animals with lower levels of brain DHA performed more poorly on spatial maze tasks using the Morris Water Maze. The n-3 deficient rats swam longer and at a higher rate, but found the platform with a longer latency. In a memory retention trial, n-3 deficient rats performed significantly worse than the n-3 adequate group, especially when deprived for three generations. Although n-3 deficient rats perform more poorly, it cannot be ascribed to lower activity or motivation as general motor activity was not different between groups and there was no difference in a progressive-ratio licking task in which animals worked for a water reward. Also, the n-3 deficient rats sampled the odors longer than the DHA-adequate animals but still made more subsequent total errors. The n-3 deficient rats were examined for changes in

brain morphology using quantitative stereological techniques. Initially, studies focused on hippocampal morphology. After fixing and cresyl violet staining, a variety of features were quantified in rats fed the n-3 deficient or adequate diets for three generations. No statistically significant differences were observed in volume, density or total number of perikaryal neurons in the hippocampus. However, perikaryal size in the septal area of the CA1 and CA2/3 fields of the hippocampus were greater in the n-3 adequate (containing DHA) group relative to the deficient group. Similar findings have now been obtained for two other brain areas, the piriform cortex and the subfornical organ. The potential implications for infant nutrition are great as it may be surmised that infants fed formulas available in North America may have similar structural changes in brain. Once these deficiencies in brain function were established relating to DHA status, it was of interest to determine whether they were reversible. For this purpose, n-3 deficiency was induced with a safflower oil-based diet over three generations. The offspring were then replete with a diet containing flax oil (supplying alpha-linolenate) and DHA at various times in development, i.e., at birth, weaning and young adulthood (7 wks) and tested on spatial task performance beginning at 9 or 13 weeks of age. Our findings were that rats replete at birth (by cross-fostering) or at weaning had substantially recovered their brain DHA by 7 weeks of age and their spatial task performance was similar to that of n-3 adequate animals. When animals were replete at young adulthood, their degree of recovery in both respects depended upon the duration of dietary repletion. When assessed at 9 weeks of age, neither brain DHA nor spatial task performance had recovered. However, when assessed at 13 weeks, DHA had substantially recovered and spatial task performance had also substantially recovered such that it was not significantly different from that of n-3 adequate animals. These are important results as they indicate that intervention with a DHA-rich diet can reverse some of the adverse consequences for brain development caused by an inadequate diet early in development. In order to expedite future studies of n-3 deficiency and to make them more relevant to the human condition, a model of deficiency based on the first generation was developed. This employs the auto-rearing method of Hoshiba for rat pups starting from the first day of life. This approach has now succeeded in producing live animals and ones extremely deficient in brain DHA when artificial milk is fed that has very low levels of n-3 fats. Rats of 29 days in age were found to exhibit a 73% loss in brain DHA. Thus infants who receive no n-3 fats from infant formula may be expected to also have a severe drop in their brain DHA during this period of rapid brain growth. This model will also provide for a great savings in the use of animals and in the time needed to produce a model of n-3 deficiency. This apparatus will make possible a new model of fetal alcohol syndrome where animals can be given alcohol from the first days of life.

RESEARCH HIGHLIGHTS: Infants in the United States who are formula-fed receive very little of certain essential fats, termed omega-3 fats. One of these, called DHA, is important for optimal brain development and is always present in human milk. Our studies in rats that are deprived of omega-3 fats during early development indicate their brain levels of DHA drops dramatically. Associated with this decline in essential brain fats is a loss in set learning in an olfactory-based task, a measure of cognition.

In the search for animal models of omega-3 fatty acid deficiency, we developed an artificial breast and artificial milk that is nearly devoid of sources of omega-3 fats. This method of "automatic feeding" allows a rat to feed from a silicone nipple containing the artificial milk from the third day of life; the animal is fed manually during the first three days. Thus we can now control the nutrient intake for essential fats throughout the entire life cycle. By four weeks of age, rats fed a diet without omega-3 sources have a profound (>70%) loss of brain DHA.

We know from past animal studies that alcohol abuse can lead to a decline in an essential brain membrane component, called DHA. Smoking and low dietary intake of a pregnant women are also factors expected to lead to a decline in fetal DHA accretion. Therefore, an investigation of the EFA status of pregnant women is being conducted with a focus upon alcohol consumption. Our first results suggest that a loss of vitamin E occurs in the maternal circulation when more alcohol is consumed.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND TO PROGRAM: One of the key actions of alcohol is to lower levels of tissue EFA, particularly omega-3 fats like docosahexaenoate (DHA). Present studies provide new models of efficient ways to produce similar deficiencies through dietary control. They further demonstrate effects on brain function through the use of behavioral tasks. An attempt is being made to extend this work to include the EFA status of pregnant women who abuse alcohol.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00262-16

Title: DESATURATION OF ESSENTIAL FATTY ACIDS USING STABLE ISOTOPE GC-MS

Staff Years: 3

Principal Investigator: N Salem Jr, PhD (NS, LMBB, NIAAA)

Other Lab Personnel: JR Hibbeln, MD
Y Lin, PhD
S Majchrzak, BS
RJ Pawlosky, PhD
BJ Wegher, MS

NIH Collaborators: None

Extramural Collaborators: Oregon Health Sciences University (W Connor, MD)

Sample Type: Human Subjects

Keywords: neurosciences, liver disease, vision/eye/ocular, Retinitis Pigmentosa

SUMMARY: Prior to the recent application of stable isotope based GC/MS methodology, little was known about human essential fatty acid (EFA) metabolism *in vivo*. Our studies focused on the metabolic capacities of infants in the first week of life and also on that of human adults. The first phase of this work defined the conversion of linoleic acid to arachidonate and also the conversion of alpha-linolenate to docosahexaenoate in infants of varying gestational ages. The somewhat surprising results were that essentially every infant was capable of both n-3 and n-6 fatty acid interconversions *in vivo*. Moreover, there was an inverse relationship of gestational age with plasma deuterium enrichment of DHA, in particular; i.e., the least developed infants had the greatest metabolic capability in this respect. This is consistent with the brain growth spurt that occurs in human fetuses during the last trimester. Infants who were small for gestational age had a somewhat diminished metabolic capacity for fatty acids but most of the variance could be explained by gestational age only. In our adult work, normal volunteers, smokers and alcoholic smokers were studied for EFA interconversions *in vivo*. Controlled diet studies indicated that increasing the long chain n-3 fatty acids in the diet led to a decrease in *in vivo* accretion of deuterated fatty acid end products in plasma. This is consistent with the well-known phenomenon of end product inhibition. Smokers produced increased amounts and had greater enrichments of deuterated AA and DHA relative to normal non-smokers. Alcoholic-smokers had a marked increase in deuterium enrichments of long chain polyunsaturates in plasma, particularly DHA. In alcoholics with liver fibrosis, deuterium enrichment of DHA in liver biopsy samples was also increased relative to alcoholics without liver histopathological findings. These results are significant, as they do not support the commonly held notion in the field that alcohol inhibits elongation/desaturation of EFA. In fact, a hypothesis where alcohol stimulates this pathway would be more consistent with our results. Our hypothesis is that alcohol leads to catabolism of long chain polyunsaturates like DHA. When the alcohol challenge is of sufficient intensity and duration, this will lead to a decrease in the tissue concentration of DHA. Metabolic processes including elongation/desaturation and transport/acylation may be increased in the alcoholic in partial compensation for the loss of these important membrane constituents. Our recent studies have examined the *in vivo* metabolism of EFA in patients with Retinitis Pigmentosa (RP). In particular, patients with Ushers II disease or non-Ushers disease were compared to normal volunteers. We observed that the amount or enrichment of deuterated n-3 fatty acid metabolites such as EPA or DHA were significantly increased in Ushers patients whereas there was a decrease relative to normal volunteers in the non-Ushers RP group. The increased metabolism in the Ushers patients with respect to DHA may be surprising as it has been hypothesized that the retinal concentration of DHA is reduced in Retinitis Pigmentosa and that this may, in part, explain some of the loss in visual function associated with this neurological disease. However, as noted above for alcoholic patients, increased metabolism may be induced by increased catabolism that is associated with the disease state. These studies point to the need for analysis of increased fatty acid catabolism or indices of lipid peroxidation *in vivo* in these patients. The opposite direction of response in the non-Ushers patients points to a quite distinct etiology

of this disease. Progress has been made during this reporting period in developing a novel multiple-isotope technique that we have termed the MultiplE Simultaneous Stable Isotopes, or MESSI, for short. This technique was invented to address the difficult problem of determining the relative efficacy of metabolism of various substrates along a pathway of fatty acid metabolism involving multiple steps. An old and intractable problem has been the direct comparison of metabolism, for example, of linoleate vs that of gamma-linolenate vs dihommo-gamma-linolenate to form arachidonate. Using the *in vivo* stable isotope approach and employing NCI GC/MS, one can simultaneously perform the deconvolution of various isotopomers of arachidonate from multiple precursors providing that suitable isotopes are selected to give a significant mass difference, e.g., 5 daltons or more. In the present experiments, rats were given an oral dose of oil containing the following isotopes: 13-C-U-18:2n6, D5-20:3n6, D5-18:3n3, 13-C-U-20:5n3. It was demonstrated that both n-6 fatty acid isotopes were converted to 20:4n6 and that they could be simultaneously measured. In the same animal, the n-3 pathway could also be assessed, both with respect to the 18-carbon and 20-carbon precursor conversions to 22:5n3 and 22:6n3. Thus, the need for four or more separate groups of animals are obviated by this approach with better control since the conditions in separate animals can never be as similar as two comparisons within the same animal at the same time. Moreover, this approach can be directly applied to human experimentation due to the use of safe stable isotopes. In particular, the approach will facilitate, indeed make possible, the study of the EFA metabolism of 18- vs 20-carbon fatty acids in human infants.

RESEARCH HIGHLIGHTS: Certain essential fats, termed omega-3 fats, are important for optimal nervous system development, particularly one called DHA. DHA is always present in human milk but formula only contains a fat that may be converted in the body to DHA. This study examined whether newborn human infants are capable of performing this conversion in their livers. It was found that they were indeed capable of forming DHA from its precursor during the first week of life, even when very premature. However, an estimation of the amounts produced suggested that it was inadequate to supply the amounts of DHA that the brain normally requires for its growth.

We know from past animal studies that alcohol abuse can lead to a decline in an essential brain membrane component, called DHA. A direct in vivo study was performed in adult alcoholics to determine how their essential fat metabolism was altered during withdrawal. It was observed that their circulating DHA was lower than smoking or non-smoking reference groups, however, their metabolism of DHA appeared to be increased, perhaps in an effort to restore lost DHA. Fat break down is also much greater in alcoholics and this may explain why the increased formation of DHA cannot keep up with demand. It is hypothesized that the loss of brain and liver DHA is related to the loss in organ function and this may be partially compensated by the preventative or therapeutic strategies of increasing dietary DHA.

A new method has been invented by which essential fat metabolism can be measured in animals or humans using stable isotope labeled compounds. Stable isotopes are ones that are not radioactive and thus are safe for human consumption. The novel development in this project has been the ability to introduce four or more of these labels simultaneously so that the efficiency of various precursors can be evaluated for their metabolic potential.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND TO PROGRAM: One of the key actions of alcohol is to lower levels of tissue essential fatty acids, particularly omega-3 fats like docosahexaenoate (DHA). Traditionally, it has been thought that alcohol accomplished this by preventing fats from being metabolized to ones used by the brain and liver. Our study shows that this is likely wrong because alcoholics on withdrawal, at least, do not respond in this manner. In fact, they respond in the opposite manner, increasing their fat metabolism. This appears to be an adaptive response to the enormous fat breakdown that is induced by chronic alcohol abuse. The combination of smoking and alcoholism accentuate this increased fat metabolism and loss of tissue essential fats. New methods will help to expedite human work for both normative nutrition and for the case of alcoholics.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00284-11

Title: ALTERATIONS IN LIPID METABOLISM IN THE NERVOUS SYSTEM BY ETHANOL

Staff Years: 4

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NIH Collaborators: None

Extramural Collaborators: None

Sample Type: Neither Human Subjects nor Human Tissues

Keywords: docosahexaenoic acid, apoptosis, Neuronal cells, phosphatidylserine, melatonin, ethanol, cytosolic PLA2, lipoxygenase, pineal, mass spectrometry

SUMMARY: We previously found that 22:6n-3 promotes the accumulation of phosphatidylserine (PS) and prevents apoptotic neuronal cell death. During this period, the effect of polyunsaturates on the survival of neuronal cells was investigated along with the underlying mechanisms of this effect. We demonstrated that 22:6n-3 prevented the apoptotic cell death of both Neuro 2A and PC-12 cells, only after a prolonged period of enrichment, suggesting that the protective effect of 22:6n-3 may be exerted as a membrane phospholipid constituent. Under conditions where PS accumulation is inhibited, 22:6n-3 enrichment was not effective. In addition to PS accumulation, growth factor signaling pathways such as Raf-1 and PI3-kinase appeared to be important for the protective effect of 22:6n-3. Translocation of Raf-1, an important upstream kinase transducing growth signaling, to membrane was significantly enhanced in 22:6n-3 enriched cells. According to *in vitro* biomolecular interaction analysis, the interaction between unilamellar vesicles of phospholipids and Raf-1 kinase required the presence of PS in the vesicle and the extent of interaction was indeed dependent on the PS composition. During n-3 fatty acid deficiency or chronic alcohol exposure, we found that the PS content can be markedly decreased, specifically in neuronal cells where 22:6n-3 was highly enriched. These results support the view that PS accumulation promoted by 22:6n-3 plays an important role in growth factor signaling and deprivation of this fatty acid either due to n-3 fatty acid dietary deficiency or to chronic alcoholism may have adverse effects on neuronal survival.

We also found that melatonin, the major product of the pineal gland, affects polyunsaturated fatty acid metabolism by down-regulating cytosolic PLA2 at gene and protein expression levels. These data suggest that polyunsaturated fatty acids may be involved in biochemical functions of melatonin.

RESEARCH HIGHLIGHTS: Docosahexaenoic acid (DHA) positively modulates the enrichment of phosphatidylserine in neuronal membranes. Deficiency of DHA or long-term ethanol treatment prevents the enrichment of PS in neuronal membranes. High levels of docosahexaenoic acid prevent the neuronal cell death in adverse conditions.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: Long-term ethanol exposure may adversely affect neuronal survival, contributing to the neuropathological effects of ethanol.

FY 2000 ANNUAL REPORT SUMMARIES

(1 OCTOBER 1999 - 30 SEPTEMBER 2000)

LABORATORY OF MOLECULAR & CELLULAR NEUROBIOLOGY

FORREST F. WEIGHT, M.D., CHIEF

DIVISION OF
INTRAMURAL CLINICAL & BIOLOGICAL RESEARCH
NATIONAL INSTITUTE ON ALCOHOL ABUSE & ALCOHOLISM
NATIONAL INSTITUTES OF HEALTH

SYNOPSIS
LABORATORY OF MOLECULAR & CELLULAR NEUROBIOLOGY
1 OCTOBER 1999 - 30 SEPTEMBER 2000

INTRODUCTION

In recent years, great progress has been made in understanding the function of the central nervous system (CNS) at the cellular and molecular level. The Laboratory of Molecular & Cellular Neurobiology (LMCN) was established to utilize this increased knowledge of neurobiology to investigate the cellular and molecular mechanisms of alcohol action in the nervous system. The investigations in LMCN are intended to provide a foundation for understanding the cellular and molecular basis of alcohol's behavioral effects, such as intoxication, anesthesia and addiction, as well as pathophysiologic phenomena such as the neurotoxicity of alcohol, that results in cerebral atrophy, and alcohol-induced alterations in neural development, that manifest as fetal alcohol syndrome.

Administratively, LMCN has three sections: the Section on Physiology, the Section on Pharmacology, and the Section on Molecular Neuroscience. Dr. Forrest Weight is Chief, Section on Physiology, and Acting Chief of the other two sections. Dr. Robert Peoples was appointed tenure-track investigator in February 1998; he is Acting Chief, Unit on Cellular Neuropharmacology, in the Section on Pharmacology. On the recommendation of the NIAAA Board of Scientific Counselors in 1998, a nationwide search for a tenure-track cellular neurophysiologist was carried out in Fiscal Year 1999; however, as the search committee did not reach a consensus on the candidates an appointment was not made. Because the NIAAA Board of Scientific Counselors also recommended recruitment of a tenure-track and/or tenured molecular neurobiologist, a combined search for a cellular neurophysiologist and/or molecular neurobiologist was planned for the end of Fiscal Year 2000; however, the research space that had been reserved for those recruitments was not available at that time, so that those recruitments are on hold until space is available.

The Section on Physiology investigated the neuronal actions of alcohol and provided further evidence that neurotransmitter receptors are cellular sites of alcohol action in the nervous system. This evidence is an important advance in alcohol research, as it permits detailed investigation of the cellular and molecular mechanisms of alcohol actions at these sites. Moreover, since neurotransmitter receptors mediate communication between neurons at synapses, the demonstration that alcohol can affect the function of these receptors suggests that these actions may underlie some of the behavioral effects of alcohol. The progress that has been made in these studies suggests that such physiological approaches will advance our knowledge of the cellular basis of alcohol actions in the nervous system.

In the Section on Pharmacology, Dr. Peoples investigated the pharmacology of alcohol actions in the nervous system. His studies have provided evidence that alcohols affect the function of NMDA receptors by acting on an extracellular site, and suggest that the "cutoff effect" for NMDA receptors is due to the insolubility of long-chain alcohols, rather than to a size exclusion mechanism.

The Section on Molecular Neuroscience used molecular biological approaches to investigate the molecular basis of alcohol action on neurotransmitter receptors. Studies in this Section have provided evidence that one of the determinants of alcohol sensitivity of neurotransmitter receptors is the molecular structure of the receptor. In addition, studies are in progress on the molecular regulation of alcohol sensitivity of neurotransmitter receptors and the molecular sites of alcohol action on these proteins. These studies hold the promise that such molecular biological approaches will advance our knowledge of the molecular basis of alcohol actions in the nervous system.

SECTION ON PHYSIOLOGY

Research studies in the Section on Physiology are directed toward elucidating the cellular mechanisms of alcohol action in the nervous system. The behavioral effects of alcohol are well known; however, the mechanisms by which alcohol produces those effects have not been established. The investigations in this Section use primarily electrophysiological methods, such as the patch-clamp technique, to study the function of membrane receptors and ion channels in neurons and neural cell-lines, and the actions of alcohol on those membrane proteins.

In experiments on voltage-gated membrane ion channels in adult mammalian neurons, a number of different types of these ion channels, which underlie the intrinsic electrical excitability of neurons, were found to be insensitive to intoxicating and anesthetic concentrations of ethanol (10 -100 mM). By contrast, a number of different types of neuronal neurotransmitter-gated membrane ion channels were found to be sensitive to this range of ethanol concentrations. This type of neurotransmitter receptor mediates fast excitatory or inhibitory synaptic transmission between neurons in the CNS. The effects of ethanol on these ion channels are summarized briefly below.

Excitatory Amino Acid-Activated Channels: Glutamate is the major excitatory neurotransmitter in the mammalian CNS. Glutamate activates at least three types of neurotransmitter-gated membrane ion channels, designated by their responses to the agonists, NMDA , AMPA and kainate.

NMDA receptor-channels mediate a slower component of synaptic excitation and are thought to be involved in several types of important neural phenomena including: cognitive function, motor control, synaptic plasticity and certain types of learning. In hippocampal neurons, ethanol inhibits the function of NMDA receptors in a concentration-dependent manner in a concentration range associated with intoxication. In addition, the potency of several short-chain alcohols for inhibiting NMDA receptor function is correlated with their intoxicating potency, suggesting that ethanol-induced inhibition of NMDA receptor function may contribute to the neural and cognitive impairments associated with intoxication. Investigation of the mechanism involved in the inhibition of NMDA receptor function by ethanol indicates that ethanol inhibits NMDA receptor function by a non-competitive mechanism that does not involve block of the ion channel, alteration of the ion selectivity of the channel or interaction with a number of regulatory sites on the receptor. Single-channel experiments indicate that ethanol inhibits NMDA receptor function by altering channel gating. In addition, straight-chain alcohols inhibit NMDA receptor function with increasing potency as the carbon chain-length is increased up to eight carbon atoms; however, straight-chain alcohols with more than eight carbon atoms do not affect NMDA receptor function. This "cutoff" for inhibition of NMDA receptor function by a series of straight-chain alcohols is similar to cutoffs reported previously for two behavioral indices of intoxication, ataxia and loss-of-righting reflex, suggesting that alcohol inhibition of NMDA receptor function may contribute to the behavioral manifestations of alcohol intoxication.

AMPA and kainate receptors (non-NMDA glutamate receptor-channels) mediate fast transmission at most excitatory synapses in the CNS. In concentrations less than 50 mM, ethanol has relatively little effect on the function of these receptors in neurons. In concentrations greater than 50 mM, however, ethanol produces a concentration-dependent inhibition of these receptors. Blood-ethanol concentrations from 50-100 mM are associated with the signs of general anesthesia. Since ethanol inhibits the function of non-NMDA glutamate receptor-channels over this concentration range, and these channels mediate fast synaptic transmission at most excitatory synapses in the CNS, it seems possible that the ethanol inhibition of these receptors may contribute to the general anesthetic effects of ethanol. This hypothesis is supported by the observations that the general anesthetic agents, trichloroethanol (the active metabolite of chloral hydrate), barbiturates and volatile general anesthetics, all inhibit the function of non-NMDA glutamate receptor-channels in an anesthetic concentration range.

Inhibitory Amino Acid-Activated Channels: The major inhibitory neurotransmitter in the brain is gamma-aminobutyric acid (GABA). Some laboratories have reported that GABA-gated membrane ion channels (GABA-A receptors) are sensitive to pharmacologic concentrations of ethanol (10 - 100 mM), whereas other labs have reported that they are not. It has been proposed that the reason for these divergent observations is that protein kinase C (PKC)-induced phosphorylation of the gamma-2-L subunit is required for this receptor to be sensitive to ethanol. We studied this hypothesis on GABA-A receptor-mediated responses in rat dorsal root ganglion (DRG) neurons (the presence of the gamma-2-L subunit was confirmed using RT-PCR) and found that GABA-A receptor-mediated responses remain insensitive to ethanol concentrations from 2.5 to 100 mM despite: (i) extracellular application of the PKC-activator, phorbol ester; (ii) raising intracellular Ca^{2+} to activate PKC; (iii) intracellular application of PKC; and (iv) extracellular application of phorbol ester plus intracellular application of protein phosphatase inhibitors to prevent breakdown of PKC. The observations do not support the hypothesis that PKC phosphorylation of the gamma-2-L subunit induces ethanol sensitivity of GABA-A receptors. We also studied the alcohol sensitivity of GABA-A receptors in mouse hippocampal neurons and found that straight-chain alcohols from ethanol to dodecanol enhance GABA responses, whereas higher alcohols have no effect. The EC50 for ethanol is 2,160 mM (approximately 20 times the lethal concentration in non-tolerant humans). The n-alcohol potency curve for enhancement of GABA-A receptor function is similar to the n-alcohol potency curve for anesthesia in tadpoles. In addition, the anesthetic potency of n-alcohols in rats more

closely reflects NMDA receptor modulatory potency for short-chain alcohols, such as ethanol, and GABA-A receptor modulatory potency for long-chain alcohols. Experiments are currently in progress on the ethanol sensitivity of GABA-A receptors in different types of central neurons.

Serotonin-Activated Channels: It has been suggested that serotonin type 3 (5-HT3) receptors may be involved in alcohol's reward mechanisms. In neurons and neural cell-lines, ethanol potentiates 5-HT3 receptor-mediated responses, at low agonist concentrations, in a concentration-dependent manner over the concentration range 10 - 100 mM. However, the potentiation by ethanol decreases with increasing serotonin concentration, suggesting that ethanol may increase the potency of serotonin action. Investigations are currently in progress to determine the mechanism involved in this ethanol-induced potentiation of 5-HT3 receptor functions. In addition, straight-chain alcohols potentiate 5-HT3 receptor function with increasing potency as carbon chain-length is increased and a "cutoff effect" has been observed; however, it appears that the "cutoff" may be affected by agonist concentration so experiments are in progress to determine the nature of the "cutoff" phenomenon for 5-HT3 receptors.

ATP-Activated Channels: Extracellular ATP (adenosine 5'-triphosphate) has been found to function as an excitatory neurotransmitter and to gate membrane ion channels in neurons in both the central and the peripheral nervous system. There are several types of ATP-gated membrane ion channels based on molecular biological cloning, as well as physiologic and pharmacologic properties of the receptors. Ethanol has been found to inhibit the function of a neuronal ATP-gated channel over a pharmacologic concentration range, with an IC50 value of 68 mM. Methanol is less potent and 1-propanol is more potent in inhibiting the ATP-activated current; however, 1-butanol and 1-pentanol are without effect on this current. In addition, the intracellular application of 100 mM ethanol does not affect the inhibition by extracellularly applied ethanol, the inhibition is not affected by the intracellular application of activators or inhibitors of G-proteins, and the inhibition is not affected by activators or inhibitors of protein kinase A (PKA) or protein kinase C (PKC). The mechanism of ethanol inhibition of these ATP-gated channels differs from the mechanism of ethanol inhibition of NMDA receptors. For NMDA receptors, ethanol decreases the maximal response (Emax) of the agonist concentration-response curve without affecting the EC50, whereas for the ATP-gated channels, ethanol shifts the ATP concentration-response curve to the right in a parallel manner, increasing the EC50 for ATP without altering Emax. Thus, the effect of ethanol on the function of ATP-gated channels appears to be a competitive type of inhibition; however, an ethanol-induced decrease in the affinity of the agonist-binding site would also result in a parallel shift to the right of the agonist concentration-response curve. To distinguish between these mechanisms, the effect of ethanol was studied on the activation and deactivation kinetics of ATP-activated current. Ethanol decreases the time-constant of deactivation of ATP-activated current, without affecting the time-constant of activation. The observations suggest that ethanol does not competitively antagonize ATP activation of the receptor, but rather ethanol has an allosteric action to decrease the affinity of the ATP binding site on the receptor. Studies are currently in progress on the physiological regulation of the ethanol sensitivity of ATP-gated channels and the effect of ethanol on ATP-activated currents in CNS neurons.

Acetylcholine-Activated Channels: The muscle-type nicotinic acetylcholine (nACh) receptor is the most extensively studied and characterized neurotransmitter-gated membrane ion channel. However, the effect of ethanol on the function of these receptor-channels has not been well characterized with modern electrophysiological techniques. Therefore, we studied the action of ethanol on this receptor, using patch-clamp recording with a very fast extracellular solution exchange system, in order to gain insight into the structure-activity relationships of ethanol action. In studies on mouse nACh receptors containing alpha-1/beta-1/delta/epsilon subunits, ethanol concentrations from 10-150 mM produce a concentration-dependent potentiation of currents activated by low concentrations of ACh. Associated with the ethanol-induced potentiation is an increased desensitization rate of the current. However, with ACh concentrations greater than 25 mM, ethanol reduces peak current amplitude, presumably due to the rapid onset of desensitization, as occurs with high agonist concentrations. Investigations on the mechanisms involved in these ethanol actions are currently in progress. In addition, the effect of ethanol on acetylcholine responses in CNS neurons is being studied.

Summary and Conclusions: The studies in the Section on Physiology have provided evidence that neurotransmitter-gated membrane ion channels are cellular sites of alcohol action in the nervous system. These studies have also shown that alcohol effects on the function of different types of neurotransmitter-gated membrane ion channels can involve different specific mechanisms. This is in contrast to the belief, for over 90 years, that alcohols exert their effects in the nervous system through a non-specific action on membrane lipids. Studies in this Section have also found that a series of straight-chain alcohols exhibits

a potency cutoff for affecting the function of all of the neurotransmitter-gated channels that have been tested. In addition, the cutoff has been found to be different for each receptor type that has been studied, suggesting that the molecular determinants of the cutoff effect may be different for different types of receptors. Studies are in progress on the physiological regulation of alcohol sensitivity of these receptors. The progress that has been made in these studies suggests that such physiological approaches will advance our knowledge of the cellular basis of alcohol action in the nervous system.

SECTION ON PHARMACOLOGY

The research in the Section on Pharmacology is directed toward elucidating the pharmacology of alcohol actions in the nervous system. As noted above, straight-chain alcohols with eight or fewer carbon atoms inhibit NMDA receptor function, whereas larger straight-chain alcohols do not affect the function of these receptors. This "cutoff effect" was thought to result from a size-exclusion mechanism; i.e., the alcohols act by binding in a hydrophobic pocket of circumscribed dimensions, so that alcohols that are larger than the size of this pocket are unable to bind in the pocket and therefore have no effect. To assess this hypothesis, Dr. Peoples tested the effect on NMDA receptor function of a series of straight-chain 1,omega-diols, which have lower hydrophobicity but slightly greater molecular volume than the corresponding straight-chain alcohols. He found that diols with nine or ten carbon atoms were able to inhibit NMDA receptor function, despite having molecular volumes greater than the corresponding straight-chain alcohols. This result suggests that the cutoff effect for NMDA receptors most probably results from the inability of long-chain alcohols to achieve adequate concentrations at the site of alcohol action due to low aqueous solubility, rather than from a size-exclusion mechanism. Dr. Peoples also performed experiments to identify the location of the site of alcohol action on NMDA receptors. He found that the intracellular application of 1-pentanol did not inhibit NMDA receptor function, nor did it alter the inhibitory effect of extracellularly applied ethanol or pentanol. In addition, the application of ethanol to the cytoplasmic face of inside-out membrane patches did not alter NMDA-activated current. The inhibitory effect of extracellularly applied ethanol was also not altered by truncation of the intracellular C-termini of the NR1/NR2B subunits of the NMDA receptor. The results suggest that the alcohol action involves an extracellular domain of the NMDA receptor.

SECTION ON MOLECULAR NEUROSCIENCE

The research activities in the Section on Molecular Neuroscience are directed toward understanding alcohol actions in the nervous system at the molecular level. This Section uses a combination of molecular biological and electrophysiological research methods to address these questions. In the studies carried out in the Section, alcohol effects have been studied on the physiology and pharmacology of recombinant neurotransmitter receptors using primarily *Xenopus* oocytes as an expression system and two-electrode voltage-clamp to study the function of these recombinant receptors. Those studies are summarized briefly below.

Recombinant NMDA Receptors: Although ethanol inhibits NMDA receptor function in a number of regions of the CNS, the sensitivity of NMDA receptors to ethanol has been found to be different in different brain regions and in different types of neurons. Cloning studies have revealed a molecular diversity of NMDA receptors and *in situ* hybridization indicates a differential distribution of different subunits throughout the brain, suggesting that differences in NMDA receptor subunit composition might be responsible for the differences in NMDA receptor sensitivity to ethanol in different types of neurons. Therefore, the ethanol sensitivity of different NMDA receptor subunits was studied using recombinant NMDA receptor subunits expressed in *Xenopus* oocytes. Various NMDA receptor subunit combinations exhibit a differential sensitivity to ethanol. The mouse heteromeric subunit combinations epsilon-1/zeta-1 and epsilon-2/zeta-1 are inhibited by 50 mM ethanol, whereas the heteromeric combination epsilon-3/zeta-1 and the homomeric zeta-1 subunits are not significantly affected by this concentration of ethanol. The sensitivity of the epsilon-4/zeta-1 subunit combination is similar to that of the epsilon-3/zeta-1 subunit combination. In addition, there are differences in the ethanol concentration-response curves for different subunit combinations. These observations are consistent with the possibility that NMDA receptor subunit composition may contribute to differences in the ethanol sensitivity of NMDA receptors observed in different brain regions and in different types of neurons. Experiments are currently in progress attempting to elucidate the molecular determinants of NMDA receptor sensitivity to ethanol and other neuroactive substances.

Recombinant Non-NMDA Glutamate Receptor-Channels: The ethanol sensitivity of non-NMDA glutamate receptor-channels was studied using recombinant non-NMDA glutamate receptor types 1-3 (GluR1-3) expressed in *Xenopus* oocytes. Ethanol inhibits the function of these receptors in a concentration-dependent manner over the concentration range 50-500 mM. The ethanol inhibition of the responses of the GluR1-2-3 heteromeric combination has an IC50 value of 176 mM, and the IC50 value for inhibition of the GluR3 subunit is 212 mM. These values are in a similar range to the ethanol sensitivity observed for non-NMDA glutamate receptor-channels in neurons. In addition, for a series of straight-chain alcohols from methanol to heptanol, the potency for inhibition of GluR receptor-mediated responses increases in proportion to the chain-length of the alcohol. However, despite increased hydrophobicity, a distinct cutoff for the inhibition of these receptors is observed for alcohols with more than seven carbon atoms for both GluR1 and GluR3 receptors. Since these two subunits have considerable homology in molecular structure, the similar cutoffs for these subunits may indicate that the molecular basis of the cutoff effect is similar for both GluR1 and GluR3.

Recombinant and Expressed GABA-A Receptors: It has been reported that GABA-A receptors from long-sleep (LS) mice are more sensitive to ethanol than GABA-A receptors from short-sleep (SS) mice, and it has been proposed that the greater ethanol sensitivity is due to the presence of the gamma-2L subunit in the GABA-A receptor. This hypothesis was based on studies in *Xenopus* oocytes expressing LS or SS mouse brain mRNA or the cRNA of mouse GABA-A receptors containing alpha-1/beta-1/gamma-2L subunits. However, we have been unable to repeat the observations on which that hypothesis was based, *viz.* we have been unable to find effects of ethanol, in concentrations from 10 - 100 mM, on GABA-A receptor-mediated responses in *Xenopus* oocytes expressing LS or SS mouse brain mRNA, or mouse GABA-A receptors containing alpha-1/beta-1/gamma-2L subunits. Our inability to confirm the observations on which the gamma-2L hypothesis is based raises questions about the molecular determinants of GABA-A receptor sensitivity to ethanol. Experiments are currently in progress attempting to elucidate the molecular basis of alcohol sensitivity of GABA-A receptors.

Recombinant nACh Receptors: The alpha7 subtype of nACh receptors is one of the most abundant nicotinic receptors in the mammalian CNS, and it has been suggested that it may be important in the addiction to nicotine. In studies on the effect of ethanol on recombinant nACh-alpha7 receptors expressed in *Xenopus* oocytes, it was surprising to find that ethanol inhibits the function of this receptor, because ethanol potentiates the function of the muscle-type nACh receptor at low agonist concentrations (see above). The inhibition of nACh-alpha7 receptor function by ethanol is of the non-competitive type, *viz.* it decreases Emax without affecting the EC50 of the agonist concentration-response curve, which is similar to the ethanol inhibition of NMDA and non-NMDA glutamate receptor function, but it differs from the response of ATP-gated channels, where ethanol increases the EC50 without affecting Emax. Studies are currently in progress on the effect of ethanol on other recombinant CNS nACh receptors.

Recombinant 5-HT3 Receptors: As noted above, studies in the Section on Physiology have shown that ethanol can potentiate 5-HT3 receptor function at low agonist concentrations in neurons and neural cell lines. However, it was also found that the 5-HT3 receptors are insensitive to ethanol in approximately 15% to 25% of those cells. Since the 5-HT3 receptor has been cloned, the recombinant receptor was expressed in *Xenopus* oocytes to study the molecular determinants of ethanol sensitivity. For the mouse recombinant 5-HT3 receptor, ethanol potentiates the response activated by low concentrations of 5-HT in all of the cells studied. Since the intracellular loop of the 5-HT3 receptor has consensus sequences for phosphorylation, and PKC activation potentiates the response of these receptors, experiments are in progress, using molecular biological methods such as site-directed mutagenesis and deletion mutation, to determine whether these consensus sequences for phosphorylation can regulate the sensitivity of 5-HT3 receptors to ethanol. It has also been found that single amino acid mutations in the N-terminal domain of the 5-HT3 receptor can alter the ethanol sensitivity of the receptor. Experiments are currently in progress to determine the mechanism involved in this alteration of ethanol sensitivity.

Chimeric Nicotinic-Serotonergic Receptor: Chimeric proteins have been extremely valuable for determining relationships between structural domains and functional properties of membrane proteins. One such construct, a chimeric receptor from two different neurotransmitter-gated membrane ion channels, with the N-terminal domain from the nACh-alpha7 receptor and the transmembrane and C-terminal domains from the 5-HT3 receptor, manifests activation by nicotinic agonists but channel specificities of the 5-HT3 receptor. Since ethanol inhibits the function of nACh-alpha7 receptors and potentiates the function of 5-HT3 receptors, this chimeric nicotinic-serotonergic receptor was used to study whether the modulatory actions of ethanol are associated with the N-terminal or the transmembrane and C-terminal domains of the receptor. Ethanol inhibits the response of the chimeric receptor in a

manner similar to that of the nACh-alpha7 receptor, suggesting that the ethanol action involves the N-terminal domain of the receptor. Experiments are currently in progress attempting to determine the molecular region of the N-terminal domain that is involved in the ethanol action on this receptor.

Summary and Conclusions: The studies in the Section on Molecular Neuroscience have provided evidence that one of the determinants of alcohol sensitivity of neurotransmitter-gated membrane ion channels is the molecular structure of the receptor. In addition, studies are in progress on the molecular regulation of alcohol sensitivity of these receptors and the molecular sites of alcohol action on these proteins. These studies hold the promise that such molecular biological approaches will advance our knowledge of the molecular basis of alcohol actions in the nervous system.

Akinshola BE, Taylor RE, Ogunseitan AB, Onaivi ES: Anandamide inhibition of recombinant AMPA receptor subunits in *Xenopus* oocytes is increased by forskolin and 8-bromo-cyclic AMP. Naunyn-Schmiedeberg's Arch Pharmacol 1999;360:242-8

Ghazanfari FA, Stewart RR: Characteristics of endothelial cells derived from the blood-brain barrier and of astrocytes in culture. Brain Research (in press)

Li C: Novel mechanism of inhibition by the P2 receptor antagonist PPADS of ATP-activated current in dorsal root ganglion neurons. J Neurophysiol 2000;83:2533-41

Li CY, Xiong KM, Weight FF: Ethanol inhibition of adenosine 5 '-triphosphate-activated current in freshly isolated adult rat hippocampal CA1 neurons. Neurosci Lett 2000;295(3):77-80

Liesi P, Stewart RR, Wright JM: Involvement of GIRK2 in postnatal development of the weaver cerebellum. J Neurosci Res 2000;60:164-73

Peoples RW, Stewart RR: Alcohols inhibit N-Methyl-D-aspartate receptors via a site exposed to the extracellular environment. Neuropharmacology 2000;39:1681-91

Xiong K, Li C, Weight FF: Inhibition by ethanol of rat $P2X_4$ receptors expressed in *Xenopus* oocytes. Br J Pharmacol 2000;130:1394-8

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Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01-AA-00007-08

Title: MOLECULAR NEUROBIOLOGY AND ALCOHOL ACTIONS

Staff Years: 9

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Sample Type: Neither Human Subjects nor Human Tissues

Keywords: neuroscience, alcohol, receptor, ion channel, oocyte, molecular biology, electrophysiology

SUMMARY: The molecular basis of alcohol action in the nervous system is poorly understood. Neurotransmitter receptors have been found to be alcohol sensitive membrane proteins. We are studying molecular determinants of alcohol sensitivity of some types of these proteins. A nicotinic-serotonergic chimera indicated that the N-terminal domain is involved in the action of ethanol on this protein (Mol Pharmacol 1996;50:1010). We found that site directed mutagenesis of single amino acids in the N-terminal domain of the 5-HT3 receptor alters the apparent agonist affinity and the ethanol sensitivity of this protein. The wild-type (WT) and mutant receptors were expressed in Xenopus oocytes and their function was studied using two-electrode voltage-clamp. The arginine at amino acid 245 was substituted with alanine, threonine, glutamic acid and lysine. The order of the EC50 values of the 5-HT concentration-response curves was R245A < R245E < R245T < WT < R245K. The order of percentage potentiation by 100 mM ethanol was R245A > R245E > R245T > WT > R245K. The percentage potentiation by ethanol inversely correlated with the EC50 values of 5-HT3 receptor-mediated currents. The observations suggest that mutation of the arginine at amino acid 245 of the 5-HT3 receptor may modulate the ethanol sensitivity of the receptor by altering the apparent agonist affinity of the receptor. The effect of mutation on ethanol sensitivity and receptor function is also being studied for other amino acids in the 5-HT3 receptor and in other ligand-gated membrane ion channels.

RESEARCH HIGHLIGHTS: This project investigates the molecular basis of alcohol actions in the nervous system using molecular biological techniques. These studies have provided evidence that the alcohol sensitivity of some types of neurotransmitter receptors is determined by the molecular structure of the receptor. In addition, studies are in progress on the molecular regulation of alcohol sensitivity of neurotransmitter receptors.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: These studies hold the promise that such molecular biological approaches will advance our knowledge of the molecular mechanisms of

alcohol action in the nervous system and provide a foundation for understanding the molecular basis of alcohol abuse and alcoholism.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00448-03

Title: Cellular Neuropharmacology and Alcohol Actions

Staff Years: 2

Principal Investigator: RW Peoples, PhD (CNP, PHARM, LMCN, NIAAA)

Other Lab Personnel: H Ren, MD

NIH Collaborators: None

Extramural Collaborators: None

Sample Type: Human Tissues, Fluids, Cells, etc.

Keywords: Alcohol Ion channel Glutamate NMDA Glycine Patch-clamp Primary culture Central Nervous System Transfection

SUMMARY: Neurotransmitter-gated membrane ion channels are among the most important target sites of alcohol action in the nervous system, although the manner in which alcohols modulate the function of these transmembrane proteins has not been established. The aim of this project is to investigate the actions of alcohols and related compounds on neurotransmitter-gated ion channels thought to be involved in producing the intoxicating effects of alcohols in nervous tissue. Previous studies have shown that the function of the N-methyl-D-aspartate (NMDA) receptor-channel, a type of receptor for the excitatory neurotransmitter glutamate, is inhibited by intoxicating concentrations of ethanol. Experiments were performed to identify the location of the site of alcohol action on the NMDA receptor in mammalian cells transfected with NR1/NR2B NMDA receptor subunits. Truncation of the intracellular C-terminal domain of the NR1 subunit did not alter ethanol sensitivity when combined with the NR2B subunit, but a similar truncation of the NR2B subunit slightly enhanced ethanol sensitivity of receptors formed from coexpression with either wild-type or C-terminally truncated NR1 subunits. 1-Pentanol applied externally potently inhibited NMDA receptors, but intracellular application of 1-pentanol did not alter NMDA receptor inhibition by externally applied ethanol or 1-pentanol. In addition, the amplitude of NMDA-activated current did not decrease during the time required for 1-pentanol to diffuse throughout the interior of the cell. Ethanol, even at high concentrations, did not inhibit NMDA receptors when bath-applied in cell-attached patches or when applied to the cytoplasmic face of inside-out membrane patches. These results appear to be best explained by an action of alcohols on the NMDA receptor-channel protein, at a site located in a domain exposed to, or only accessible from, the extracellular environment. Experiments in this unit have also been directed toward identification of the precise molecular site and mechanism of action of alcohols on the NMDA receptor, as well as the physiological regulation of glycine receptor-ion channels.

RESEARCH HIGHLIGHTS: The *N*-methyl-D-aspartate (NMDA) receptor is a protein that plays an important role in producing the intoxicating effects of alcohol in the brain. Although this protein is exposed on both the inner and outer surfaces of cells in the brain, deletion of most of the protein that is exposed to the inside of the cell did not change its sensitivity to alcohol. In addition, NMDA receptors in isolated membrane patches were not inhibited by alcohol applied to the intracellular side. These findings agree well with previous results from this unit, and establish that the site of alcohol on the NMDA receptor is on a region of the protein that is exposed to the outside of the cell.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: Because the NMDA receptor is thought to be among the most important sites in the brain through which alcohol produces its intoxicating effects, determining the site and mechanism of action of alcohol on this receptor are of great importance. These studies constitute important steps toward this goal, as well as toward the larger goal of understanding the molecular basis of alcohol intoxication.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00479-17

Title: SYNAPTIC MECHANISMS AND ALCOHOL ACTIONS

Staff Years: 5

Principal Investigator: FF Weight, MD (PHYS, LMCN, NIAAA)

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Extramural Collaborators: CNRS, Strasbourg, France (J Dupont, PhD)

Sample Type: Neither Human Subjects nor Human Tissues

Keywords: neuroscience, alcohol, neuron, synapse, receptor, ion channel, electrophysiology

SUMMARY: The cellular basis of ethanol action in the nervous system is poorly understood. Ethanol can affect the function of neurotransmitter receptors; however, the receptors in the central nervous system that are involved in the behavioral effects of ethanol have not been established. P2X receptors in bullfrog dorsal root ganglion neurons were inhibited by pharmacological concentrations of ethanol (Neurochem Intl 1999;35:143). P2X receptors are ligand-gated membrane ion channels that are activated by extracellular ATP. We studied the effect of ethanol on P2X4 receptors, the most common P2X receptor in the mammalian central nervous system. P2X4 receptors were expressed in Xenopus oocytes and their function was studied using two-electrode voltage-clamp. We found that the amplitude of current activated by 1 uM ATP was decreased by ethanol in a concentration-dependent manner over the concentration range 1-500 mM. The concentration of ethanol that produced 50% inhibition (IC50) of current activated by 1 uM ATP was 58 mM. Ethanol inhibition of ATP-activated current was not dependent on membrane potential from -60 to +20 mV, and ethanol did not change the reversal potential of ATP-activated current. Ethanol, 50 mM, shifted the ATP concentration-response curve to the right, increasing the EC50 for ATP from 9.1 to 16.0 uM, but ethanol did not reduce the maximal response to ATP. The results suggest that ethanol may inhibit P2X receptors by decreasing the apparent affinity of the binding site for ATP. Since the P2X4 receptor is the most abundant P2X subunit in the brain, these receptors could be important effectors of ethanol action in the central nervous system. Experiments are in progress studying the molecular basis of ethanol action on P2X4 receptors and the effect of ethanol on P2X receptors in central neurons.

RESEARCH HIGHLIGHTS: This project investigates alcohol actions on neuronal neurotransmitter receptors using electrophysiological techniques. These studies have provided evidence that neurotransmitter receptors are cellular sites of alcohol action in the nervous system. These studies have also demonstrated mechanisms by which alcohol affects the function of different types of neurotransmitter receptors. In addition, studies are in progress on the physiological regulation of alcohol sensitivity of neurotransmitter receptors.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: The studies suggest that the physiological approaches used in these studies will advance our knowledge of the cellular mechanisms of

alcohol action in the nervous system and provide a foundation for understanding the cellular basis of alcohol abuse and alcoholism.

Reporting Period:	10/01/1999 – 09/30/2000
Project Number:	Z01 AA00480-17
Title:	NERVE CELL EXCITABILITY AND ALCOHOL ACTIONS
Staff Years:	0.6
Principal Investigator:	FF Weight, MD (PHYS, LMCN, NIAAA)
Other Lab Personnel:	RR Stewart, PhD P Zhu, PhD
NIH Collaborators:	None
Extramural Collaborators:	Emory University School of Medicine, Atlanta (MB Luskin, PhD)
Sample Type:	Neither Human Subjects nor Human Tissues
Keywords:	neuroscience, alcohol, neuron, excitability, ion channel, electrophysiology

SUMMARY: Alcohol is classified pharmacologically as a central nervous system depressant. The cellular mechanisms that underlie this alcohol-induced depression of nervous system excitability, however, are poorly understood. This project investigated excitability mechanisms in neuronal precursor cells. Precursor cells from the anterior subventricular zone (SVZa) of neonatal rat forebrain express neuron-specific markers and divide while migrating along the path to the olfactory bulb, where they differentiate into granule and periglomerular interneurons. SVZa cells also express neuron specific tubulin and divide in vitro. Voltage-dependent membrane ion channels have been studied in SVZa cells after 1 day in culture (J Neurophysiol 1999; 81:95). In this study, the responses of both voltage-dependent channels and neurotransmitter receptors were determined for SVZa cells in both culture and slices. Whether in culture or in slice, SVZa cells displayed voltage-dependent sodium and potassium currents, were sensitive to the neurotransmitter GABA (1-300 uM), but were insensitive to 100 uM NMDA, 100 uM kainate, 24 uM glutamate, 100 uM ATP or 100 uM acetylcholine. Thus, many of the properties of SVZa cells observed after 1 day in culture are observed in SVZa cells in slice. Although GABA inhibits neurons in the adult CNS, during development GABA depolarizes SVZa cells and newly differentiating neurons. In addition, GABA has been shown to regulate cell proliferation and migration, as well as to act as a trophic factor in other types of cells. In the future, we plan to examine the role of GABA in the migration and differentiation of SVZa cells, and the effect of ethanol on GABA responses in these cells.

RESEARCH HIGHLIGHTS: This project investigates alcohol actions on neuronal excitability mechanisms using electrophysiological techniques. These studies have indicated that in most cases voltage-gated membrane ion channels, which underlie the intrinsic electrical excitability of neurons, are relatively insensitive to intoxicating and anesthetic concentrations of alcohol.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: The studies suggest that the physiological approaches used in these studies will advance our knowledge of the cellular mechanisms of alcohol action in the nervous system and provide a foundation for understanding the cellular basis of alcohol abuse and alcoholism.

FY 2000 ANNUAL REPORT SUMMARIES

(1 OCTOBER 1999 - 30 SEPTEMBER 2000)

LABORATORY OF NEUROGENETICS

DAVID GOLDMAN, M.D., CHIEF

DIVISION OF
INTRAMURAL CLINICAL & BIOLOGICAL RESEARCH
NATIONAL INSTITUTE ON ALCOHOL ABUSE & ALCOHOLISM
NATIONAL INSTITUTES OF HEALTH

SYNOPSIS
LABORATORY OF NEUROGENETICS
1 OCTOBER 1999 – 30 SEPTEMBER 2000

The role of the laboratory (LNG) is to understand neural mechanisms in alcoholism, and the major paradigm is the study of interindividual variation. The first aim is to relate genotype to complex behavioral phenotype, with the goal of identifying vulnerability and protective alleles responsible for the substantial heritability of alcoholism. A critical observation is that individuals are differentially vulnerable to alcoholism, even in societies, such as the SW Indians we have studied, where the prevalence of alcoholism is highest and the effects of alcohol are most pernicious. Differential vulnerability could indicate the existence of innate differences, environmental differences, or a combination of both. However, family and twin studies on large, epidemiologically ascertained collections of twins (Kendler et al; Martin et al) have established that a substantial component of this variance is attributable to genes. Identification of these alleles will lead to better understanding of mechanisms of vulnerability and gene-environment interactions, new molecular diagnostic markers to individualize treatment, and new molecular targets for intervention. Thus the ultimate objective of our research activities is to lessen the suffering of affected patients, families, and communities by making major scientific discoveries that lead to an understanding of alcoholism.

Major accomplishments:

- LNG is largely responsible for analysis of this complex disorder in isolates and well-defined populations that are genetically and environmentally more homogeneous.

- LNG is responsible for the systematic evaluation of genetic factors on alcoholism in American Indians and for defining and characterizing the impact of alcoholism in American Indian communities, which on a worldwide basis are among the most afflicted by alcoholism but neglected by research. These studies extend from analysis of phenotype and transmission to a whole genome linkage scan identifying putative locations of vulnerability genes.

- LNG plays a leading role in identification of functional variants in neurogenetic candidate genes. We screen a large sample to detect both common and uncommon variants, screen in relevant clinical populations, and functionally characterize alleles *in vitro.* Common functional variants LNG has discovered or for which it has demonstrated *in vitro* functionality include serotonin, dopamine, and opioid receptor variants. Several are important targets in neurogenetics.

- LNG has been the groundbreaking genetics laboratory for studies relating brain gene polymorphisms to *in vivo* intermediate phenotypes such as brain imaging findings. The polymorphism/function associations are congruent with the known molecular neurobiology of the transmitters and proteins and include dopamine transporter genotype/dopamine transporter density in striatum, serotonin transporter genotype/serotonin transporter density in raphe nucleus, and COMT genotype/metabolic activity in frontal lobe.

- LNG is a principal exponent of case/control, sib-pair linkage, and TDT studies to relate alleles to behavior. Case/control findings that may stand the test of time and in which LNG has been a major contributor include serotonin transporter/anxiety, serotonin receptor 5HT2A/clozapine response, tryptophan hydroxylase/suicidality-impulsivity, serotonin receptor 5HT1B/antisocial alcoholism, $GABA_A$ alpha 6/benzodiazepine and alcohol sensitivity, and COMT/frontal lobe function.

- For case/control associations in neuropsychiatry genetics--best illustrated by the DRD2 controversy--LNG had a critical role in raising level of quality and stringency through its use of more incisive procedures in replication studies. These included careful clinical evaluation and testing of predictions through the extended phenotype, ethnically matched patients and controls, accurate determination of population frequencies of alleles and genotypes, attention to the problem of arbitrarily combining genotypes, sib-pair linkage and TDT, and a focus on the functional alleles at these loci.

- LNG has been at the forefront of the use of haplotype-based methods for linkage and population analyses. We provide one of the most widely used programs for haplotype estimation, adopted Templeton=s method for cladistically-directed haplotype clustering in a study detecting evidence for

a Y chromosome locus in alcoholism, and used haplotypes to trace the origins and influences of selection on ALDH2 Glu487Lys, a polymorphisms with a known role in alcoholism.

- LNG is a leading exponent of gene/environment interactions in alcoholism, including the use of intermediate phenotypes and cross-population studies. LNG is largely responsible for showing that the common, stable low voltage alpha EEG trait [LVA] identifies a subtype of alcoholism associated with anxiety disorders, and has collected EEG and cell lines in a large Plains Indian family where definitive gene identification analyses can be performed. In addition to family linkage datasets from tribes with high and low rates of alcoholism, the gene/environment interaction studies encompass the collection of the first large multi-population dataset specifically suited for evaluation of gene/environment interaction: the Ten Tribes Study.

- LNG has identified an endogenous oxidative DNA lesion, 8,5'-cyclo-deoxyadenosine. This lesion is repaired by nucleotide excision repair (NER) and is a strong block to gene expression in mammalian cells. Therefore this is an excellent candidate for the DNA lesion that causes neurodegeneration in patients with xeroderma pigmentosum (XP). Also, this DNA lesion may play a role in other neurodegenerative conditions associated with increased oxidative stress, including alcohol-related brain damage.

A central theme of LNG is use of functional variants [candidate alleles] in genetic linkage studies, and the validation and explanation of linkage findings by tracing genes linked to behavior back through brain physiology [see intermediate phenotype discussion below]. LNG and a few other laboratories have specifically focused on variation in neurogenetic candidate genes, and have found that such genes have as high abundance of polymorphism, including frequent missense variant, as genes expressed elsewhere in the body. Variation within the coding sequences of 18 dopamine and serotonin GPCRs carefully scanned for sequence variation across several hundred individuals was summarized (Cravchik & Goldman, 2000). Because many of these non-synonymous variants had been evaluated for *in vitro* functionality, these two receptor families offer a preview of the genetic variation of neurochemical individuality. In Caucasians, 55% (10 of 18) of the dopamine and serotonin receptor coding sequences contained protein amino acid substitutions or deletion/insertions with allele frequencies of $\geq$0.01, defining them as polymorphic. Average heterozygosity (chance of encountering a heterozygous locus) was 15% across these eighteen coding sequences, and 94 of 100 Caucasians would express at least one variant altering dopamine or serotonin receptor structure. LNG discovered and demonstrated *in vitro* functional significance for a substantial number of all of the candidate alleles presently available for psychiatric genetics. LNG is essentially the only laboratory in the field of alcoholism research that has used candidate alleles in a major way, and those studies have included candidate alleles at DRD2, COMT, 5HT2A and the serotonin transporter. Candidate alleles we discovered and/or "functionated" include DRD2 Ser311Cys, HTR2A His452Tyr, OPRM1 Asn40Asp, HTR1A, and HTR2C Ser23Cys (Okada et al).

Ongoing SNP screening is proceeding in a large [N=500] sample enriched for clinical and ethnic diversity. The major methodologies are dHPLC and sequencing. The major methodology for high throughput genotyping is the 5'exonuclease assay.

For *in vivo* function, LNG has collected four human datasets, has collected six datasets with intermediate phenotypes through close collaboration, and has three such datasets either underway or in advanced planning. Several of these are of outstanding size and structure for genetic analysis, and most of the datasets include intermediate phenotypes. The datasets are:

1) Bethesda EEG/ERP/heart rate variability/personality dataset, N≈350, structure families and population (Enoch and Goldman)
2) Plains Indian alcoholism and EEG/ERP/heart rate variability/personality dataset, N≈450, structure large pedigree (Enoch and Goldman)
3) SW alcoholism and Indian personality, N=600 (Robin and Goldman)
4) Finn alcoholism and personality, N=850, population and pedigrees (Linnoila & Goldman).

In close collaborations, we have worked with other investigators to design and carry out collections of new datasets and presently collaborators from NIMH, NIDCR and the Jackson Foundation work directly in LNG as guest researchers. In collaborative studies, LNG plays a major role in study design, is the site of organization and preparation of cells and DNA and is the primary site for genetic analyses. The existing intermediate phenotype datasets that have been collected collaboratively are:

1) alcohol response, N≈50, selective genotyping (M Schuckit, UCSD & Goldman)
2) benzodiazepine response, N≈80 (D Cowley, U Seattle & Goldman)

3) Executive cognitive function, N≈150, schizophrenia (A Malhotra, Hillside Hospital & Goldman)
4) Executive cognitive function, N≈800, sib pairs and population, schizophrenia (M Egan, D Weinberger, NIMH & Goldman)
5) Executive cognitive function, N≈150, head injury (R Lipsky, Jackson Found & Goldman)
6) *in vivo* dopamine and serotonin transporter, N≈25 (A Heinz, NIMH & Goldman)

Dataset collection underway or in advanced planning:
1) A new dataset for thermal pain threshold and personality, N ≈ 400, is substantially complete (Dionne, NIDCR & Goldman)
2) Concerning pharmacogenetics, a collection for naltrexone/sertraline response is underway in an Alaska Native tribe, N ≈ 200 (O'Malley, Yale & Goldman)
3) a collection for naltrexone/acamprosate response, N ≈ 1300, is in the advanced planning stages with COMBINE (Anton et al).

Other collaborative datasets are:
1) obsessive-compulsive disorder (D Murphy, NIMH & G Cassano, Pisa)
2) anorexia-nervosa (W Kaye, Pittsburgh & G Cassano, Pisa),
3) seasonal affective disorder (N Rosenthal & T Wehr, NIMH)
4) bipolar disorder (G Cassano, Pisa).

Investigators from NIMH (D Weinberger & M Egan) are working as guests in our laboratory and as part of a close collaboration on schizophrenia. For neuroimaging, we have primarily collaborated with D Weinberger (NIMH) and A Heinz (formerly of NIMH). For studies on human postmortem brain we have collaborated with NIMH (D Weinberger & J Kleinman). For complementary studies in nonhuman primates LNG has created a library of some 200 fibroblast lines on carefully phenotyped Rhesus macaques with JD Higley (LCS, NIAAA).

Themes in genetic analysis of behavior have included the use of candidate alleles, genetic isolates, TDT and sib-pair strategies to avoid false positives due to ethnic stratification, and *in vivo* functional genomics to validate and elucidate meaning of linkage findings. Biochemical, physiological and histological phenotypes are useful for identifying genetically distinct subgroups of common, complex biomedical disorders. LNG has helped to develop phenotypes that may represent true intermediate, or mediating, phenotypes in alcoholism. These include:
- alcohol sensitivity/genes involved in neuronal sensitivity to alcohol
- CSF monoamine metabolites/monoamine genes
- low voltage alpha EEG/genes involved in the neurochemistry of anxiety and dysphoria
- executive cognitive function as measured by Wisconsin Card Sort and other methods.

In addition, we have used other traits, such as psychiatric comorbidity, which narrow the phenotype even though they do not closely represent intermediate phenotypes.

The alcohol response phenotype was developed by M Schuckit, who found that relatively alcohol-naive sons of alcoholics tended to show diminished subjective, endocrine, and motor responses to alcohol. In long-term follow-up, alcohol-insensitive subjects were at enhanced risk for alcoholism. In collaboration with Dr. Schuckit, we are selectively sampling, with cell lines and DNA, the individuals from the upper and lower third of his prospective sample. Some 50 DNAs were used in an association study to obvious candidate alleles for alcohol sensitivity, and we have also done the same with a somewhat parallel dataset collected with D. Cowley. That dataset used the intermediate phenotype of benzodiazepine response as evaluated by eye movements. The findings include preliminary associations of alcohol response to HTTLPR and alcohol and benzodiazepine response to $GABA_A$ alpha 6 (Iwata et al, 1999; Schuckit et al, 1999).

The LVA EEG trait is an abundant [0.10], stable, neurophysiologic trait that can be qualitatively classified and appears to be transmitted in autosomal dominant fashion. Heritability of the corresponding quantitative trait, alpha power, is >0.9. LNG reported and then replicated a phenotype/phenotype association between LVA and alcoholism (Enoch et al, 1995 & 1999). We found the frequency of LVA to be about four times higher in alcoholics as compared to the general population. Alcoholics with anxiety disorders were still more likely to have LVA. LVA is a relatively common trait and considerable genetic heterogeneity may be present, thus reducing power to detect linkage without a large pedigree. A large Plains Indian family dataset should be capable of yielding significant linkage results for EEG and ERP

phenotypes, and to evaluate relationship of neuropsychological phenotypes to alcoholism in American Indians.

Drugs have become available that improve ability of alcoholics to maintain abstinence (O'Brien et al, Anton et al, O'Malley et al). Disulfiram had a direct counterpart in ALDH2 Glu487Lys that creates a natural blockade of the same metabolic enzyme. New drugs, particularly sertraline and naltrexone, can also direct us to molecular targets which could be either sources of variation in treatment response or sources of differential vulnerability. Two principal gene targets are the serotonin transporter, sertraline, and the mu opioid receptor, naltrexone, and other gene targets are also directly inferred: HTR1A and HTR1B autoreceptors, postsynaptic serotonin receptors, opioid propeptides, and various genes involved in the function of interacting neurotransmitters such as dopamine. A major collaboration led by Dr. O'Malley (Yale) addresses the pharmacogenetics of naltrexone and sertraline response among alcoholics in a NW Indian tribe. We found a common, nonconservative mu opioid receptor variant Asn40Asp that Kreek and colleagues reported alters affinity of the receptor for endomorphin.

Regarding Type I and population stratification errors in association studies and as especially well-illustrated by the DRD2 story, e.g., Goldman et al, 1998, LNG played an invaluable role in pointing out the problem of multiple testing created by testing various groupings of genotypings at a locus and testing of multiple phenotypes, and we have used population isolates, the TDT method and family-based linkage methods to address the problem of ethnic background. For related haplotypes, LNG used cladistic hierarchies with appropriate corrections for multiple testing, as developed by Templeton (see J Long report and Kittles et al, 1999). We have also been able to initially or subsequently evaluate linkages in more than one dataset. Examples include:

- HTR1B/antisocial alcoholism: sib-pair linkage in Finns and SW Indians
- HTR2A/anorexia nervosa: association in Univ Pittsburgh and Univ Pisa datasets, associations by other groups; and the related finding HTR2A/OCD: association in NIMH and Univ Pisa datasets
- TPH/suicidality: association in two Finnish datasets
- COMT: frontal lobe function: association to this intermediate phenotype in NIMH schizophrenics and controls, Hillside Hospital controls, and Jackson Foundation head injury patients.

While replication is uncertain, validation occurs through allele identification and the discovery of predicted biological correlates.

Our candidate gene studies have focused on loci already implicated by multiple threads of evidence in particular phenotypes and frequently the allele being tested is functional. Thus the prior probability of these markers to be in genuine association is substantially higher, probably by several orders of magnitude, than with random markers. Where a previous linkage was to a nonfunctional marker, for example the DRD2 Taq1A, this laboratory has helped spearhead efforts to identify the functional alleles at the locus, for example the DRD2 Cys311Ser (Gejman et al, 1994) which blocks signal transduction (Cravchik et al) and has an allele frequency of 0.16 in the SW Indians (Goldman et al, 1997). We have then used the functional allele for more stringent evaluation of the original hypothesis (Goldman et al, 1997). LNG was one of the first genetics laboratories investigating psychiatric disease to extend mouse QTL and gene knockout findings to the human. A strong mouse alcohol preference QTL (Buck and colleagues) in the region of HTR1B and the subsequent finding that HTR1B knockout mice (Hen et al) drank more alcohol and showed increased aggression prompted us to perform sib-pair linkage to the nearest behavioral homologue in the human, namely antisocial alcoholism. As reported (Lappalainen et al, 1998), evidence for linkage was found in both the SW Indian dataset as well as the Finns. The latter is a linkage dataset strongly enriched with antisocial alcoholic probands. As a terminal autoreceptor, HTR1B is a likely candidate to modulate serotoninergic action in either impulse control or reward. However, studies in LNG and elsewhere have so far yielded no HTR1B variant likely to be functionally significant. Therefore, HTR1B and this region of Chromosome 6 are being studied in collaboration with University of Colorado (J Sikela & M Ehringer). New markers will be evaluated individually and as haplotypes in linkage and case-control association in the SW Indian and Finn datasets.

Knowledge of *in vitro* functionality also leads to direct *in vivo* follow up in the human as well as predictions for the effects on appropriate intermediate phenotypes. We followed up (Mazzanti et al, 1998) on results with the serotonin transporter HTTLPR by Lesch, who had found the s allele impaired transcription and was associated with anxiety, a clinical problem which responds to drugs which inhibit the serotonin transporter. We obtained independent support for the anxiety linkage in a different type of dataset: Finnish sib-pairs evaluated with the TPQ. We also found association of HTTLPR to Seasonal Affective Disorder, a psychiatric disorder in the anxiety/dysphoria spectrum (Enoch et al). By brain imaging, HTT density in brain was shown to directly correlate with HTTLPR genotype (Heinz et al, 2000), and with the

further interesting result that there may be long-term disturbance of HTT density in the brains of alcoholics.

The $GABA_A$ alpha-6 gene is a second example of convergence between genetic findings in rodent and human. Korpi et al detected a rat $GABA_A$ alpha-6 amino acid substitution altering the sensitivity of this receptor to alcohol and benzodiazepines and this substitution predicts the difference in alcohol sensitivity observed between alcohol accepting and non-accepting rats. $GABA_A$ gene clusters on human chromosomes 4 and 5 are implicated by linkage studies in the human (Long et al) and in the rodent (Portland group), respectively. We detected a common, non-conservative amino acid substitution $GABA_A$ alpha-6 Pro385Ser and obtained preliminary evidence for involvement in alcohol (Schuckit et al, 1999) and benzodiazepine sensitivity (Iwata et al, 1999).

Executive cognitive functions localized to the frontal lobe are thought to be impaired in several psychiatric diseases: alcoholism, ADHD, and schizophrenia. Val158Met, a common COMT variant, leads to a four-fold reduction in the activity of the enzyme. Due to the effect of dopamine to improve executive cognitive performance, Val158 and Met158 were candidate alleles for variation in frontal lobe cognitive function. Three TDT linkage studies (Kunugi et al; Li et al; Li et al) had detected evidence for Val158Met in schizophrenia and some evidence for a schizophrenia locus had been found near 22q11 (e.g., Pulver et al). LNG formed a close collaboration with NIMH (D Weinberger & M Egan) to collect a very large TDT/sTDT schizophrenia dataset with cognitive intermediate phenotypes. Wisconsin Card Sort performance was evaluated versus COMT genotype in 75 controls, 184 schizophrenics, and 222 siblings of schizophrenics (Egan et al), with the result that a remarkable allele-dosage relationship was found to perseverative errors in both the schizophrenia patients and the controls. This finding was directly expanded by a study of frontal lobe metabolic activity. Five val/val, six val/met and five met/met individuals were evaluated using blood oxygen level dependent [BOLD] fMRI during the N-back task, which accesses these prefrontal cognitive functions. As predicted, during this memory task the Val158 allele was associated with increased metabolic activity in frontal lobe consistent with the hypothesis of diminished cortical efficiency. Furthermore, in a TDT analysis, the ancestral Val158 allele was preferentially transmitted the schizophrenic offspring, as had been observed in the three previously reported TDT studies. Finally, we also observed association of Met158 to improved prefrontal cognitive performance in two other datasets: a control dataset (Malhotra et al) and a head injury dataset (Lipsky et al). Thus, the Val158 allele appears to compromise relevant prefrontal function and may be a susceptibility gene for schizophrenia and other diseases involving cognitive executive functions.

Isolates: The definition and utility of genetic isolates are poorly understood despite a general perception that it is an advantage to conduct studies in such populations. The greater cultural and environmental homogeneity of isolates may be the most critical advantage for their use in complex diseases. Isolate is a shorthand but imprecise description for populations relatively well defined and non-admixed as compared to other populations. For example, we showed that the Finnish population, a classic example of an isolate, has a dual Asiatic/N European genetic signature inconsistent with a single founding event but consistent with a variety of cultural clues within Finland (Kittles et al, 1998). Evidence for reduction of diversity occurs in the Finnish Y chromosome indicating a male-specific bottleneck because there was no reduction of diversity in autosomal or mitochondrial DNA (Kittles et al, 1999). In contrast, we found multiple American Indian populations reduced in autosomal STR diversity (Urbanek et al, 1996).

LD relationships differ between populations. The implication is that opportunities vary between populations to discover linkages and to home in on genes. Unless using the actual functional allele, some populations are better for detection of an initial association signal and others are better for isolation of functional alleles. Replication can be problematic in a different population. Certain functional polymorphisms including ALDH2 Glu487Lys, DRD2 Ser311Cys, and HTTLPR s/l, show dramatic inter-population differences in allele frequency. By conducting whole genome linkage scans in isolates we have been able to empirically verify p values by random assignment and random transmission of alleles through the defined structure of the pedigree (Long et al, 1998 - SW Indian linkage scan). Some of the most powerful results we obtained regarding the DRD2 Taq1A controversy were from isolates: Taq1A allele frequencies and LD differed several fold across populations, and ethnically matched controls from the SW Indian and Finnish populations did not differ from alcoholics derived from the same populations in A1 allele frequency (Goldman et al, 1992; 1993).

Alcoholism in neglected populations: Alcoholism and its consequences are pervasive in most American Indian populations. For the further development of treatment and prevention, it is vital to establish the role and identity of the causative factors in those populations. Because of the wide variation in

prevalence of alcoholism, which LNG is indeed for the first time stringently documenting, it is critical to understand the role of gene/environment interactions in American Indian populations. Despite the magnitude of the alcoholism problem in American Indians, neither a genetic transmission study nor a genetic linkage study had ever been performed. Also, systematic data on phenotype, including cross-population studies needed to address speculations about the reliability and meaning of the alcoholism diagnosis in American Indians, were largely unavailable. Other than LNG, the data collected using structured or semi-structured diagnostic interviews had been derived from a single group of investigators at the University of Colorado (Shore, Manson et al). Whether Indian alcoholics were misclassified binge drinkers was still an open question.

LNG has comprehensively addressed the problem of alcoholism in American Indians in three family linkage datasets:

1) SW tribe (Robin & Goldman)
2) Eastern Oklahoma tribe with low prevalence of alcoholism a field study in progress (Long)
3) Plains Indian tribe with neuropsychological data (Enoch & Goldman);

and also in a large cross-population study in progress--the Ten Tribes Study--that systematically compares 300 randomly, sampled subjects from each tribe (coPIs Dr. M Koss, Univ Arizona & Goldman). LNG is also the genetics collaborating laboratory on the first pharmacogenetic study on alcoholism treatment response to naltrexone and sertraline, the study of Alaska Natives, is led by Dr. S O'Malley (Yale).

A genome scan for the SW tribe detected two potential new loci for alcoholism: the DRD4 region at the chromosome 11p telomere, and the $GABA_A$ cluster site in the chromosome 4p centromeric region. The SW Indian family sample (N=582) comprises a sizeable fraction of a Southwestern tribe with a high rate of alcoholism (85% of males, >50% of females). This family was systematically interviewed and substantial evidence for familial transmission of alcoholism was found, then it was evaluated for genetic linkage in collaboration with investigators from NIDDK (W Knowler, R Hanson, P Bennett) using a large (N=517) panel of DNA markers.

Studies on the SW tribe were also representative of the meaning and consequences of alcoholism in the tribe because:

- the very large family was ascertained solely on the basis of structure and availability,
- the family is representative of the overall tribal population in coefficient of relationship and demography, and
- CAGE scores do not differ from a random sample of 3112 individuals studied collaboratively by NIDDK (Saremi et al, in press).

Our evidence from the SW tribe (Robin et al, 1998) is that binge drinking is neither benign nor beneficial, that alcoholism is familial, and that the same patterns of psychiatric comorbidity seen in the general U.S. population (National Comorbidity Survey) are seen in Indian alcoholics. Almost all of the large fraction of the population that were binge drinkers were also alcoholics, and binge drinkers tended to become alcoholic at a younger age. Regardless of whether binge drinkers met criteria for alcoholism, they were dramatically worse off in each of the four symptom categories evaluated in the SADS-L: social, work, violence/lawlessness and physical (Robin et al, 1998). These results may help lay to rest the misconception that drinking, particularly binge drinking, is other than deleterious to American Indians regardless of whether binge drinking is culturally determined or culturally congruent.

BIBLIOGRAPHY
LABORATORY OF NEUROGENETICS
1 OCTOBER 1999 – 30 SEPTEMBER 2000

Bergen A, Wang C-Y, Nakhai B, Goldman D: Mass allele detection (MAD) of rare 5HT1A structural variants with allele specific amplification and electrochemi-lumlinescence detection. Human Mutations (in press)

Brooks PJ: Brain atrophy and neuronal loss in alcoholism: A role for DNA damage? Neurochemistry International 2000;37(5-6):403-12

Cardozo-Pelaez F, Brooks PJ, Stedeford T, Song SJ, Sanchez-Ramos J: DNA damage, repair, and antioxidant systems in brain regions: A correlative study. Free Radical Biology and Medicine 2000;28(5):779-85.

Cravchik A, Goldman D: Neurochemical Individuality: Genetic diversity among human dopamine and serotonin receptors and transporters. Arch Gen Psych 2000;57(12):1105-14

Enoch M-A, Harris CR, Goldman D. Does a reduced sensitivity to bitter taste increase the risk of becoming nicotine addicted? Addictive Behaviors (in press)

Evans J, Reeves B, Platt H, Liebenau A, Goldman D, Jefferson K, Nutt D: Impulsiveness, serotonin genes and repetition of deliberate self harm (DSH). Psychological Medicine 2000;30(6):1327-34

Funabashi T, Kleopoulos SP, Brooks PJ, Kimura F, Pfaff DW, Shinohara K, Mobbs CV: Changes in estrogenic regulation of estrogen receptor alpha mRNA and progesterone receptor mRNA in the female rat hypothalamus during aging: an in situ hybridization study. Neuroscience Research 2000;38(1):85-92

Goldman D: Genetic heterogeneity and genetic etiologies for alcoholism and substance abuse. Ann Rev Drug Abuse & Addictions (in press)

Goldman D: Identifying alcoholism vulnerability genes. Alcohol Clin Exp Res (in press)

Heinz A, Goldman D: Genotype effects on neurodegeneration and neuroadaptation in monoaminergic neurotransmitter systems. Nurochemistry International 2000;37(5-6):425-32

Heinz A, Goldman D, Jones DW, Palmour R, Hommer D, Gorey JG, Lee KS, Linnoila M, Winberger DR: Genotype influences *in vivo* dopamine transporter availability in human striatum. Neuropsychopharmacology 2000;22(2):133-9

Heinz A, Jones DW, Mazzanti C, Goldman D, Ragan P, Hommer D, Linnoila M, Weinberger DR: A relationship between serotonin transporter genotype and in vivo protein expression and alcohol neurotoxicity. Biological Psychiatry 2000;47(7):643-9

Iwata N, Virkkunen M, Goldman D: Identification of a naturally occurring Pro385-Ser385 substitution in the GABA(A) receptor alpha 6 subunit gene in alcoholics and healthy volunteers. Molecular Psychiatry 2000;5(3):316-9

Johnstone LE, Brown CH, Meeren HKM, Vuijst CL, Brooks PJ, Leng G, Russell JA: Local morphine withdrawal increases c-fos gene, fos protein, and oxytocin gene expression in hypothalamic magnocellular neurosecretory cells. Journal of Neuroscience 2000;20(3):1272-80

Kaye WH, Lilenfeld LR, Berrettini WH, Strober M, Devlin B, Klump KL, Goldman D, Bulik CM, Halmi KA, Fichter MM, Kaplan A, Woodside DB, Treasure J, Plotnicov KH, Polllice C, Rao R, McConaha CW: A search for susceptibility loci for anorexia nervosa: Methods and sample description. Biological Psychiatry 2000;47(9):794-803

Linnoila M, Virkunnen M, Tokola R, Nielsen D, Goldman D: Alcoholic violent offenders: behavioral, endocrine, diurnal rhythm, and genetic correlates of low CSF 5HIAA concentrations. In: Paoletti R, Vanhouttem P, Saxena P (eds), Serotonin from Cell Biology to Pharmacology and Therapeutics. Milan:Kluwer (in press)

Kolman C: Molecular Anthropology: Progress and Perspectives on Ancient DNA Technology. In: Papiha, Deka, Chakraborty (eds). Genomic Diversity: Applications in Human Population Genetics. New York:Kluwer, 1999, 183-200.

Kolman CJ, Tuross N: Ancient DNA analysis of human populations. American Journal of Physical Anthropology 2000;111(1):5-23

Long JC, Lorenz JG: Genetic polymorphism and American Indian origins, affinities, and health. In: Rhoades, E (ed). The Health of American Indians and Alaska Natives. Baltimore:Johns Hopkins Univ Press 2000, 122-37

Vanakoski J, Mazzanti C, Naukkarinen H, Virkkunen M, Goldman D: An abundant proneurotensin polymorphism, 479A>G, and a test of its association with alcohol dependence in a Finnish population. Alcoholism-Clinical & Experimental Research 2000;24(6):762-5

Williams RC, Long JC, Hanson RL, Sievers ML, Knowler WC: Individual estimates of European genetic admixture associated with lower body-mass index, plasma glucose, and prevalence of type 2 diabetes in Pima Indians. American Journal of Human Genetics 2000;66(2):527-38

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: ZO1AA 00016-08

Title: GENE MAPPING AND LINKAGE STUDIES WITH SHORT TANDEM REPEAT (STR) MARKERS

Principal Investigator: JC Long, PhD (SPGL, LNG, NIAAA)

Other Lab Personnel: J Brooks, MS
C Kolman, PhD
JG Lorenz, PhD
E Moore
RL Vallejo, PhD

NIH Collaborators: NIAAA, HN (D Goldman, MD)
NIDDK, PECRB (PH Bennett, MB, FRCP)
NIDDK, PECRB, DAE (RL Hanson, MD)
NIDDK, PECRB, DAE (WC Knowler, MD, DrPH)

Extramural Collaborators: None

Sample Type: Interviews, Questionnaires, or Surveys only

Staff Years: 6.67

Keywords: gene mapping (human), neurosciences, drinking patterns/causes, molecular genetics

SUMMARY: We are searching for genetic loci that contribute to the predisposition to alcoholism and related behaviors by conducting genetic linkage and mapping analyses using over 500 highly polymorphic DNA marker loci. These loci span all of the non-sex chromosomes at an average interval less than 10 centimorgans. To date, we have completed over 300,000 locus typings, primarily on American Indians and Finns. A whole autosomal genome scan for genetic linkage to alcohol dependence in a Southwestern American Indian tribe was performed. The best evidence for linkage is seen with D11S1984 on chromosome 11p, in close proximity to several candidate genes with neurobiological functions. These candidate genes include the DRD4 dopamine receptor gene, the tyrosine hydroxylase gene and the tryptophan hydroxylase gene. Good evidence for linkage is also seen with D4S3242 on chromosome 4p, nearby the beta1 GABA receptor gene. The chromosome 11 findings were followed up by genotyping a high-resolution map on an expanded sample of subjects from the same Southwestern Indian population. The high-resolution genetic map includes polymorphisms within the DRD4 and tyrosine hydroxylase genes as well as STR markers closely linked to these candidate genes. We are also developing statistical approaches and software for identifying which specific polymorphisms, from a set of closely linked loci, are responsible for altering an individual's vulnerability to disease.

In Finns, association between alcohol dependence and Y-chromosomes was statistically significant. Interestingly, there is no association between Y- chromosomes and antisocial personality disorder after the comorbid effects of alcohol dependence were removed. However, we find evidence for genetic linkage and association between antisocial personality disorder (ASPD) occurring with alcoholism and the chromosome 6 serotonin receptor gene HTR1B. We also find evidence for linkage and association between ASPD with alcoholism and a polymorphism in the closely linked marker locus D6S286. These findings are confirmed by multipoint linkage analyses and by independent observations in the Southwestern Indian sample.

We are conducting a two-stage full autosomal genome linkage scan on the Finnish families. This approach is being taken in order to minimize laboratory analyses while retaining statistical power. In Stage I, 282 individuals are being genotyped for a panel of 256 microsatellite loci. These marker loci span the autosomal genome at an average density of 13.2 cM (largest gap = 25 cM). The 282 individuals belong to 112 informative full sib-ships from 89 pedigrees. Most loci in this panel segregate tetra-nucleotide repeat alleles. More than 100 loci have been typed on the Stage I sample since January 1, 2000. Genotyping is being performed on Perkin Elmer/Applied Biosystems 373A and 377 DNA

sequencers using routine methods. Two standard DNA samples are run on every gel in order to facilitate calibration of amplicon size estimates across gels. In addition, 1 out of every 22 DNA samples is a quality control sample whose identity is hidden from the laboratory genotyping personnel. Our genotyping error rate is less than 1.5% based on over 750 duplicated typings of these quality control samples. Our success rate of PCR amplification exceeds 95%. Two-point and multi-point linkage analyses are being performed on these data using a linkage method that simultaneously utilizes information from unaffected, discordant and affected sib-pairs.

In Stage II, the genomic regions identified in Stage I with the most promising evidence for loci linked to alcoholism and related phenotypes will be selected for follow-up. New microsatellite loci will be added to the existing marker map to increase the marker density in the most promising regions. In addition, statistical power will be enhanced by genotyping all family members. This will provide more precise estimates of allelic identity by descent. It will also provide many pairs of distant relatives who contribute linkage information to the variance-components and pedigree-based linkage methods.

RESEARCH HIGHLIGHTS: This year, we collected about 75% of the laboratory typings necessary for a full genome linkage scan in families of antisocial alcoholics. This is a severe alcohol related phenotype characterized by aggressive and impulsive behavior that often occurs with antisocial personality disorder. This form of alcoholism is distinguished by its high heritability. Genetic analysis may eventually lead to a better understanding of the behavioral and developmental underpinnings of this trait.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: It is well known that alcoholism is transmitted in families in a complex manner. Nonetheless, it has been difficult to identify either the contributing genes or specific environmental factors. This work has contributed to identifying the chromosomal regions that contain predisposing genes. Ultimately, we should be able to identify these genes and the mechanisms leading to alcoholism.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00017-08

Title: POPULATION GENETICS OF NATIVE AMERICAN TRIBES

Staff Years: 0.75

Principal Investigator: JC Long, PhD (SPGL)

Other Lab Personnel: C Kolman, PhD
JG Lorenz, PhD
FC Romero, MA

NIH Collaborators: NIAAA, HN (D Goldman, MD)

Extramural Collaborators: Indian Health Service (C North, MD)

Sample Type: Interviews, Questionnaires, or Surveys only

Keywords: population research, ethnicity, molecular genetics

SUMMARY: The purpose of this work is to ascertain the numbers of alleles, allele frequencies, and allele frequency differences among American Indian tribes. The genetic systems being typed are the same as those being used currently in our genetic linkage analyses. Approximately 30 individuals from each of 20 tribes, collected primarily at the Albuquerque Indian Hospital between the years 1992 and 1994, are being analyzed. Various tests for allele frequency differences between tribal groupings, based on cultural and linguistic affinities, are being performed. In order to more fully quantify isolate structure and exploit such populations for linkage analyses, we have developed a maximum likelihood method to characterize populations by their levels of gene identity. We have applied this method to microsatellite typings for three American Indian and three European populations. Low gene identity was observed in Europeans, approximately 28%. By contrast, gene identity was higher in all American Indian populations, about 39%. We have studied local patterns of gene diversity in 7 samples from populations in the Southwest and Alaska. These populations all speak closely related Athabascan languages, despite their dispersed geographic locations. When compared to non-Athabascan speaking neighbors, we do not find a strong tendency for these populations to comprise a unified gene pool. Rather, geographic proximity is the best predictor of the genetic relationship. In the past year, we have studied distribution in these populations of genetic polymorphism in unique sequences associated with genes that are related to neurobiology and alcohol metabolism. We have also been examining these polymorphisms in native Asian populations. This information is important to genetic linkage and disease association studies on American Indians because failure to account for population diversity can result in false evidence for linkage and allelic heterogeneity among groups can create spurious associations with disease.

RESEARCH HIGHLIGHTS: Linkage analyses indicate that alleles at the alcohol dehydrogenase (ADH) gene cluster may influence vulnerability to alcoholism in American Indians and others. In a survey of 5 tribes we failed to find the *ADH2*2* allele that is most commonly associated with resistance to alcoholism in Chinese, Japanese and other East Asians. Moreover, we performed a detailed analysis of genetic polymorphism spanning the entire ADH gene cluster in American Indians and Asians (including Chinese, Mongolians and Siberians). In a large sample from one tribe, we were unable to show any association between these genetic variants and alcoholism or a variety of traits related to the symptoms of alcoholism or the physiological response to drinking alcohol.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: This research shows that there are genetic differences within and among American Indian tribes and between American Indians and the peoples of Asia. It is important to be aware of these patterns of genetic differences so that results from one group are not inappropriately superimposed on others.

Reporting Period: 1 October 1999 - 30 September 2000

Project Number: Z01 AA00019-08

Title: ALDH2 DEFICIENCY--POPULATION GENETICS AND RELATIONSHIP TO PHENOTYPE

Staff Years: 0.5

Principal Investigator: D Goldman, MD (HN, LNG, NIAAA)

Other Lab Personnel: L Akhtar, MS
M Enoch, MD
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K Xu, MD, PhD

NIH Collaborators: NIAAA, LNG, SPGL (JC Long, PhD)

Extramural Collaborators: None .

Sample Type: Neither Human Subjects nor Human Tissues

Keywords: population research, ethnicity, gene mapping (human), molecular genetics, drinking patterns & causes

SUMMARY: In Orientals, ALDH2 deficiency due to the common polymorphism Glu487Lys frequently causes a flushing reaction after alcohol consumption and this aversive reaction is responsible for lower rates of alcoholism in individuals with the inactive Lys487 allele. R. Peterson identified a series of additional markers at ALDH2. By restriction enzyme analysis, SSCP, and sequencing, haplotypes characteristic for the two ALDH2 alleles were identified. This analysis has shed light on the origins and functional role of the Lys487 allele, which appears to have originated once, on a single haplotype lineage, and spread among East Asian populations. Furthermore, although it probably originated on a single genetic background, haplotype analysis reveals that the mutation was sufficiently ancient for additional mutations to have occurred subsequently. The results on ALDH2 haplotypes are most compatible with an effect of selection to maintain the Oriental ALDH2 variant, Glu487Lys. We are investigating the relationship of ADH and ALDH functional alleles to alcoholism and other substance abuse phenotypes. These genotype/phenotype relationships are being studied in combination with other loci, for example the OPRM1 receptor locus in opioid addiction.

RESEARCH HIGHLIGHTS: We have shown, through the analysis of ALDH2 haplotypes (constellations of genetic markers), that the functional ALDH2 variant causing the common enzyme deficiency in Orientals is ancient in origin and has probably been brought to high frequency by natural selection.

SIGNIFICANCE TO BIOMED RESEARCH AND TO THE PROGRAM: Evidence for selection to maintain a high frequency of the ALDH2 variant strongly implies that it has a role in physiology, even in the absence of alcohol intake.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00025-04

Title: TRANSMISSION AND GENETICS OF ALCOHOL DISORDERS IN A NATIVE AMERICAN TRIBE

Staff Years: 0.75

Principal Investigator: JC Long, PhD (SPGL, LNG, NIAAA)

Other Lab Personnel: E Moore

NIH Collaborators: NIAAA, LNG, HN (D Goldman, MD)

Extramural Collaborators: Center for Human Behavior Studies, Weatherford OK (B Albaugh, MSW)

Sample Type: Interviews, Questionnaires, or Surveys only

Keywords: gene mapping (human), neurosciences, drinking patterns/causes, molecular genetics

SUMMARY: Using a whole genome scan, we will assess genetic linkage to alcoholism and associated psychiatric disorders in Choctaw American Indians. Choctaw is a large Eastern North American Indian tribe with over 30,000 enrolled members living within tribal boundaries in Oklahoma. By contrast to neighboring American Indian tribes that have high prevalence of alcoholism, this tribe stands out because alcoholism has a low prevalence, about 1% of females and 10% of males. By studying American Indians in the context of low alcoholism, we can expect to reveal differences in the roles of genetic and/or environmental determinants of alcoholism. Genetic analysis is to be conducted using two samples from the tribe: a small random sample (N=100) and large extended families (N=200 tested subjects). The sampling design accommodates case-control association analyses and non-parametric two- and multi-point linkage methods. In order to perform the analyses outlined above, individual psychiatric interviews and blood samples have been collected. Research diagnoses are made from the psychiatric interviews and DNA for genotyping is being extracted from the blood samples. For the linkage analysis, we expect to type up to 2000 unique sequence DNA polymorphisms, spanning the entire human genome, as the technology becomes available. Database checks and epidemiological analyses are now under way and we are assessing the reliability of psychiatric diagnoses made using different systems, e.g., DSM-III-R, DSM-IV and ICD 10. We are also examining individual differences in cultural experience and how they relate to vulnerability to alcohol-related problems.

RESEARCH HIGHLIGHTS: We have documented a Native American tribe with a low prevalence of alcoholism and related mental disorders. This is partially related to a strong religious community and low exposure to alcohol.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: There are many genetic and environmental factors that contribute to risk for alcoholism. It may be possible to identify these factors by studying the transmission of alcoholism in semi-isolated communities such as Native American tribes. We can expect that each community will have fewer genetic and environmental factors contributing to risk and that these may be apparent by contrasting disease occurrence patterns in different communities.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00083-07

Title: DNA DAMAGE, DNA REPAIR AND ALCOHOL

Staff Years: 4.2

Principal Investigator: PJ Brooks, PhD (MN, LNG, NIAAA)

Other Lab Personnel: K Bhuiya, BS
D Goldman, MD
A Kang
G Lorenzo
CA Marietta, MS
Y Ni, MD, PhD

NIH Collaborators: NCI, DB (J Robbins, MD, PhD)
NIEHS, LSB (RW Sobol, PhD)
NIEHS, ECM (KR Tindall, PhD)

Extramural Collaborators: Mt Sinai School of Medicine (A Cedarbaum, PhD)

Sample Type: Human Tissues, Fluids, Cells, etc.

Keywords: neurosciences, molecular genetics, transgenic animals, cancer, cirrhosis, health and behavior

SUMMARY: Alcohol consumption produces a variety of pathological effects, including fetal alcohol syndrome, liver and brain damage and an increased risk of cancer of the upper GI tract. The association between ethanol consumption and cancer suggests that alcohol intake results in effects on genomic DNA. Mechanisms by which ethanol can produce DNA damage are: 1) direct adduction of DNA by acetaldehyde, the major metabolite of ethanol; 2) the generation of DNA damaging oxygen radicals by cytochrome P450 2E1 (CYP2E1), which is induced by ethanol in liver and brain. To test the hypothesis that alcohol metabolism results in DNA damage, we have created CHO cell lines, which express biologically relevant levels of CYP 2E1, ADH4 (the form of ADH specifically produced in upper GI tract) or both enzymes. The cells contain a mutational target (the APRT gene), which makes them highly sensitive for detecting mutations produced by cross-linking agents and oxygen radicals. Our prediction is that growing these cells in presence of EtOH will increase mutation frequency. The level of DNA repair activity in target tissues is expected to be a crucial determinant of alcohol-induced DNA toxicity. Therefore, we have also placed the EtOH metabolizing enzymes in CHO cells, which lack specific DNA repair pathways. This will allow us to identify which of the several DNA repair pathways play a role in protecting cellular DNA from genomic damage to EtOH metabolites. Analogous studies are being carried out in mice to assess whether the lack of specific DNA repair pathways makes them more susceptible to EtOH related tissue pathologies. We are also studying N2-ethyl deoxyguanosine, the major DNA adduct produced by acetaldehyde. This adduct is undetectable in normal liver but accumulates in the DNA of mice fed alcohol. We are assessing whether the adduct is a substrate for DNA repair and what type of repair is involved as well as assessing the mutagenicity of this adduct in mammalian cells. We are also focusing on the generation and repair of a specific type of oxidative DNA lesion called cyclo-dA. We have shown that cyclo-dA is a substrate for repair by the nucleotide excision repair pathway, and is a strong block to gene expression in mammalian cells. We are developing sensitive assays for detecting this lesion in DNA obtained from tissue samples, to study the relationship between the formation of this lesion and neurodegenerative disease.

RESEARCH HIGHLIGHTS: We have identified a novel type of DNA damage that interferes with the ability of human cells to use genes. Because this type of damage results from oxygen radicals that are generated in human cells constantly, it may play a role in neurodegenerative disease and aging of the brain.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: We have identified a novel type of DNA damage that interferes with the ability of human cells to use genes. Because this type of damage

results from oxygen radicals that are generated in human cells constantly, it may play a role in neurodegenerative disease and aging of the brain. Since alcohol abuse generates oxygen radicals, this lesion play a role in the loss of neurons observed in the brains of chronic alcoholics.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00280-11

Title: GENETIC STUDIES OF EEG AND ERP TRAITS RELATED TO ALCOHOLISM

Staff Years: 1.8

Principal Investigator: MA Enoch, MD (HN, LNG, NIAAA)

Other Lab Personnel: D Goldman, MD
CR Harris, BA
KV White, ME

NIH Collaborators: None

Extramural Collaborators: Washington University School of Medicine, St Louis (JW Rohrbaugh, PhD)

Sample Type: Interviews, Questionnaires, or Surveys only

Keywords: health & behavior, neurosciences, drinking patterns & causes, electrophysiology/EEG, molecular genetics

SUMMARY: Alcoholism heritability has been established in both men and women, but, as for other complex psychiatric diseases, it has proved difficult to map genes. We have identified an intermediate marker for alcoholism vulnerability, the low voltage alpha (LVA) EEG, which is a normal, trait-like heritable variant of the resting EEG, present in 4-11% of the population, in which the alpha rhythm is virtually absent. We now have a complete data set, including EEG and ERP phenotypes, blind-rated DSM-III-R diagnoses, psychometric tests and DNA on 247 individuals from Bethesda, MD. We have shown that in these subjects, LVA is associated with alcoholism, particularly when accompanied by anxiety disorders (Enoch et al 1995,1999). We have found that LVA individuals have reduced amplitude P300 ERPs, further strengthening our argument for the association of LVA with alcoholism vulnerability. In order to obtain sufficient power to map genes for alcoholism, the focus of this study shifted to a Plains American Indian tribe, which has a high prevalence of alcoholism. We now have EEGs and ERPs on 374 tribal members from large pedigrees and have almost completed the data set of blind-rated DSM-III-R psychiatric diagnoses and DNA from these individuals. Preliminary studies confirm the relationship of LVA with alcoholism. We will soon be in a position to start analyses for mapping genes for alcoholism.

This project was formerly titled "Genetic studies of the electroencephalogram and event-related potentials."

RESEARCH HIGHLIGHTS: We have succeeded in collecting a full data set of: EEG/ERP analyses, psychiatric diagnoses, psychological testing and DNA on approximately 360 members of a Plains American Indian tribe who come from essentially one large pedigree. Preliminary analyses confirm the association of low voltage alpha EEG with alcoholism that we found in Caucasians.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: We now have a large data set on 360 individuals who are all inter-related. This will allow us to map genes for alcoholism and nicotine addiction.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00281-11

Title: ALCOHOLISM IN AMERICAN INDIANS

Staff Years: 2

Principal Investigator: D Goldman, MD (HN, LNG, NIAAA)

Other Lab Personnel: L Akhtar, MS
MA Enoch, MD
RW Robin, PhD
SG Schamberger
K Xu, MD, PhD

NIH Collaborators: NIAAA, LNG, SPGL (JC Long, PhD)
NIDDK, PECRB (PH Bennett, MB, FRCP)
NIDDK, PECRB, DAE (RL Hanson, MD)
NIDDK, PECRB, DAE (WC Knowler, MD, PhD)

Extramural Collaborators: Yale University School of Medicine (S O'Malley, PhD)

Sample Type: Human Subjects

Keywords: population research, rural health, neurosciences, health & behavior, molecular genetics, gene mapping (human)

SUMMARY: The problem of alcoholism in American Indians has been comprehensively addressed in three family linkage datasets: 1] SW tribe; 2] Eastern Oklahoma tribe with low prevalence of alcoholism - field study in progress directed by Dr. Long; and, 3] Plains Indian tribe with neuropsychological data [EEG] - analytical studies in progress; and also in a large cross-population study in progress - the Ten Tribes Study which systematically compares 300 randomly sampled subjects from each tribe. In addition, LNG is the genetics collaborating laboratory on the first pharmacogenetic study on alcoholism treatment response [to naltrexone and sertraline]. S. O'Malley leads this study, on Alaska Natives. The whole genome scan for the SW tribe detected two potential new loci for alcoholism: the DRD4 region at the chromosome 11p telomere and the region of the GABA(A) cluster near the chromosome 4p centromere. The SW Indian sample is a large family (N=582) comprising a sizeable fraction of a Southwestern tribe with a high rate of alcoholism (85% of males, greater than 50% of females). This family was systematically interviewed, substantial evidence for familial transmission of alcoholism was found, and then it was evaluated for genetic linkage, in collaboration with investigators from NIDDK (W Knowler, R Hanson, P Bennett) and, using a large (N=517) panel of DNA markers which covered all human chromosomes except the sex chromosomes: X and Y. The SW tribe studies were informative for the meaning and consequences of alcohol-related diagnoses in American Indians. The results are representative because 1] the very large family was ascertained solely on the basis of structure and availability, 2] the family is representative of the tribe in coefficient of relationship and demography, and 3] CAGE scores do not differ from a random sample of 3112 individuals studied collaboratively by NIDDK [Hanson et al]. The evidence from the SW tribe is that binge drinking is neither benign nor beneficial, that alcoholism is familial, and that the same patterns of psychiatric comorbidity seen in the general United States population [National Comorbidity Survey] are seen in Indian alcoholics. The majority of the large fraction of the population who were binge drinkers was also alcoholics, and binge drinkers tended to become alcoholic at a younger age. Regardless of whether binge drinkers met criteria for alcoholism, they were dramatically worse off in each of the four symptom categories evaluated in the SADS-L: social, work, violence/lawlessness and physical. We found the same heavy clusterings of psychiatric disorders with alcoholism as previously observed in the general population of the U.S. These results may help lay to rest the misconception that drinking, particularly binge drinking, is other than deleterious to American Indians regardless of whether binge drinking is culturally determined or congruent.

Formerly titled: *Molecular Genetic Studies on Alcoholism in American Indians – Southwestern Tribe*

RESEARCH HIGHLIGHTS: Three putative genome locations for genes influencing alcoholism were identified, each at the location of a prominent candidate gene or genes. The locations were chromosome 11p – dopamine D4 receptor, chromosome 4p – GABAA receptor cluster and chromosome 4q – alcohol dehydrogenase gene cluster.

SIGNIFICANCE TO BIOMED RESEARCH AND TO THE PROGRAM: Identification of genes for alcoholism vulnerability can lead to new drug targets and more accurate targeting of therapies.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00290-10

Title: MOLECULAR GENETIC STUDIES OF SEROTONIN FUNCTION

Staff Years: 1

Principal Investigator: D Goldman, MD (HN, LNG, NIAAA)

Other Lab Personnel: L Akhtar, MS
MA Enoch, MD
C Mazzanti, PhD
SG Schamberger

NIH Collaborators: NIAAA, LMCN (FF Weight, MD)
NIAAA, LNG, MG (GL Jenkins, MS)
NIAAA, LNG, MG (DA Nielsen, PhD)
NIAAA, LNG, SPGL (JC Long, PhD)
NIAAA, LCS, NN (JD Higley, PhD)
NIMH, CPB (NE Rosenthal, MD)
NIMH, LCS (DL Murphy, MD)
NCI, LVC (M Dean, PhD)

Extramural Collaborators: Karolinska Hospital, Stockholm (M Asberg, MD)

Sample Type: Human Subjects

Keywords: population research, neurosciences, gene mapping (human), molecular genetics, drinking patterns & causes

SUMMARY: Studies on individuals and animals with genetic defects in serotonin function can shed light on the role of this neurotransmitter on behavior and on the role of milder functional variants in serotonin genes in predisposing individuals to psychopathologies and to alcoholism. We are identifying probands for family studies by measuring the serotonin metabolite 5-HIAA in cerebrospinal fluid and by identifying individuals with amino acid substitutions in genes involved with serotonin function. Two 5HT1A variants are rare amino acid substitutions (Gly22Ser and Val28Ile), one conservative and one nonconservative. The 5HT2C variant is a common (allele frequency=0.18) nonconservative substitution (Cys23Ser). Two 5HT2A amino acid substitutions (Ala477Val and His452Tyr) have allele frequencies of 0.01 and 0.09. Rare serotonin transporter and 5HT7 amino acid substitutions were also discovered. Three of these amino acid substitutions were shown to alter the functional properties of the corresponding receptor. 5HT1A Gly22Ser when expressed in CHO-K1 cells dramatically altered desensitization and down regulation of these receptors. 5HT2C Cys23Ser in oocytes and COS-7 cells decreased ligand binding. 5HT2A His452Tyr impaired signal transduction in platelets from subjects with the 452Tyr allele. For association and direct gene analysis, we collected more than 40 cell lines from each of the following populations: anorexia nervosa (collaboratively with W. Kaye), obsessive-compulsive disorder (D. Murphy), low CSF 5-HIAA with Type II alcoholism (M. Linnoila, M. Virkkunen, M. Eggert), and seasonal affective disorder (N. Rosenthal, N. Ozaki). The detected polymorphisms are converted to PCR RFLPs or allele-specific amplification markers for ease of analysis. Using the CEPH reference pedigrees and the polymorphisms at these genes, each gene is genetically mapped to its chromosomal location. For direct gene analysis, we mainly use single-strand conformational polymorphism (SSCP) analysis and direct sequencing. Association of a TPH polymorphism with suicidality in impulsive alcoholic Finns was replicated. Sib-pair linkage of 5HT1B to antisocial alcoholism was found in Finns (J. Lappalainen) and replicated in Southwestern American Indians. The serotonin transporter promoter variant 5-HTTLPR that was previously linked to neuroticism was linked to the two anxiety-related subscales of the TPQ in a sib-pair analysis (C. Mazzanti), partially replicating an earlier finding. In a series of publications, we have shown that the 5HT2A -1438G>A promoter variant is linked to anxiety related conditions, including OCD, seasonal affective disorder, anorexia nervosa and anxiety-related scales from the Tridimensional Personality Questionnaire.

Formerly titled: *Molecular Genetic Studies of Disturbed Serotonin Function*

RESEARCH HIGHLIGHTS: We have shown that the 5HT2A -1438G>A promoter variant is linked to anxiety-related conditions including OCD, SADS and anorexia nervosa.

SIGNIFICANCE TO BIOMED RESEARCH AND TO THE PROGRAM: Anxiety is frequently a component in alcohol abuse. Identifying links to its causes may aid in identifying links to alcohol abuse.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00293-04

Title: ROLE OF THE SEROTONIN TRANSPORTER PROMOTER POLYMORPHISM IN PSYCHIATRIC DISORDERS

Staff Years: 1.5

Principal Investigator: D Goldman, MD (HN, LNG, NIAAA)

Other Lab Personnel: DJ Bozak, MS
A Ellini, BS
C Mazzanti, PhD
CL Sokolsky, BA
J Vanakoski, MD, PhD

NIH Collaborators: NIMH, CPB (NE Rosenthal, MD)
NIMH, CBDB (D Weinberger, MD)
NIMH, LCS (DL Murphy, MD)

Extramural Collaborators: University of Pisa, Italy (G Cassano, MD)

Sample Type: Interviews, Questionnaires, or Surveys only

Keywords: neurosciences, health & behavior research, eating disorders, drinking patterns & causes, gene mapping (human), molecular genetics

SUMMARY: Dysfunctions in serotonergic pathways may underlie several psychiatric disorders. The serotonin transporter (5-HTT) plays a critical role in the termination of serotonergic neurotransmission by Na-dependent uptake of serotonin by the presynaptic neuron. 5-HTT also represents the initial site of action of certain antidepressant drugs and neurotoxins. A functionally significant polymorphism, 5HTTLPR, was identified in the 5-HTT promoter. The polymorphism affects *in vitro* 5-HTT transcription and, ultimately, 5-HTT function. We have shown that the "s" allele is also associated with lower *in vivo* serotonin transporter availability in brain. This was accomplished using B-CIT in a SPECT study of normal controls and alcoholics. Frequency of the 5-HTTLPR was identified in a variety of clinical psychiatric populations including alcoholics; linkage and association studies were performed. Positive linkage was detected between 5-HTTLPR and the two anxiety-related personality traits available on the TPQ, at least partially replicating the reported association of this variant to behavior (see bibliography). In contrast, no association was found in Italian patients with obsessive-compulsive disorders, panic disorders and eating disorders. However, two additional disease-specific findings were made: 1) in a collaboration with A. Malhotra and D. Pickar, 5-HTTLPR was found to be significantly associated with BPRS-rated psychoticism in schizophrenia and 2) in a collaboration with N. Rosenthal, HTTLPR was significantly linked with seasonal affective disorder and seasonality rating in SAD patients.

RESEARCH HIGHLIGHTS: The 5HTTLPR promoter was identified in a variety of psychiatric populations including alcoholics.

SIGNIFICANCE TO BIOMED RESEARCH AND TO THE PROGRAM: These discoveries may lead to identification of causes of alcoholism.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00294-04

Title: EVOLUTION AND VARIATION OF MACACA 5HT1A

Staff Years: 0.75

Principal Investigator: D Goldman, MD (HN, LNG, NIAAA)

Other Lab Personnel: P Guillot, PhD

NIH Collaborators: NIAAA, LNG, MG (GL Jenkins, MS)
NIAAA, LNG, MG (DA Nielsen, PhD)
NIAAA, LCS, NN (JD Higley, PhD)

Extramural Collaborators: University of New York, Stoneybrook (PT Mehlman, PhD)

Sample Type: Neither Human Subjects nor Human Tissues

Keywords: neurosciences, molecular genetics, gene mapping (nonhuman)

SUMMARY: 5HT1A is the intronless coding locus (1266 base pair - 422 amino acids) for a G protein-coupled serotonin receptor with a typical 7-transmembrane structure, located on chromosome 5 in humans. Previous work in this laboratory discovered two variants (Biochem Biophys Res Commun 1995;210(2):530-6), characterized their frequency and distribution in human populations (Human Mutation 1996;7:135-43) and investigated their functional effects (Neuropsychopharmacology 1997;17:18-26). In order to assess the polymorphic spectrum of this locus in a primate animal model heavily used in neuroscience research, we cloned and sequenced the highly conserved 5HT1A gene from four macaque species (Macaca fascicularis, Macaca maura, Macaca mulatta and Macaca nemestrina) and from the vervet monkey (Cercopithecus aethiops). Both interspecific and intraspecific sequence variations have been discovered, the interspecific variation supporting the known phylogeny of Macaca, while the intraspecific variation will be characterized in a large group of Macaca mulatta, for which serotonin metabolites and behavioral data exist, in order to assess potential association between serotonin receptor variants and behavior.

RESEARCH HIGHLIGHTS: We are examining Macaca Mulatta to assess potential associations between serotonin receptor variants and behavior.

SIGNIFICANCE TO BIOMED RESEARCH AND TO THE PROGRAM: This may lead to identification of variants, which predispose to alcohol abuse.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00295-04

Title: Y CHROMOSOME POPULATION GENETICS

Staff Years: 0.5

Principal Investigator: D Goldman, MD (HN, LNG, NIAAA)

Other Lab Personnel: RA Aragon, BS
P Guillot, PhD
MQ Radel, MD

NIH Collaborators: NIAAA, LNG, SPGL (JC Long, PhD)

Extramural Collaborators: Natal Institute of Immunology (M Hammond, PhD)

Sample Type: Human Subjects

Keywords: population research, drinking patterns & causes, gene mapping (human), molecular genetics

SUMMARY: The estimation of the mutation rates of Y chromosome microsatellite loci, human Y chromosome phylogeny and human population divergence dates are the goals of this project. Approximately 500 individuals, drawn primarily from 5 Asian and 11 Native American population samples, are being genotyped at 9 microsatellite loci and 5 non-repetitive loci with known ancestral state, all located on the non-recombining, non-pseudoautosomal region of the human Y chromosome. Haplotypes will be constructed from the collected genotypes and population genetic parameters including heterozygosity and allelic repeat unit variance statistics will be calculated to compare population diversities and haplotype/population associations. Phylogenetic analysis using distance (population variance) and parsimony (interhaplotypic distance) methods will construct networks of evolutionarily-related populations and haplotypes. Linkage disequilibrium statistics will be calculated to estimate microsatellite mutation rates using population-modeling approaches adapted from autosomal linkage disequilibrium mapping methods. These analyses will contribute to an understanding of the dispersal and migration of ancestral Asian populations into Asia and the Americas and will describe the relationships among descendent populations through the combined population genetic, phylogenetic and linkage disequilibrium analyses performed.

RESEARCH HIGHLIGHTS: Haplotypes will be constructed from the collected genotypes and population genetic parameters, in order to compare population diversities and haplotype/population associations.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: This research will contribute an understanding of the dispersal and migration of ancestral Asian populations into Asia and the Americas and the direct effect on descendant populations.

Reporting Period:	10/01/1999 – 09/30/2000
Project Number:	Z01 AA00296-04
Title:	ALCOHOL DEPENDENCE AND CHROMOSOME 11P15.5
Staff Years:	0.5
Principal Investigator:	D Goldman, MD (HN, LNG, NIAAA)
Other Lab Personnel:	DP McKeane, BS
NIH Collaborators:	NIAAA, LNG, SPGL (JC Long, PhD)
Extramural Collaborators:	None
Sample Type:	Interviews, Questionnaires or Surveys only
Keywords:	population research, neurosciences, health & behavior, gene mapping (human), molecular genetics, drinking patterns & causes, ethnicity

SUMMARY: In order to follow-up a linkage finding on chromosome 11p15.5 to alcohol dependence, two short tandem-repeat marker panels for semi-automated fluorescent genotyping containing 15 loci distributed over the distal 20 cM of chromosome 11p15.5 have been created. One panel (six loci) has been typed in approximately 500 psychiatrically interviewed individuals from a Southwest American Indian tribe. A second panel (nine loci) has been optimized and typing will begin soon. In addition, two coding polymorphisms and one promoter polymorphism at DRD4, a candidate gene for involvement in vulnerability to alcohol dependence, have been typed in order to develop haplotypes at this locus, distal to the short-tandem repeat locus linked in the whole genome linkage scan. Linkage using haplotypes constructed from these markers has confirmed the primary linkage finding in a second panel of Southwest American Indian sib-pairs, indicating that a locus contributing to alcohol dependence is in the 11p15.5 region.

RESEARCH HIGHLIGHTS: A candidate gene for involvement in vulnerability to alcohol dependence has been typed along with two coding polymorphisms and one promoter polymorphism at DRD4, to develop haplotypes for the second panel.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: Haplotypes developed from markers used in the study has confirmed the primary finding indicating that a locus contributing to alcohol dependence is in the 11P15.5 region.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00297-04

Title: VARIATION OF Y CHROMOSOMAL GENES AND RELATIONSHIP TO BEHAVIOR

Staff Years: 0.2

Principal Investigator: D Goldman, MD (HN, LNG, NIAAA)

Other Lab Personnel: P Guillot, PhD

NIH Collaborators: NCI, LVC (SJ O'Brien, PhD)

Extramural Collaborators: University of New York, Stoneybrook (PT Mehlman, PhD)

Sample Type: Human Subjects

Keywords: population research, ethnicity, molecular genetics, gene mapping (human)

SUMMARY: Sequence variation in genes within the non-pseudoautosomal region of the Y chromosome within and between Hominidae and other species can elucidate Y chromosome evolution and paternal human population history and can help make sense of phenotypic variation in the male. The sequence of the coding region of the RPS4Y locus, a ribosomal protein gene, was determined in 4 non-human primate species and in 59 individuals from three human populations. Sequence analysis of RPS4Y in the Hominidae suggests that the RPS4Y protein is under relaxed functional selection compared to its highly conserved homolog, RPS4X, and predicts that the gene transposed to the Y chromosome approximately at the prosimian-simian divergence. Sequence variation at RPS4Y was detected both within and between human populations. One RPS4Y variant, C711T, appears to be the first common coding sequence polymorphism on the Y chromosome. The coalescent of human sequences (175,000 $\pm$ 125,000 years) at this locus is similar to estimates derived from different Y loci and different human population samples. The ethnographic distribution of this Y chromosome substitution identifies a paternal lineage ancestral to Asian and Native American populations. Other genes in the non-pseudoautosomal region of the Y chromosome are being evaluated for variation and relationship to behavior.

Formerly titled: *Evolution and Variation of RPS4Y*

RESEARCH HIGHLIGHTS: Identification of a coding area on the Y chromosome that may lead to identification of a relationship to behavior.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: Identification of sequence variation in genes within the non-pseudoautosomal region of the Y chromosome can elucidate Y chromosome evolution and paternal human population history and can help make sense of phenotypic variations in the male that may lead to identification of coding areas and their relationship to behavior.

Reporting Period:	10/01/1999 – 09/30/2000
Project Number:	Z01 AA00298-04
Title:	MU OPIOID RECEPTOR POLYMORPHISMS AND ALCOHOL DEPENDENCE
Staff Years:	0.2
Principal Investigator:	D Goldman, MD (HN, LNG, NIAAA)
Other Lab Personnel:	None
NIH Collaborators:	NIAAA, LNG, SPGL (JC Long, PhD) NIDCR, DIR, PNMB (R Dionne) NIDCR, DIR, PNMB (M Iadarola)
Extramural Collaborators:	University of Helsinki, Finland (M Virkkunen, MD) Yale University School of Medicine (S O'Malley, PhD)
Sample Type:	Interviews, Questionnaires, or Surveys only
Keywords:	drinking patterns & causes, craving/addictions, molecular genetics, gene mapping (human), nonalcoholic drug abuse & dependence

SUMMARY: The mu opioid receptor is implicated in the reward, tolerance and withdrawal effects of alcohol and other drugs of abuse. We directly sequenced the human mu opioid receptor locus, OPRM1, to detect natural variation that might affect the function of this receptor or be associated with psychiatric phenotypes related to opioid function. Four DNA sequence variants were found: three amino acid substitutions (Ala6Val [rare], Asn40Asp [frequency 10%], Ser147Cys [rare]) and one intronic variant (IVS2+691G/C [frequency 50%]). OPRM1 alleles, genotypes and haplotypes from three psychiatrically characterized population samples (N = 791) were used to perform association and sib-pair linkage analyses to alcohol dependence. There was no significant association or linkage between OPRM1 and alcohol dependence in any of the population samples. These results and power calculations strongly suggest that variation at the mu opioid receptor is not involved in vulnerability to DSM-III-R Alcohol Dependence. Variation is being investigated for possible association to response to opiate pharmacotherapy and to variation in opioid function, in collaboration with Stephanie O'Malley, Yale University. A study on inherited differences in nociception has been initiated with Ray Dionne & Michael Iadarola of the National Institute of Dental and Craniofacial Research, NIH. A large-scale case-control association study on opioid addiction has been completed and these results are in preparation for publication.

RESEARCH HIGHLIGHTS: There is no significant association or linkage between OPRM1 and alcohol dependence in any of three population samples. These results strongly suggest that variation at the mu opioid receptor is not involved in vulnerability to DSM-III-R Alcohol Dependence.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: Variation is being investigated for possible association to response to opiate pharmacotherapy as well as variation in opioid function.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00299-04

Title: TRYPTOPHAN 2,3-DIOXYGENASE--CANDIDATE GENE FOR SEROTONIN METABOLISM DISORDERS

Staff Years: 0.1

Principal Investigator: MA Enoch, MD (HN, LNG, NIAAA)

Other Lab Personnel: D Goldman, MD
CR Harris, BA

NIH Collaborators: NIMH, LCS (DL Murphy, MD)

Extramural Collaborators: University of Pisa, Italy (G Cassano, MD)

Sample Type: Human Tissues, Fluids, Cells, etc.

Keywords: behavioral research, health & behavior, neurosciences, molecular genetics, gene mapping (human), drinking patterns & causes

SUMMARY: Genetic defects in the enzymes involved in serotonin metabolism may contribute to a wide range of neuropsychiatric diseases, from eating disorders, obsessive-compulsive disorder and alcoholism to autism. Tryptophan, obtained only from the diet in humans, is converted to serotonin by tryptophan hydroxylase, or to kynurenine by tryptophan 2,3-dioxygenase (TDO2). Both enzymes are rate limiting in their respective pathways. The purpose of this study is to screen the TD02 gene for polymorphisms, assess functionality and search for disease associations in 350 individuals, primarily using single-strand conformational polymorphism (SSCP) analysis. Most of the coding region (11 of the 12 exons) and short regions of the introns were successfully amplified and screened across populations with anorexia or bulimia nervosa, obsessive-compulsive disorder, autism, major depression, suicidality, impulsivity and alcoholism and subjects were enrolled in a tryptophan depletion study. No associations were found for polymorphisms in introns 5, 6 and 11 or for a variant in exon 7 (A to C, 749 Asn to His). In the SSCP screening of the promoter region, no polymorphisms were found in the regions of two TATA boxes. An A to C variant was detected in the putative glucocorticoid site but was not associated with disease. However, in the promoter region of GTT repeats, a GTT insertion was found that may be associated with impulsivity and novelty seeking but not with alcoholism. Three further polymorphisms have now been found in the promoter region and are in the process of being sequenced and screened across populations.

RESEARCH HIGHLIGHTS: Full analysis of the variants in this gene is underway.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: Unable to determine until results have been analyzed.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00300-03

Title: 5HT2A RECEPTOR PROMOTER POLYMORPHISM, -1438G/A AND SEROTONIN DYSFUNCTION

Staff Years: 0.1

Principal Investigator: MA Enoch, MD (HN, LNG, NIAAA)

Other Lab Personnel: D Goldman, MD
CR Harris, BA

NIH Collaborators: NIMH, CPB (NE Rosenthal, MD)
NIMH, LCS (DL Murphy, MD)

Extramural Collaborators: University of Pisa, Italy (G Cassano, MD)

Sample Type: Human Tissues, Fluids, Cells, etc.

Keywords: genetics, serotonin receptor, association, anorexia nervosa, obsessive-compulsive disorder, depression

SUMMARY: Brain serotonin dysfunction is implicated in a range of heritable diseases including alcoholism, mood disorders, eating and obsessive-compulsive (OCD) disorders. An important starting point for understanding innate vulnerability to such diseases is to identify variation in genes involved in serotonin function. The 5-HT2A receptor gene is thought to contribute to appetitive behaviors and to anxiety, and is one site of action of antipsychotics, hallucinogens and anti-depressants. 5-HT2A receptor densities are higher in individuals with depression and suicide attempts. In this study we replicated an earlier finding of an association of the 5-HT2A promoter polymorphism -1438G/A with anorexia nervosa. In addition we showed that the association extends to OCD but not to bulimia nervosa, a disorder in which obsessive and perfectionistic traits are less manifest (Enoch et al, 1998). We have now shown that the promoter polymorphism is associated with OCD in women but not men (Enoch et al, Biol Psychiatry, in press). We have also demonstrated that this 5-HT2A polymorphism is associated with a particular personality type: low novelty seeking and high harm avoidance, again in women but not in men. We have shown an association of the -1438A variant allele with Seasonal Affective Disorder, a condition in which depression recurs in the winter and remits in the spring (Enoch et al, 1999). Association studies are being completed in other data sets and preparations are being made for functional studies of this promoter polymorphism.

RESEARCH HIGHLIGHTS: We have replicated an earlier finding that a variant in the promoter region of the gene for the serotonin 2A receptor (-1438G/A) is associated with anorexia nervosa but not bulimia nervosa. We have also shown that it is associated with obsessive-compulsive disorder (OCD) in women but not in men.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: The results suggest that the variant in this gene may predispose individuals to the development of the perfectionistic and obsessional type of personality that underlies both anorexia nervosa and OCD, but is not so manifested in bulimia. This may eventually lead to more specific treatments for these disorders.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00301-02

Title: HIGH THROUGHPUT GENOTYPING & GENOTYPE CELLS FOR SINGLE NUCLEOTIDE POLYMORPHISMS

Staff Years: 3

Principal Investigator: D Goldman, MD (HN, LNG, NIAAA)

Other Lab Personnel:
L Akhtar, MS
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AJ Marko, BS
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S Nagarajan, BS
M Repik, BS
J Rho, BA
KK Robinson, BS
J Rudolph, PhD
D Shaw
S Vattikuti, BS
SK White, BS
KC Wolfe, BS
K Xu, MD, PhD

NIH Collaborators: None

Extramural Collaborators: HM Jackson Foundation, Rockville (R Lipsky, PhD)

Sample Type: Human Tissues, Fluids, Cells, etc.

Keywords: automated genotyping, single nucleotide polymorphisms

SUMMARY: *High Throughput SNP Identification*--LNG plays a leading role in the identification of functional variants in neurogenetic candidate genes. This involves high throughput detection of sequence variants of genes expressed in the brain, with a particular focus on detection of both common and uncommon variants, screening in relevant clinical populations and functional characterization. Common functional variants LNG has discovered or for which it has demonstrated *in vitro* functionality include serotonin, dopamine and opioid receptor variants. Each is a major target in the fields of behavior and neuropharmacology. Studies on neurogenetic candidate genes have found that such genes also have a high abundance of polymorphism, including frequent missense variants. Variation within the coding sequences of 18 dopamine and serotonin GPCRs carefully scanned for sequence variation across several hundred individuals has been summarized. Because many of the non-synonymous variants at these serotonin and dopamine receptors had also been evaluated for *in vitro* functionality, these two receptor families offer a preview of the genetic variation of the brain, i.e., "*neurochemical individuality*." In Caucasians, 10 of 18 (55%) of the dopamine and serotonin receptor coding sequences contained protein amino acid substitutions or deletion/insertions with allele frequencies of $\geq$0.01, defining them as polymorphic. The average heterozygosity (chance of encountering a heterozygous locus) is about 15% across these 18 coding sequences. The likelihood of one individual expressing every one of the most prevalent dopamine and serotonin receptor alleles is 0.06; 94 of 100 Caucasians would be expected to express at least 1 variant altering dopamine or serotonin receptor structure. Variant detection is performed in up to 1000 neurogenetic candidate genes for alcoholism and related behaviors, evaluating both coding sequence and sequence domains proximal to coding sequence and likely to alter transcription or mRNA processing. The screening panel is composed of 477 genomic

DNAs enriched for clinical and ethnic diversity, enabling immediate and accurate estimation of allele frequencies of novel polymorphisms with frequency p >0.05 in Southwestern Indians and Caucasians from which our primary linkage data sets are derived. Genes for screening are prioritized on the basis of 1] postulated role in alcohol reward or response, including treatment response, 2] sensitivity of the gene product to alcohol, 3] implication of gene by whole genome or candidate gene linkage study, 4] mouse QTL or knockout finding, 5] availability of sequence, and 6] known sequence variation. As implemented, dHPLC detects > 90% of single nucleotide substitutions in amplicons 200-450 bp in size and is thus equivalent to DNA sequencing in sensitivity. Variants are confirmed and characterized by semi-automated DNA sequencing on an ABI377.

High Throughput Genotyping--Key issues are cost, accuracy and flexibility for adding new polymorphisms. Of the variety of potential choices, we selected 5'-exonuclease and developed assays for a series of single nucleotide substitution polymorphisms. The principle of the 5' nuclease assay is that two allele-specific probes are designed such that they bind to their cognate allele target at the temperature of annealing of the amplification primers. During amplification, the 5'exonuclease activity of Taq polymerase cleaves the quenching dye from the allele-specific probe, enabling the allele-specific signal dye [Vic or Fam] to fluoresce. Genotypes are read automatically and immediately following PCR. 5'nuclease assays are optimized on the ABI7700 in 96-well plates, thus the fluorescence of the two allele-specific probes is followed in real-time. The 5'nuclease assay works without further modification for > 80% of SNPs we have evaluated. Optimized assays are read on an end-point reader [Cytofluor 4000] in 384-well plates handled with an automated pipetting station [Hydra]. Genotypes are called automatically using a principal components analysis software package [Partek] and uploaded into the Access database on the server. Genotyping error rates are readily calculated from triplicates and are <0.5%. Reagent cost is $0.80/assay [$0.31 for primer/probe and $0.49 for master mix containing polymerase etc.]. LNG is evaluating a single-base extension procedure in which the amplified and single-base extended DNA is hybridized using locus-specific Zipcodes, enabling sorting of multiplexed genotypes on color coded microbeads. The locus-specific microbeads are resolved using a flow-sorter [Luminex]. The two alleles at a locus are distinguished by the single-base extension, which can be performed in multiplex and requires a minimum of locus-specific reagents [one additional locus-specific, zipcoded oligonucleotide]. This technology currently enables flexible multiplexing with up to 100 different addresses [50 loci], and it is anticipated that the number of addresses will be expanded to 1000 on the Luminex device with the addition of a blue laser.

RESEARCH HIGHLIGHTS: Studies on neurogenetic candidate genes have found that such genes also have a high abundance of polymorphism, which included frequent missense variants.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: Many of the non-synonymous variants found at the serotonin and dopamine receptors have been evaluated for *in vitro* functionality. The two receptor families offer a preview of the genetic variation of the brain or "*neurochemical individuality*."

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00302-01

Title: RELATIONSHIP OF CANDIDATE GENES AND ALLELES TO BEHAVIOR

Staff Years: 4

Principal Investigator: D Goldman, MD (HN, LNG, NIAAA)

Other Lab Personnel:
BD Berezdivin, BS
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M Repik, BS
J Rho, BA
J Rudolph, PhD
D Shaw
SK White, BS
KC Wolfe, BS
K Xu, MD, PhD

NIH Collaborators:
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NIAAA, LNG, SPGL (JC Long, PhD)
NIMH (R Vakkalanka)
NIMH, CPB (NE Rosenthal, MD)
NIMH, CRSB (MF Egan, MD)
NIMH, CBDB (D Weinberger, MD)
NIMH, CBDB (BS Kolachana)
NIMH, LCS (DL Murphy, MD)
NIDR, PNMB (RA Dionne, PhD, DDS)
NIDR, PNMB (MJ Iadarola, PhD)

Extramural Collaborators: HM Jackson Foundation, Rockville (R Lipsky, PhD)

Sample Type: Human Subjects

Keywords: candidate genes and behavior

SUMMARY: Candidate allele and gene hypotheses developed from pharmacological and genetic findings are tested. For example, testing of the functional OPRM1 variant Asn40Asp in alcoholism, opioid, dependence and naltrexone response or evaluation of HTR1B markers in antisocial alcoholism to follow up mouse QTL and knockout results. We also attempt to replicate or validate prior linkage findings, e.g., by evaluating the non-transducing DRD2 Cys311 variant in alcoholism. Finally we followed up on whole genome linkage findings. Candidate gene linkage investigations have included DRD2 Ser311Cys/alcoholism, HTR1B/antisocial alcoholism, SLCA4/anxiety, and COMT/Schizophrenia.

Phenotype, sampling framework, genetic structures [e.g. TDT, sib-pairs], and power--Assessment is generally with structured interview, and usually with intermediate phenotypes and/or additional end-phenotypes. The Finnish dataset was ascertained from criminal alcoholic probands and is thus enriched for so-called Type II alcoholism. Southwestern (SW) Indian, Plains Indian, Ten Tribes, and Finnish datasets are derived from isolates; psychiatric interviewed controls are available from source populations. Remaining datasets are almost entirely Caucasian; some 980 psychiatrically interviewed Caucasian population controls are available. The SW Indian dataset includes 600 sib-pairs, the Finnish dataset used in the SLCA4 study included 366 sib-pairs, and the Plains Indian EEG dataset contains a large

quad enabling quantitative sib-pair linkage and TDT. Both schizophrenia datasets [Egan & Weinberger, Malhotra] enable TDT/sTDT analysis. Power for case/control association is dependent on allele frequency, association strength, and desired level of alpha. The functional alleles we test have moderate [HTR2C, HTR2A, mu opioid receptor: 0.1] or high [SLCA4 and COMT: 0.4] frequencies, and the functional polymorphisms known to affect alcoholism ADH2 Arg47His and ALDH2 Glu487Lys have 5-10 fold effects. Therefore, our smallest datasets [n=50] have 58% power at the p <0.01 level; power is essentially 100% for larger datasets available for alcoholism [several datasets], schizophrenia, OCD, anorexia nervosa, SAD, and bipolar. For lower effect size, power at p <0.01 remains high [>0.8] for the association strength of 0.1 in the larger datasets.

Relationship of candidate genes and alleles to behavior:

DRD2--The DRD2 dopamine receptor, "Reward Deficiency Gene" hypothesis, was critically tested in SW Indians. The study was done with three DRD2 markers: the Taq1A marker previously implicated, STR, and Ser311Cys, which impairs signal transduction and which is far more abundant [0.16] in this particular population as compared to Caucasians [0.03]. Because it impairs function, Cys311 also serves as a surrogate for unknown DRD2 alleles that attenuate function to a similar extent or by the same mechanism [transduction]. There was no sib-pair linkage nor was there association, although the dataset included 15 Cys311/Cys311 homozygotes. Previously, LNG reported the early negative results for DRD2 and alcoholism in ethnically matched, psychiatrically interviewed alcoholics and controls, in severe alcoholics and in alcoholics with parental alcoholism. LNG also identified large ethnic differences in DRD2 allele and haplotype frequencies and participated in the discovery of Ser311Cys. Subsequently, the COGA sib-pair linkage study [Edenburg et al] has also failed to detect a DRD2 linkage signal. These results suggest there is scant evidence for a substantial role for DRD2 variation in alcohol vulnerability. To monitor functional variants at DRD2 locus, LNG has developed high throughput assays for Ser311Cys and -141DelT.

Serotonin transporter--A functional serotonin transporter promoter polymorphism was associated with dimensionally measured anxiety. Quantitative linkage in 366 Finnish sib-pairs linked this polymorphism to the two anxiety-related subscales of the TPQ [Mazzanti]. Functionality was further pursued using *in vivo* B-CIT SPECT imaging of transporter density. In controls, but not alcoholics, the lower transcribing s allele was indeed associated with lower brain transporter density. We speculate that alcoholics may experience sustained changes in serotonin transporter function, e.g., due to alcohol-induced serotonin release or effects of withdrawal.

5HT1B receptor--We built on findings from mouse genetic models to identify a potential role for the 5HT1B receptor in a subtype of alcoholism. HTR1B was implicated as a potential alcohol preference gene by location of a mouse alcohol preference QTL and subsequent discovery that the HTR1B knockout mouse exhibited increased aggression and preference for alcohol. As a terminal autoreceptor, 5HT1B modulates serotonin release and was an excellent candidate for variation in alcohol preference and aggressivity, independent of the mouse findings. Aggressive and impulsive behaviors are represented in two psychiatric diagnoses - ASPD and IED [Intermittent Explosive Disorder] and alcohol preference is a component of alcoholism. For HTR1B, we used G861C [described by us] and the closely linked D6S284 STR. We studied 640 Finnish subjects including 350 sib-pairs [220 unaffected, 79 discordant and 51 affected] and 418 SW Indians including 305 sib-pairs [223 unaffected, 71 discordant and 11 affected pairs] and classified for the presence/absence of antisocial alcoholism [DSMIII-R alcoholism plus either ASPD or IED]. This single-locus study detected evidence for i.b.d. linkage in both datasets [p=0.04 and p=0.01] and association to HTR1B was also found in Finns.

COMT (catechol-O-methyltransferase), COMT Val158Met and Frontal lobe function: Intermediate phenotype--Executive cognitive functions (ECF) localized to the frontal lobe are thought to be impaired in several psychiatric diseases: alcoholism, ADHD and schizophrenia. Patients with schizophrenia, and to a lesser extent their healthy siblings, have deficits in working memory and ECF and show excess cortical activity with fMRI [and are thus said to be inefficient] during these tasks. It has therefore been proposed that frontal lobe ECF represent an important intermediate phenotype. Dopamine enhances prefrontal cortical efficiency during frontal lobe tasks. Val158Met, a common COMT variant, leads to four-fold reduction in enzyme activity and was thus an excellent candidate gene for variation in frontal lobe cognitive function. Also, three TDT linkage studies had detected evidence for Val158Met in schizophrenia and some evidence for a schizophrenia locus had been found near 22q11. Wisconsin Card Sort performance was evaluated versus COMT genotype in 75

controls, 184 schizophrenics and 222 siblings of schizophrenics, with the result that a remarkable allele-dosage relationship was found to preserve errors in both the schizophrenic subjects and the controls. This finding was directly expanded by a study of frontal lobe metabolic activity in individuals evaluated using blood oxygen level dependent [BOLD] fMRI during the N-back task, which accesses prefrontal cognitive functions. As predicted, the Val158 allele was associated with increased metabolic activity in frontal lobe, consistent with the hypothesis of diminished cortical efficiency. Furthermore, in a TDT analysis, the ancestral Val158 allele was preferentially transmitted to the schizophrenic offspring, as had been observed in the three previously reported TDT studies. Thus, the Val158 allele appears to compromise relevant prefrontal function and may be a susceptibility gene for schizophrenia and other diseases involving ECF.

RESEARCH HIGHLIGHTS: The DRD2 dopamine receptor alleles and haplotypes frequencies have identified large ethnic differences. LNG participated in the discovery of Ser311Cys, but failed to detect a linkage signal for DRD2 variation in alcohol vulnerability.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: To attempt to replicate or validate prior candidate genes and allele linkage findings as well as to follow up on the entire genome linkage findings.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00303-01

Title: SNP Function -- In Vitro and In Vivo

Staff Years: 6.3

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Sample Type: Human Subjects

Keywords: single nucleotide polymorphisms

SUMMARY: LNG is a groundbreaking laboratory for studies relating brain gene polymorphisms to in vitro function as well as in vivo functional measures obtained by brain imaging, and through collaborations with NIMH. The polymorphism/function associations are congruent with the known molecular neurobiology of the transmitters and proteins and include dopamine transporter genotype/dopamine transporter density in striatum, serotonin transporter genotype/serotonin transporter density in raphe nucleus, and COMT genotype/metabolic activity in frontal lobe. Functional variants are the endgame of positional cloning and offer an invaluable tool for selecting the most appropriate phenotypes for linkage studies, for candidate gene hypothesis testing and for improving the prior probability of linkage. LNG is screening noncoding sequences for effects on transcription and RNA processing, and we are screening certain coding variants of GPCRs for altered ligand affinity, signal transduction and receptor down regulation. In vitro Due to neuroadaptive processes, phenotypic effects of functionally significant alleles may be difficult to discern except in cellular expression systems in which the genetic background on which the various alleles are assayed is identical. We have used transiently transfected cos-7 [mouse kidney] cells and stably transfected CHO-K1 [Chinese hamster ovary] cells for this purpose and are currently using transiently transfected HEK [human embryonic kidney] cells to compare the human HTR5a Pro15 and Ser15 alleles. 5HT1A is a somatodendritic autoreceptor on serotonin neurons in the raphe nuclei and is also expressed postsynaptically with highest densities in the limbic system. Pharmacobehavioral studies have revealed a significant role for 5HT1A in irritable aggression. We detected two rare amino acid substitutions in the amino-terminal, extracellular domain. Gly22Ser [rare allele 0.002] was observed in three Finnish Caucasians. Ile28Val [rare allele 0.005] is

present in various populations. In vitro expression studies detected no effect of these alleles on ligand binding or transduction. The effect of these substitutions on receptor down-regulation was explored because two naturally occurring amino terminal substitutions of the homologous b2 adrenergic receptor affect down-regulation. The Ser22 allele was ineffective in down-regulating the receptor via 24 hr exposure to the agonist 8-OH DPAT. We detected two missense substitutions in an intracellular loop of the 5HT2A receptor: Ala447Val [allele frequency 0.007] and His452Tyr [allele frequency 0.093. Due to the high frequency of Tyr452 and the expression of authentic 5HT2A in platelets, we were able to compare the functional properties of the His452 and Tyr452 alleles in eight His452/Tyr452 heterozygotes to eight His452/His452 homozygotes matched for sex, age and diagnosis. After 10 mM serotonin, calcium mobilization was reduced in His452/Tyr452 heterozygotes and the decay of stimulated intracellular levels was prolonged. These observations were replicated in transiently transfected cells. A 5HT2C Cys23Ser variant we detected has an allele frequency of 0.13. The variant amino acid residue is again thought to be located in the amino terminal extracellular domain. HTR2C is X-linked; therefore, 13% of males are hemizygous for Ser23 and 87% are hemizygous for Cys23. Both alleles were individually expressed in two highly distinctive cellular environments: cos-7 kidney cells and Xenopus oocytes. In both expression systems, the Ser23 allele showed diminished affinity for MCPP and affinity for 5HT was diminished in cos-7 cells. Other variants which we have detected which alter ligand affinity are the common m opioid OPRM1 receptor variant Asn40Asp, and the DRD2 dopamine polymorphism Ser311Cys, which alters affinity and transduction. In vivo The functionality of the serotonin transporter [SLCA4] polymorphism, which is associated with anxiety and alters in vitro transcription was pursued in vivo. If the mechanism of the SLCA4 linkage to anxiety was to alter transcription, an important validating step would be to demonstrate an effect of the polymorphism on serotonin transporter density in human brain. B-CIT SPECT imaging was used for genotype/transporter density studies of both the dopamine transporter [visualized in striatum] and serotonin transporter [quantitated in midbrain]. These studies were led by A. Heinz and were collaborative with NIMH; LNG was the genetics component. First, we found a significant relationship of dopamine transporter allele to dopamine transporter density. The direction of this association was congruent with dopamine transporter allele associations to ADHD and with the relationship of the allele associated with reduced DAT density to cocaine-induced paranoia. Next SLCA4 was found to be related to serotonin transporter density in 42 alcoholics and controls. Serotonin transporter density in midbrain was evaluated in a two-way ANOVA with genotype and diagnosis as predictor variables. In controls, the lower transcribing s allele was indeed associated with lower transporter density. In alcoholics, there was no relationship of genotype to transporter density. We speculate that alcoholics have sustained changes in transporter function, for example due to alcohol-induced serotonin release or effects of withdrawal. We and others detected TDT linkage of schizophrenia to the functional COMT polymorphism Val158Met, but we have extended this finding to executive cognitive performance and in vivo brain metabolic activity. Wisconsin Card Sort performance was evaluated versus COMT genotype in 75 controls, 184 schizophrenics, and 222 siblings of schizophrenics, with the result that a remarkable allele-dosage relationship was found to perseverative errors in both schizophrenia patients and controls. This finding was directly expanded by a study of frontal lobe metabolic activity. Genotyped individuals were evaluated using blood oxygen level dependent [BOLD] fMRI during the N-back task, which accesses these prefrontal cognitive functions. As predicted, during this memory task the Val158 allele was associated with increased metabolic activity in frontal lobe - consistent with the hypothesis of diminished cortical efficiency. Thus, the Val158 allele appears to compromise relevant prefrontal function and may be a susceptibility gene for schizophrenia and other diseases involving cognitive executive functions.

RESEARCH HIGHLIGHTS: *In Vitro* expression studies detected no effect of alleles on ligand binding and transduction. A study of frontal lobe metabolic activity was performed *in vivo* using blood oxygen level dependent fMRI during the N-back task, which accesses prefrontal cognitive functions. During the memory task, the Val158 allele was found to be associated with increased metabolic activity in frontal lobe. It is consistent with the hypothesis of diminished cortical efficiency.

SIGNIFICANCE TO BIOMED RESEARCH AND TO PROGRAM: Brain gene polymorphisms related to *in vitro* function as well as *in vivo* function, offers an invaluable tool for selecting the most appropriate phenotypes for linkage studies, for candidate gene hypothesis testing and for improving the prior probability of linkage.

Reporting Period: 10/01/1999 – 09/30/2000

Project Number: Z01 AA00304-01

Title: Response Mechanisms of Candidate Genes and Alleles to Behavior

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NIH Collaborators: None

Extramural Collaborators: HM Jackson Foundation, Rockville (R Lipsky, PhD)

Sample Type: Human Subjects

Keywords: alcohol treatment response

SUMMARY: Alcohol and benzodiazepine response A strategy of LNG is to test the candidate alleles against intermediate phenotypes including alcohol response and benzodiazepine response, which are predictive of vulnerability. Datasets for these two intermediate phenotypes have been developed in collaboration with their acknowledged experts: M. Schuckit who has prospectively followed a cohort of 450 men and has available psychophysiological and subjective measures of response as well as clinical outcome, and D. Cowley, who had available sensitive and accurate eye-movement measures of benzodiazepine response [using the paradigm originally developed and brought to Seattle by D. Hommer, now Acting Chief of LCS]. Thus far we have reported preliminary, but positive results with a common, non-conservative GABAA a6 amino acid substitution discovered by LNG as well as with the functional serotonin transporter polymorphism HTTLPR described by P. Lesch [Schuckit et al; Iwata et al]. The GABAA a6 gene represents an example of where we have been able to find convergence between a rodent genetic finding and the human. Korpi et al detected a GABAA a6 amino acid substitution altering the sensitivity of this receptor to alcohol and benzodiazepines and this substitution predicts the difference in alcohol sensitivity observed between alcohol accepting and non-accepting Finnish rats. The GABAA clusters on human chromosomes 4 and 5 are implicated by linkage studies in the human [LNG, Long et al] and in the rodent [Portland group], respectively. Nevertheless, except for the GABAA a6 result in the rat, a6 might not have been the first GABAA receptor we evaluated in the human because the GABAA a6 is restricted in its expression to cerebellum. As discussed above, LNG was able to detect a common, non-conservative amino acid substitution Pro385Ser and obtain preliminary evidence for involvement in alcohol and benzodiazepine sensitivity The alcohol response dataset is being expanded according to M.Schuckit's resources to collect these individuals. Pharmacogenetics of treatment response A radical change in alcoholism research is that several pharmacological treatments have recently become available that improve the ability of alcoholics to maintain abstinence. The previously available drug - disulfiram - had a direct counterpart in the ALDH2 Glu487Lys variant which created a natural blockade of the same metabolic enzyme. The new drugs, particularly sertraline and naltrexone, could also point directly to molecular targets which could be either

sources of variation in treatment response or sources of differential vulnerability. Two principal gene targets are the serotonin transporter [sertraline] and the m opioid receptor [naltrexone], and other gene targets are also directly inferred: HTR1A and HTR1B autoreceptors, postsynaptic serotonin receptors, opioid propeptides, and various genes involved in the function of interacting neurotransmitters such as dopamine. LNG is focusing efforts in sequence variant detection towards these genes and by initiating a major collaboration led by Dr. S. O'Malley towards the pharmacogenetics of naltrexone and sertraline response among alcoholics in a NW Indian tribe and towards the pharmacogenetics of naltrexone and acamprosate in the COMBINE multicenter study. LNG's molecular studies have already yielded a common, nonconservative m opioid receptor variant that M. Kreek and colleagues have reported alters affinity of the receptor for endomorphin. Ten Tribes Study LNG is a leading exponent of gene/environment interactions in alcoholism, including the use of cross-population studies. The Ten Tribes Study is a gene/environment interaction study encompassing the collection of the first large multi-population dataset specifically suited for evaluation of gene/environment interaction: the Ten Tribes Study. This study, which is approximately 2/3 complete involves the collection of DNA and psychiatric interview [AUDADIS] data on 300 demographically sampled individuals from 10 different American Indian tribes, including tribes with widely disparate rates of alcoholism and alcohol associated problems. This dataset will be used to study the effects of social and historical determinants on alcoholism, effects of alcoholism on communities [especially rates and types of trauma], and interaction of genetic factors with environmental loadings and thresholds.

RESEARCH HIGHLIGHTS: Our molecular studies have yielded a common, nonconservative mu opioid receptor variant that reportedly alters affinity of the receptor for endomorphin.

SIGNIFICANCE TO BIOMED RESEARCH AND TO THE PROGRAM: A radical change in alcoholism research is that several pharmacological treatments have recently become available to improve the ability of alcoholics to maintain abstinence. A strategy is created to test the candidate alleles against intermediate phenotypes, which are predictive of vulnerability.