ABSTRACT

Maternal alcohol consumption is the leading known cause of mental retardation in the Western world. The fetal alcohol spectrum disorder (FASD) including fetal alcohol syndrome and alcohol-related neurodevelopmental disorder causes a life-time disability to the victim. The goal of this project is to test therapeutic agents that can protect against FASD, particularly the central nervous system. The activity dependent trophic factor and their associated peptides has been known to be extremely potent as protectant against oxidative stress and alcohol induced fetal demise.

The goals of this project will be to (a) test the protective/ neurotrophic effects of ADNF-like peptides against alcohol compromised neurons, particularly the serotonin, and excitatory neurons in the limbic system. Due to extremely dynamic change in neural development, the effect of ADNF-like peptides will be tested against alcohol exposure at pre- and neo-natal stages of mice equivalent to the human 1st/2nd and 3rd trimesters respectively. (b) Second, we will study the mechanism of ADNF-like peptide protection by analyzing its gene expression in a selective paradigm.

The Aim 1 will test the neuroprotective and /or neurotrophic effect of ADNF-like peptides against alcohol neurotoxicity to developing serotonin (5-HT) and hippocampal neurons by study their apoptosis, number of matured neurons, and degree of 5-HT and glutamate innervation in the hippocampus with an established alcohol liquid diet model at E7- E18, equivalent to 1st and 2nd trimesters of human gestation. The Aim 2 will determine the neuroprotective and neurotrophic effect of ADNF-like peptide on an alcohol induced apoptosis, neurogenesis, synaptogenesis of the limbic system with binge ethanol exposure at a neonatal stage equivalent to 3rd trimester of human gestation.

SPECIFIC AIMS

Testing FASD Therapeutic Agents in C57/BL Mouse Model

The long-term goal of this project is to test therapeutic agents that can protect against fetal alcohol spectrum disorder (FASD). Two activity dependent trophic molecules, the Activity Dependent Neurotrophic Factor (ADNF) and Activity Dependent Neuroprotective Protein (ADNP) released by astrocytes upon stimulation by vasoactive intestinal peptide (VIP) (Brenneman, Hauser et al. 1998; Gozes, Bassan et al. 1999) are known to be extremely potent protective (Gressens, Marret et al. 1997) (Zamostiano, Pinhasov et al. 1999) and neurotrophic agents (Gressens, Hill et al. 1993) (Gozes, Davidson et al. 1997), particularly in developing nervous tissue. The short peptides of ADNF and ADNP, SAL and NAP, respectively, have been shown to have substantial effects on preventing alcohol induced fetal demise (Spong, Abebe et al. 2001). This proposed study will test the native form, L-form, of NAP and SAL on the cascade of midline forebrain and brainstem damage induced in prenatal and neonatal mouse models of ethanol exposure. The C57Bl/6 (B6) mouse liquid diet prenatal alcohol model and the binge exposure neonatal model have been well-characterized, have less intrusive stress than other models, are inexpensive, and have been effective in testing

NAP and SAL (preliminary data). The goals of this project will be to (a) test the protective/ neurotrophic effects of NAP/SAL against alcohol compromised neurons—monoaminergic, serotonin, and excitatory glutaminergic (Glu) neurons in the limbic system with alcohol exposure at stages of pre- and neonatal of mice equivalent to the human 1st/2nd and 3rd trimesters and (b) study the mechanism of NAP/SAL protection by analyzing its gene expression in a selective paradigm.

<u>Specific Aim 1</u>. Tests the hypothesis that NAP/SAL is neuroprotective and / or neurotrophic against alcohol neurotoxicity to developing neurons with respect to apoptosis or differentiation using alcohol exposure via consumption of a liquid diet by dams from E7- E18, the equivalent of first and second trimesters of human gestation.

Apoptosis will be determined using caspase-3 activity assay at E18. The number of mature neurons will be determined by Neu-N-positive (mature neuronal marker) of pyramidal neurons in hippocampus at E18 and P45, and 5-HT-immunostaining (im) neurons in raphe at E15 /E18 and P45 using stereological counting. Neurogenesis will be tested using bromo-deoxy-uridine (BrdU) injections at E16 and E17 and examination at E18. The neurotrophic effect of NAP/SAL on 5-HT and glutamate fibers / terminals will be assayed with immunocytochemical staining of 5-HT fibers and glutamate transporters at fibers/ terminals in the hippocampus at E18 and P45.

<u>Specific Aim 2</u>. Determine the neuroprotective and neurotrophic effect of NAP/SAL on an alcohol induced injury of the limbic system with binge ethanol exposure at a neonatal stage equivalent to 3rd trimester of human gestation.

Alcohol (s.c, 2.5g/kg twice/day, peak blood alcohol concentration-- BAC ~300mg/dl) and NAP treatments (along the same time course) will be given at P7. The neuroprotective effect, particularly with respect to hippocampus, subiculum, medial frontal and anterior cingulate cortices and the limbic related thalamus and basal ganglia, will be tested by caspase-3 activity assay, caspase-3 immunostaining, and terminal deoxynucleotidy transferase-mediated dUTP nick end labeling (TUNEL) 6 hrs after last alcohol treatment, in the limbic system. The glutamate terminals will be assayed by immunocytochemical staining glutamate transporter at terminals in the above regions at P7 and P45. Collaboratively, the behavior component of this paradigm, especially the deficit of hippocampal function will be studied in Dr. C. Goodlett's Aim f.

B. BACKGROUND AND SIGNIFICANCE

Maternal alcohol consumption is the leading known cause of mental retardation in the Western world. The most severe consequences of maternal alcohol abuse are fetal alcohol syndrome (FAS) and alcohol-related neurodevelopmental disorder (ARND) (see (Stratton, Howe et al. 1996)), both of which are associated with substantial cognitive and behavioral deficits. FAS/ ARND includes microencephaly, particularly in limbic system, basal ganglia, corpus callosum and cerebellum. A major reduction and misposition of neuronal populations as a result of cell death or failure of proper migration in the brain are associated with ethanol's neuroteratogenesis. The spectrum of disorders that result from prenatal ethanol exposure, including FAS, ARND, alcohol related behavioral disorders, and alcohol related birth defects, are referred to as fetal alcohol spectrum disorder (FASD)

Mechanisms of FAS

Many mechanisms have been identified as potential candidates mediating alcohol-induced teratogenesis, but no single mechanism has been established with certainty as being responsible for specific phenotypic effects of fetal alcohol exposure (Goodlett and Horn 2001) (West, Chen et al. 1994) (Schenker, Becker et al. 1990). Effects may be due to direct actions of ethanol on the fetus or indirect actions mediated through maternal factors, such as effects on metabolism or placental function. It is probable that multiple mechanisms operate concurrently or are initiated at different stages of development or at different thresholds of ethanol exposure. Candidate mechanisms include:

- Induction of apoptosis (Olney, Ishimaru et al. 2000) (Kotch and Sulik 1992) (Kotch, Chen et al. 1995) (Cartwright and Smith 1995) (Cartwright, Tessmer et al. 1998) (West, Goodlett et al. 1990; Zhang, Rubin et al. 1998)
- Excessive formation of reactive oxygen species and oxidative stress (Kotch, Chen et al. 1995; Chen and Sulik 1996; Guerri 1998; Henderson, Chen et al. 1999)
- Suppression of the production of retinoic acid—a morphogenesis regulator—at critical stages due to inhibition of Class IV ADH conversion of retinol to retinal (Haselbeck and Duester 1998)
- Inhibition of cell-cell interactions mediated by the L1 cell adhesion molecule (Charness, Safran et al. 1994) (Ramanathan, Wilkemeyer et al. 1996).
- Suppression of neural proliferation (Miller 1988; Luo, West et al. 1999) (Pantazis, Dohrman et al. 1992; Resnicoff, Sell et al. 1993)
- Disruption of the release and action of trophic and growth factors (Dow and Riopelle 1985) (Walker, Lee et al. 1992) (Heaton, Swanson et al. 1992) (Heaton, Paiva et al. 1993) (Roivainen, Hundle et al. 1994; Roivainen, Hundle et al. 1995) (Hundle, McMahon et al. 1997) (Resnicoff, Sell et al. 1993)
- Inhibition of NMDA receptors (Bhave and Hoffman 1997) (Ikonomidou, Bittigau et al. 2000)
- Potentiation of GABA receptors (Ikonomidou, Bittigau et al. 2000)
- Disruption of multiple pathways of signal transduction (Diamond and Gordon 1997)
- Oxidative stress as an important factor in ethanol-induced cell death (Kotch, Chen et al. 1995) (Mitchell, Paiva et al. 1999) (Luo, West et al. 1999)

ADNF/ ADNP as a Therapeutic Agent

FASD is a highly preventable disorder. Methods of prevention include intervention with alcohol drinking and antagonism of the alcohol neurotoxicity. In light of the limited knowledge on the mechanisms of FASD to date, neuroprotection and neurotrophism are likely to provide promising approaches to antagonize alcohol neurotoxicity. Vasoactive intestinal peptide (VIP) and its activity dependent release of molecules known as Activity-Dependent Neuroprotective Protein (ADNP) are known to be extremely potent neural trophic (Gressens, Hill et al. 1993) (Gozes, Davidson et al. 1997) or protective agents (Gressens, Marret et al. 1997) (Zamostiano, Pinhasov et al. 1999). Two peptides associated with ADNF, ADNF14 (VLGG SALLRSIPA) and SAL (SALLRSIPA, also called ADNF-9), have been identified as extremely potent in neurotrophic function. The ADNF14 is very similar to a section of heat shock protein 60 (VLGGGCALLPRCIPA), a stress protein supporting cell survival (Lindquist and Craig 1988). SAL corresponds to the carboxy terminus of ADNF14. Antibodies against ADNF-9 identified an additional, structurally related, glia-derived ADNP. A short peptide

form of ADNP, NAP (NAPVSIPQ), was identified as the most potent neuroprotectant, effective in fentomolar quantities (Gozes, Giladi et al. 2000). Two unique features, one being that ADNP and ADNF exert effects at the femtomolar level (Gozes and Brenneman 1996), the other being that they have been effective in growth enhancement in embryos, render them potentially useful agents for treating FAS. The extremely low effective concentration allows for maternal administration without using a large dose. The growth enhancement in the embryonic stage allows for prevention of microencephaly and brain retardation in the early stages.

(1) On Neuroprotection

There are two recognized biological functions of the ADNP, ANPF, and the derived peptides NAP and SAL, respectively. First, they provide a wide-range of neuroprotection against the following:

- Excitotoxic lesions induced by ibotenic acid lesion--- (VIP and ADNF14) (Gressens, Marret et al. 1997) (Gressens, Marret et al. 1999)
- Excitotoxicity by stabilizing intracellular calcium levels and abrogating the increased oxyradical production and mitochondrial dysfunction(Guo, Sebastian et al. 1999)
- Oxidative stress (100 microM H_2O_2) by protecting PC12 cells (Offen, Sherki et al. 2000; Steingart, Solomon et al. 2000)
- Tetrodotoxin electrical blockade induced apoptosis (Gozes and Brenneman 1996)
- NMDA Excitotoxicity (Brenneman, Glazner et al. 1998)
- Beta-amyloid peptide (Brenneman, Glazner et al. 1998)

The mechanism of action of these peptides on anti-excitotoxicity is complex and not clear to date. Known cellular actions include increased expression of nuclear factor kappaB (Gozes, Davidson et al. 1997; Glazner, Boland et al. 1999; Glazner, Camandola et al. 2000) and heat shock protein-60 (hsp-60) (Zamostiano, Pinhasov et al. 1999; Glazner, Camandola et al. 2000), reduction in reactive oxygen species, and inhibition of oxidative stress (Glazner, Boland et al. 1999). They act through a cAMP-independent mechanism, as neither forskolin, an adenylate cyclase activator, nor pituitary adenylate cyclase-activating peptide, provided VIP-like protection (Gressens, Marret et al. 1997). ADNF-14 requires protein kinase C and mitogen-associated protein kinase kinase activation to protect the developing mouse brain against excitotoxicity (Gressens, Marret et al. 1999).

(2) On Neurotrophism

ADNF/ ADNP also provide a wide-range of neurotrophism in the following ways:

- As neurotrophic factor for survival--antiserum to activity-dependent neurotrophic factor produces neuronal cell death in CNS cultures (Gozes, Davidson et al. 1997).
- Preventing degeneration of neuronal circuits at synaptic level (Guo and Mattson 2000)
- Increasing neurite outgrowth --ADNF14 has neurotrophic actions, at the level of 0.001-0.1 fM, significantly enhancing the neurite outgrowth of dorsal root gangalial cells mediating through protein kinase A and CREB (White, Walker et al. 2000);

- Inducing downstream trophic factor--ADNFs were found to cause secretion of neurotrophin 3 (NT-3), and both proteins regulate NMDA receptor subunit 2A (NR2A) and NR2B (Blondel, Collin et al. 2000).
- Promoting glutamate response – promoting neuronal differentiation by acting directly on neurons to promote glutamate responses and morphological development in hippocampal neurons in culture (Blondel, Collin et al. 2000).

The last two sets of data suggest that the VIP-ADNF-NT-3 neuronal-glial pathway regulates glutamate responses from an early stage in the synaptic development of excitatory neurons and may also contribute to the known effects of VIP on learning and behavior in the adult nervous system (Blondel, Collin et al. 2000).

Effects of ADNF/ ADNP PEPTIDES on FAS

Pretreatment of pregnant dams with an equimolar combination of NAP and SAL prevented alcohol-induced fetal death and growth abnormalities in a high dose intraperitoneal alcohol injection in mouse. (Spong, Abebe et al. 2001). Pretreatment with NAP alone resulted in a significant decrease in alcohol-associated fetal death; whereas ADNF-9 alone had no detectable effect on fetal survival after alcohol exposure, indicating a pharmacological distinction between the peptides. Biochemical assessment of the fetuses indicated that NAP and SAL prevented the alcohol-induced decreases in reduced glutathione which protects against oxidative stress (Fernandez-Checa, Garcia-Ruiz et al. 1998). Peptide efficacy was evident with either 30-min pretreatment or with 1-h post-alcohol administration.(Spong, Abebe et al. 2001). Further investigation of VIP, the upstream inducer of ADNF, indicated that alcohol treatment can decrease the expression and immunoreactivity of VIP in both maternal and fetal tissues (Spong, Auth et al. 2002). Moreover, NAP prevents this action of ethanol on VIP.

The question "*Can neuroprotective peptides protect against brain damage in ARND*?" is immediately raised. In collaborating with Dr. Spong, we began to investigate ADNF's effects in a liquid diet model of ARND in C57Bl/6 mice. The mice have the advantage of consuming alcohol ad lib. We demonstrated that continuous access to ethanol liquid diets over midgestation, producing peak BACs of 60-140mg/dl, causes microencephaly, hydrocephalus, cortical thinning, perforation of neural tube midline tissue, delayed occlusion of the ventral canal, and reduction of serotonin neurogenesis in the raphe in late gestation. These deficits can be prevented by treatment with SAL or NAP (see Preliminary data). Given the complexity and dynamics of the neurodevelopment, this observation in turn leads to the next two major questions: *How does it work?* and *When does it work?*

Charness et al found that NAP antagonizes ethanol inhibition of L1-mediated cell adhesion in NIH/3T3 cells (Wilkemeyer, Menkari et al. 2002) and prevented ethanol teratogenesis in mouse whole embryo culture (Chen, Charness et al. 2003). This exciting finding prompted his team to investigate the active site of NAP on alcohol-mediated inhibition of cell adhesion. As most trophic factors are effective in a delimited time window and within specific brain regions, the mechanism of NAP /SAL protection against alcohol toxicity is expected to be complex and stage dependent. Given the appearance of ADNF in early developing brain, and that susceptibility to alcohol's teratogenic effect occurs during neurulation, a prenatal model of alcohol exposure during the period of brain development comparable to the human 1st and 2nd trimester, including neurulation and extending over peak periods of neurogenesis will most effectively detect

the trophic effects. Our preliminary observation indicates that this model is sensitive to the beneficial effects of NAP and SAL. The teratogenic effect of alcohol, regardless of the mechanisms, results in growth retardation and neuronal loss in the nervous system, which is most likely a result of compromised neurogenesis or excessive apoptosis leading to cell death. To understand the effect of NAP / SAL on ameliorating or reversing ARND describe above, we asked the question "*Can NAP / SAL Protect the Nervous System From Excessive Apoptosis, Retarded Neurogenesis, or Neurite Outgrowth in Our Proven Prenatal Alcohol Liquid Diet Model of C57Bl/6 Mice?*" In the current application, we are especially interested the potential for NAP to protect the embryonic and fetal development of the serotonin neurons in midline raphe, the medial frontal and cingulate cortices, and the hippocampus (*Hypothesis and Specific Aim 1*), including the neurotrophic effect of NAP / SAL on nerve fiber growth of 5-HT and glutamate projections in the cortices and hippocampus.

Cell death and deficits in brain growth elicited by alcohol treatment during development are believed to be associated, in part, with severe oxidative damage. NAP and SAL have been shown to exhibit anti-oxidative and anti-apoptotic actions *in vitro*. The mechanism of the protective effects of

ADNF against alcohol-mediated degeneration is unknown. The pre- and postnatal mechanisms of neurodegenerative effects of alcohol on the developing nervous system is a function of the maturation of the various neural systems and thus, most likely, mechanistically different. Alcohol mediates a great deal of cell death through Glu-NMDA-inhibition and $GABA_A$-enhancement during the peak development of NMDA and $GABA_A$ receptors in early postnatal rodent and 3rd trimester human development (Olney, Wozniak et al. 2001). The limbic system contains the highest density of glutamate neurons and NMDA receptors and the limbic-associated basal ganglia has the largest population of GABA and $GABA_A$-bearing neurons. We next ask the question "*Does NAP / SAL Protect Developing Neurons in the Limbic and Associated Systems which Peak at Early Postnatal Stages of Mice Equivalent to the 3rd Trimesters of Human?*" (*Hypothesis and Specific Aim 2*). The alcohol exposure in neonatal mice through subcutaneous delivery is a reliable model with predictable apoptosis in limbic forebrain regions (Ikonomidou, Bittigau et al. 2000; Olney, Tenkova et al. 2002; Olney, Tenkova et al. 2002). The global analysis of the effects of ethanol, and the protective effects of NAP and SAL, on these brain regions in our UO1 combined with the analyses of the behavioral consequences in Aim 3 of Dr. Goodlett's UO1 will be of great value in defining the therapeutic potential of these agents and understanding the mechanisms of both toxicity and protection.

SIGNIFICANCE

Fetal alcohol spectrum disorder (FASD) is a prevailing social-biological disease which occurs prior to birth and sustains lifelong impact to the individual and to society. To date, there is no cure for this disorder. A small, naturally produced substance in the body called Activity Dependent Neurotrophic Factor "ADNF" is known to enhance fetal growth and its related agents recently showed a potent effect on protecting fetal mice from demise in womb. Our study will determine whether these agents can prevent

abnormalities for those surviving birth. This early intervention, if proven effective, will have tremendous potential for prevention of FAS. One of the most attractive features of ADNF-like related agents is their extreme potency; if proven effective on preventing FASD, they have great potential for pharmaceutical application.

C. PRELIMINARY DATA

C1. Alcohol-Induced Microencephaly and Hydrocephalus in the Mouse Liquid Diet Model

Alcohol treatment in liquid diet form [20% ethanol-derived calories (20%EDC, peak blood alcohol level, BAC, 10-60mg/dl over days) or 25 % EDC (measure peak BAC, 40-140 mg/dl over days)] in C57BL mice through midgestation, reduces the embryonic body weight, brain weight and total brain dimensions (forebrain volume) in both ALC groups at the end of midgestation. Many brain regions have decreased volume: the basal ganglia, diencephalons, septal nucleus, and, to a greater degree, the hippocampus, and the amygdala (Figure 1). Alcohol also reduced the thickness of the medial frontal and cingulate cortices (shown in C6, Figure 5). The reduction is brain-wide in the emerging brain areas with the limbic system being most severely affected. The ventricles are enlarged. Two other features of this model are that (1) no differences are found between the pair-fed (PF) and Chow-fed (Chow) control groups and (2) the reduction in brain volume is ethanol-dose dependent. This liquid-died alcohol model produced microencephaly and hydrocephalus that mimics human ARND caused by daily alcohol consumption during mid-gestation pregnancy.

C2. Liquid Diet Alcohol Compromised Neural Tube Midline Tissue and Development of Serotonin Neurons

In our study, alcohol exposure resulted in a high frequency of compromised neural tube in the midline underneath the wrapping membrane, in the following ways: (1) approximately 60% of embryos at E13 and 20% at E15 showed perforation of the floor plate in telencephalic and diencephalic vesicles, (2) approximately 70-80% of embryos failed to complete the formation of neural tissue at the roof of the neural tube as the alcohol exposure continued to E15, and (3) approximately 60-80% of embryos showed delayed “occlusion” of the ventral canal by newly formed ventral nestin-positive neuroepithelial cells and S100□-positive glia in brainstem at E15. The PF and Chow showed few or none of these deficits. The compromised neural tube midline (cNTM) occurs near ventricles in E13
and E15, but is later completed at E18. In all cases, the cNTM was accompanied by an enlarged ventricle, and dose-dependent brain weight reduction. These results suggest that moderate alcohol treatment imposes a high frequency of cNTM at fetal stages.

Moderate fetal alcohol exposure causes neural tube development problems involving delayed formation of midline tissue. The midline of the neural tube at the roof and floor plates is known to mediate timely trophic induction for neural differentiation. Prenatal midline deficits also have the potential to affect the development of midline

neurons such as raphe, septal nuclei, and the timely crossing of commissural fibers such as the corpus callosum.

We recently have found in mice that alcohol liquid diet consumption during pregnancy resulted in incomplete-neural-tube-fusion (ventral canal occlusion) which hinders the development of midline cells such as 5-HT neurons(Zhou, Sari et al. 2001). At the birth of 5-HT neurons (E11), the 5-HT immunoreactive (5-HT-im) neurons are often missing fibers that project medially towards the ventricle in the Alcohol treated group (ALC) as compared with PF and Chow. At E13, there are fewer 5-HT-im neurons in either dorsal or median raphe of ALC as compared with PF or Chow; furthermore, neurite outgrowth and migration of the 5-HT neurons are also compromised with alcohol exposure (Zhou 2002). We thus demonstrated that fetal alcohol exposure compromised 5-HT development as early as the time of 5-HT neuronal birth. The deficit of 5-HT neurons continues through E15, E18, and to postnatal day (P) 45 (Zhou, Sari et al. 2001) (Sari, Powrozek et al. 2001) (Sari and Zhou 2003). Since 5-HT is a signal for development of many forebrain neurons, the disruption of 5-HT in early life may have consequences on brain development that extend beyond those seen in the 5-HT system. In the mature brain, 5-HT in hippocampus helps modulate memory, and in basal ganglia helps mediate motor function. A compromised 5-HT system has wide-ranging consequences, ranging from sudden infant death syndrome in infants (Kinney, Filiano et al. 2001) to affective disorders in adults (Coccaro and Murphy 1990).

C3. L-SAL Antagonizes Ethanol-induced Embryonic Microencephaly and Hydrocephalus

We have previously shown that brain weight, brain size and forebrain area are reduced, and midline neural tube development is compromised in B6 mice treated with a 25% ethanol derived calorie (EDC) diet. A therapeutic approach using SAL was tested in our experimental paradigm with alcohol (ALC, 25%EDC, from E7-E14), PF, Chow, and ALC supplemented with injections of L-SAL (ALC/SAL, 20µg/day, i.p.) along the time course of alcohol consumption. L-SAL was found to protect against alcohol induced body and brain weight loss. The body weight increases from the level of the ALC. The brain weight increased in ALC/SAL from the level of ALC ($P<0.05$) to levels comparable to PF and Chow (Figure 2). The brain dimension (circumference) also increased in ALC/SAL vs ALC ($p<0.01$) to a level comparable to PF and Chow, while the enlargement of lateral ventricle inducement seen in ALC was reduced in ALC+SAL (Zhou, Sari et al. 2003).

C4. L-SAL Antagonizes Fetal Alcohol Compromised Neural Tube Midline and the Size Brain Areas

25%EDC alcohol treatment compromised neural tube development, leaving openings along the third ventricle and preventing the fusion (occlusion) of the ventral canal in the brainstem (Figure 3c). A delayed formation of raphe (where 5-HT neurons are born) was evident. SAL treatment reduced the size of the opening in the floor plate of the neural tube, and promoted the occlusion of ventral canal in brainstem that leads to

to formation of the raphe (Figure 3d, and lower panel). This may have important consequences on protecting neurogenesis of 5-HT neurons in the region.

There were general increases in the size of numerous actively developing brain regions including the basal ganglia*, septal nucleus**, diencephalons*, hippocampus**, and amygdala**, as well as increases in the cortical thickness of the medial frontal** and cingulate cortices* in ALC/SAL as compared to ALC (*=p<0.05; **=p<0.01); these increases reached the levels of PF and Chow groups. Treatment with L-SAL was found to have widespread effects, reducing alcohol-induced-deficits at E15. Fetal body weight increased in ALC/SAL from the level of ALC (P<0.01), but not to the level of PF and Chow. The ability of SAL to antagonize the alcohol retardation of growth of forebrain and midline neural tube at midgestation, without inducing noticeable abnormalities, confirms it has strong potential for use as a therapeutic agent to prevent fetal alcohol teratogenesis.

C5. L-NAP Protect Against Fetal Alcohol Compromised Body and Brain Weight

NAP treatment in 20ug/day to pregnant dams also protected against alcohol-compromised fetal body and brain weights. The fetal body weight at E15 was increased in ALC/NAP from the level of ALC (P<0.01), but did not reach that of PF and Chow controls. The brain weights were increased in ALC/NAP above the level of ALC (P<0.01), and reached the level of PF and Chow controls (Figure 4).

C6. L-NAP Antagonizes Fetal Alcohol Compromised Microencephaly and Hydrocephalus

In our 25%EDC alcohol liquid diet studies (from E7-E15), the cortical thickness was reduced in ALC as compared with PF and Chow. Treatment with L-NAP 20ug/day along the alcohol treatment time course significantly increased medial frontal and cingulate cortical thickness as compared to those of alcohol-treated; the medial frontal cortex increased to the PF as well as Chow control level. (Figure 5). Furthermore, the alcohol induced enlargement of lateral ventricle at E15 was prevented by administration of NAP (Figure 6).

C7. NAP protect against the alcohol induced brainstem cell death in culture

The NAP protection against alcohol induced neural cell death is also demonstrated in our brainstem as well as cortical cell culture derived from UBI-GFP/BL6 mouse which ubiquitously express green fluorescence protein (GFP) in all neural cells (Schaefer, Schaefer et al. 2001). The GFP-expressing neural cell culture has an advantage for analyzing live cells in real time under various treatments. Fetal brainstem or cortical cells were let to grow in 16-chamber-slides (coated with laminin) for two days in B27 enriched neural basal medium before subjected to treatment. Alcohol (300mg/dl alcohol, kept constant with large reservoir of same dose alcohol) and / or NAP ($5x10^{-11}$M) were added to the culture for the next 5 days. Alcohol reduced the number of brainstem (Figure 7) or cortical cells greatly in many with condensed nuclei (Figure 7, red arrows). Addition of NAP in the alcohol treated culture protects against the alcohol induced cell death and reduced the number of cells with condensed nuclei.

C8. Caspase-3 activity

The caspase-3 activity assay (see Method) is adopted for two purposes—provides a sensitive quantitative analysis of apoptosis in a
brain region, and a substitute when caspase-3 staining is not feasible in the prenatal stage. We are able to measure the caspase-3 activity in a small amount of brain tissue, as in E18. In the liquid alcohol model with 25% EDC alcohol from E7 to E18, the caspase-3 activity is increased in ALC as compared to PF and Chow in the brainstem and the forebrain at E18 (Figure 8).

C. Gene expression analysis in brain

Dr. Edenberg, a consultant on this project who directs the Center for Medical Genomics at Indiana University School of Medicine (the site at which the microarray studies will be conducted), has extensive experience in use of Affymetrix GeneChips® to analyze gene expression in brain. Because of the complexity and tight regulation of brain function, gene changes in brain are usually modest, often less than 1.5 fold. Careful experimental design and analysis and use of multiple biologically independent samples

allows for robust detection of even these small changes. In previous experiments “Gene expression in brain: a window on ethanol dependence, neuroadaptation, and preference” (Hoffman, Miles et al. 2003), Dr.Edenberg has shown that small changes in gene expression in specific areas of the rat brain can be robustly detected, and confirmed by a subsequent experiment. The experimental design proposed here is based upon this experience.

D. RESEARCH DESIGN AND METHODS

Specific Aim 1. Tests the hypothesis that NAP and SAL are neuroprotective and /or neurotrophic against alcohol neurotoxicity in developing neurons with respect to apoptosis, or differentiation using an alcohol liquid diet model at E7--E15/E18, in B6 mice, a period of brain development equivalent to that of first and second trimester of human gestation.

Apoptosis will be determined by caspase-3 activity assay on E18. The number of mature neurons will be determined by Neu-N-positive (mature neuronal marker) of pyramidal neurons in hippocampus at E18 and P45, and 5-HT-immunostaining (im) neurons in raphe at E15/ E18 and P45 using stereological counting. Effects on neurogenesis will be tested using BrdU injections in E16 and E17 and examined at E18. The neurotrophic effect of NAP on 5-HT and glutamate fibers / terminals will be assayed with immunocytochemical staining of 5-HT fibers and glutamate transporters at fibers/ terminals in the hippocampus at E18 and P45..

[Rationale]

Our studies show that NAP and SAL protect against the alcohol-induced reductions in brain weight and brain size resulting from either decreased cell acquisition or excessive neural death in midgestation. It is plausible that NAP and SAL achieve this end through decreasing apoptosis. This aim will test, for the first time, the antagonism of individual peptides against 25% EDC liquid diet induced apoptosis. We are aware that a “normal” amount of apoptosis occurs in each developmental stage, and a quantitation will be required for analysis. Since caspase-3 immunostaining is not optimal at E18, a sensitive and quantitative assay of apoptosis activity will be performed. The final neuronal number will be determined by counts of differentiated pyramidal and 5-HT neurons at E15 (The 5-HT neurons reach their peak number of raphe at E15). At this stage, alcohol characteristically delays the raphe formation (failure of occlusion of ventral canal) leaving behind a cleft. Upon BrdU injection at E16 and E17, the hippocampus will be examined for BrdU labeling at E18, as most of pyramidal neurogenesis occurs prenatally. All parameters will also be examined at P45 to assess long-term effects in young adults. The dose of 20ug/day NAP/SAL, used to reduce fetal demise previously, will be our first choice. If the effect of 20ug/day is marginal, larger doses will be tried.

[Experimental design]

The NAP will be tested in B6 mice using a liquid-diet alcohol consumption model. Nulliparous females will be mated with males for two hours, and the presence of a sperm plug will determine E0. The time-pregnant mice will be divided on E7 (168 hours post-coitus, PC) into 6 treatment groups --Chow, Alcohol (ALC; liquid diet with 25% ethanol derived calories, EDC), pair-fed (PF), ALC+NAP, ALC+ random sequence of NAP (ALC+rNAP), and PF+NAP alone. If NAP is not effective, SAL will be used instead. The random sequence control, if found not functional, can be deleted in this later study.

The NAP (saline for control groups) will be administered intraperitoneally (20ug/0.2ml/day) 30 minutes before the dark cycle (when liquid diet is changed daily) until E15 or E18, at which time all embryos will be perfused for analysis. An additional time point, P45, will be used for long-term observation. All postnatal litters will be surrogate-fostered to a normal B6 dam to prevent neglect-care by the drinking dam. One fetus from each litter will be used for each analysis-- caspase-3 activity (at E18), 5-HT and glutamate transporter immunoreactivity (at E15 and P45), and BrdU (at E18) and NeuN (at E18 and P45) staining. The stained cells will be stereological counted; the density of the 5-HT and glutamate fibers/terminals will be measured by densitometry as described in the Methods section. The average brain weight from each litter will be taken as one sample value. According to our pilot study and power analysis, n=7-9 dams per group will be required to detect the differences between groups. The embryonic brain, after weighing, will be fixed, sectioned for various staining and Nissl-counterstained for examination.

For 6 treatment groups, 3 time points, and 9 dams per group, a total of 162 dams will be needed. With a 10% attrition / unexpected loss in the procedures, a total of 180 dams will be used. We estimate 240 attempted pregnancies will be needed for to produce adequate usable dams. If an increased dose of NAP/SAL is required, 40 dams will be added for the pilot test (see Interpretation and Pitfall).

[Data analysis]

Four treatments in each time course, brain weight, caspase-3 activity, 5-HT neurons in raphe, and BrdU and NeuN neurons in pyramidal layer of hippocampus, and 5-HT / glutamate transporter fibers /terminals in hippocampus will be compared independently in each time course. Measurements will be compared among treatments with ANOVA, and between treatments with post-hoc Fisher's PLSD.

[Interpretation and Pitfalls]

The increase of caspase-3 (activity) in ALC (as compared to PF and Chow) and the extent of its reversal to a varying degree in ALC+NAP litters will indicate protection against the apoptotic effect of alcohol.

The reduction of pyramidal NeuN-immunoreactive (-im) neurons (E18 or P45) in the hippocampus in ALC and reversal from reduction in ALC+NAP/SAL indicates a protective effect of NAP/SAL against alcohol's neurotoxicity. If the alcohol compromises the neurogenesis of hippocampal neurons, the number of BrdU positive cells in the pyramidal layer of the hippocampus at E18 will be reduced in ALC as compared with PF and Chow. If NAP/SAL protects against the alcohol compromised neurogenesis, the number of BrdU neurons in the pyramidal layer of ALC+NAP/SAL embryos will increase from the level of ALC. Together, this supports our previous observation of size reduction of hippocampus by alcohol and its reversal by NAP/SAL.

A similar reduction and reversal in the number of 5-HT-im neurons in raphe, and the compromised versus completed formation of raphe via occlusion of ventral canal of brainstem indicate the protection of NAP against the neural toxicity of alcohol in the brainstem.

The 5-HT-im and glutamate transporter-im fibers / terminals in the hippocampus will indicate the neurotrophic aspect of the ADNP for neurite outgrowth and synaptogenesis.

If the protective effect in the above parameters extended to P45, the young adult stage, it would indicate the therapeutic effect of the NAP / SAL likely offers life-long protection against fetal alcohol toxicity.

The ineffectiveness of the random-sequence NAP / SAL (e.g., ALC+rNAP) in reversing parameters from the level of ALC will indicate the specificity of the NAP / SAL from other peptide sequence with same amino acid composition. The normal general morphology and number of NeuN-im neurons in hippocampus and 5-HT neurons in raphe in the Saline+NAP / SAL brain indicate that NAP / SAL itself does not have adversary effects on brain development. NAP / SAL has not been reported in the literature thus far to have detrimental effects when administered.

If 20ug/day of NAP / SAL is not adequate to protect the alcohol-imposed reduction of measurements, a double dose, 40ug/day, of NAP / SAL will be used. In addition, a combination of NAP and SAL will be used to test if a mixture has superior effects to the single peptide. If these conditions are to be adopted, up to 50 more dams will be added to the experimental design.

<u>Specific Aim 2</u>. Tests the hypothesis that in B6 neonatal mice, NAP and SAL will have neuroprotective and neurotrophic effects on on damage to limbic system structures induced by binge ethanol exposure on P7, during a neonatal stage of brain development comparable to that of the third trimester of human gestation.

Alcohol (s.c, 2.5g/kg twice/day, peak BAC ~300mg/dl) and the NAP treatments (along the same time course) will be given at P7. The neuroprotective effect, particularly with respect to hippocampus, subiculum, medial frontal and anterior cingulate cortices and the limbic related thalamus and basal ganglia, will be tested by caspase-3 activity assay, caspase-3 immunostaining, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) 6 hrs after last alcohol treatment in the limbic system. The glutamate terminals will be assayed by immunocytochemical staining glutamate transporter (GluT) at terminals in the above regions at P7 and P45. The neurogenesis (in hippocampus mainly granular neurons of dentate gyrus) will be monitored by BrdU injection twice (50mg/kg) over the 24 hrs, and the number of newly generated neurons or the matured neurons will be determined by stereological counting BrdU-labeled and NeuN-im cells, respectively, in the highest apoptotic regions at P7 and P45.

[Rationale]

To study the effect of alcohol exposure during the period of brain development comparable that of the human 3rd trimester, the alcohol exposure must occur during the neonatal period in mice. We adopted the subcutaneous delivery of alcohol into neonatal mouse which has been established to demonstrate the neurodegeneration in B6 mice (Olney, Tenkova et al. 2002; Olney, Tenkova et al. 2002). This acute episode of binge alcohol exposure involves administering 2 injections of alcohol to B 6 mice on P7, 2.5g/kg each injection, two hrs apart, We have found that this protocol achieves peak BACs of ~250-300mg/dl, and it has been shown to cause apoptosis in several forebrain regions (Ikonomidou, Bittigau et al. 2001).

Apoptosis will be examined in three ways: caspase-3 activity, caspase-3 staining, and TUNEL. The caspase-3 activity assay provides a sensitive quantitation of apoptosis; caspase-3 and TUNEL (DNA breakage at adjacent section) staining are

correlated with apoptosis at the cellular level. Both enzyme and DNA breakage should appear during our time course when apoptosis occurs. The effect on neurogenesis and the number of neurons will be examined in the short (P7) and long-term (P45) models.

The area with highest apoptotic activity at P7 will be counted for mature neurons (e.g. cingulate cortex, hippocampus). Pyramidal neurons in the mouse hippocampus are born prenatally, while granular neurons in dentate gyrus are born perinatally. The birth of granular cells into dentate gyrus will be revealed by incorporation of BrdU into chromosome and detected by immunocytochemistry. The measurement of NeuN-im neurons will reveal how many neurons reached maturity in the pyramidal and granular layers as a function of binge alcohol neonatal treatments. The pyramidal neurons are predominantly born prenatally, therefore, the toxicity of alcohol and the protection of NAP will mostly address the differentiated neurons in the CA area, and the neurogenesis of granular cells in the dentate gyrus. Lastly, the excitatory circuit, specifically the glutamate fibers terminals in the above regions, will be analyzed; this tests the effect of alcohol and NAP/SAL on glutaminergic neuronal differentiation.

[Experimental design]

NAP and SAL will be tested in B6 mice using the P7 2-injection model of alcohol binge exposure. The litter will be culled to 8 pups, with equal numbers of males and females when possible. The litters will be divided on P6 into 5 treatment groups --Saline, Alcohol in saline (ALC; 2.5g/kg twice a day, two hours apart, s.c.), ALC+NAP, ALC+random sequence of NAP (ALC+rNAP), ALC+SAL, Saline+SAL, and Saline+NAP. (If ALC+rNAP is found to be nonfunctional, ALC+rSAL will not be tested). The peptides (saline for control groups) will be administered (3 ug/0.2ml, s.c., twice per day along with the time course of alcohol treatment). All treatments will be done on P7 (see Methods section). Pups of each subgroup will be processed for caspase-3 activity, caspase-3 single staining, or caspase-3/ TUNEL double staining (at adjacent sections) 6 hrs after the last alcohol treatment. For BrdU assay, BrdU will be injected 50mg/kg (i.p.) 0 and 4 hours after the first alcohol treatment. The pups from each litter will be divided into two time-course subgroups for perfusion at P7 and P45. Pups for BrdU and NeuN staining will be perfused 6 hours after their last alcohol treatment or at P45. The stained cells will be counted with stereology; the density of the 5-HT and glutamate fibers/terminals in the highest apoptosis brain regions (hippocampus, subiculum, cingulate cortex) will be measured by densitometry as described in the Methods section. Brain weight will be taken prior to analyses.

Our study and power analysis indicate n=7-10 dams per group will be required to detect the differences between groups. For 7 treatment groups, 2 time courses, and 8 litters per group, a total of 112 dams will be needed. With a 20% attrition in the procedures, a total of 150 dams will be used. We estimated 180 dams will be needed to produce a usable litter size. If an increased dose of NAP or SAL is required, an additional 40 dams will be tested.

[Data analysis]

Similar analyses to those detailed in Specific Aim 1 will be done in Aim 2. Four treatments in each time course, brain weight, caspase-3 activity, caspase-3 staining, BrdU, and NeuN in selective regions will be compared independently. Measurements will be compared among treatments with ANOVA, and between treatments with post-hoc Fisher's PLSD.

[Interpretation and Pitfalls]

Increases of caspase-3 activity and number of caspase-3-im and $TUNEL^+$ cells are expected in the alcohol-treated group. A significant reduction of the three parameters in ALC+NAP/SAL from the level of ALC will indicate a protective effect of NAP/SAL.

The reduction of BrdU-im cells in the dentate gyrus (granular cells) of ALC at P7 and its reversal in ALC+NAP indicates protective effect of NAP/SAL on the neurogenesis of granular cells. The reduction of pyramidal NeuN-im neurons in ALC together with amelioration / reversal in ALC+NAP at P7 indicates the protective effect of NAP on differentiated neurons in the hippocampus. The antagonizing effect at P7 indicates the immediate response to the NAP/ SAL, and accordingly, changes in BrdU and Neu-N parameters on P45 indicates that the effects at P7 last into the young adult.

Reductions of the Glu-terminals examined with GluT staining in limbic system (hippocampus..etc) of ALC will indicate the inhibition of differentiation of glutamate neurons by alcohol. Alternatively, the amount of GluT fiber terminals can reflect the number of Glu neurons in the local limbic area when the adjacent sections to the GluT show increase caspase-3-TUNEL staining. The reversal of reductions in GluT terminal density by NAP/SAL will indicate a protective / neurotrophic effect of the NAP/SAL for neuronal differentiation.

Similar to the Aim 1, the random-sequence NAP / SAL (e.g., ALC+rNAP) serves as control to indicate that the protective effect of NAP / SAL is not due to extra supply of peptides in general, , and is an effect of the specific NAP or SAL functional sequence

The above effects together indicate that NAP is effective in antagonizing the alcohol neurotoxicity in binge drinking pattern at neonatal stage equivalent to the 3rd trimester in humans. The analysis of NAP and SAL in this aim will also determine if NAP (or SAL) is (more) suitable for genetic study in Aim 3.

In addition to our anatomical analysis, we will collaborate with Dr. C. Goodlett in his U01 Aim 3 studies of alcohol-induced deficits in hippocampal-dependent variants of eyeblink classical conditioning. Those studies test the prediction that the P7 binge alcohol treatments in B6 mice (as used in this component) will induce deficits in reversal of discriminative eyeblink classical conditioning, a learning function for which hippocampal interactions with the cerebellum is necessary, while sparing acquisition of the initial discrimination learning, a learning function that depends only on the essential cerebellar circuit and does not require normal hippocampal function.

METHODS

Animal and breeding

The C57BL mice (Harlan, Inc., Indianapolis, IN) will be housed in the IU Laboratory Animal Research Center (LARC), in rooms maintained at 22º C, humidity of 30%, with a 12-hr:12-hr light:dark cycle (on at 0700 hr). Upon arrival, the mice breeders will be individually housed in the IU Laboratory Animal Research Center vivarium. They will be acclimated for at least one week, maintained with ad lib chow and water with a 12-hr:12-hr light:dark cycle.

Only nulliparous females will be used for mating. Individual female mice will be placed in the cage of a designated male, and removed after 2 hours. Sperm plug detection will be designated as embryonic day 0 (E0 or hour 0). Within a breeding cohort, females will be mated in subgroups separated by about 2-3 days. This will facilitate contemporaneous pair-feeding (below), which requires a 2-3 day lag in gestation between the ethanol-consuming dam and its matched pair-fed dam. To control for potential variation in gestational duration, gestational ages will be followed. For convenience, the postnatal studies designate age in terms of postnatal day, i.e., P7, but postnatal ages are defined based on the day of sperm plug detection, not from the day of birth (i.e., E19 = P0; E26 = P7).

Prenatal Alcohol Liquid Diet Model.

Within a cohort of breeders, pregnant females will be weighed daily and on E7 assigned pseudo-randomly (matching for weight) to one of three gestational treatment groups: Ethanol liquid diet (ALC) delivering 25% ethanol-derived calories (EDC) as the sole source of nutrients; Pair-fed control (PF), yoked individually to a ALC dam and given matched daily amounts of isocaloric liquid diet, with maltose-dextrin substituted for ethanol; or *ad lib* chow and water (Chow). The Chow group will have free access to standard mouse chow and water at all times during gestation. The fortified liquid Sustacal diet is the same as that used in our Preliminary Studies, and follows the published protocols reported by Middaugh and colleagues (Middaugh and Boggan 1995). The diet contains 237 ml of chocolate-flavored Sustacal (Mead Johnson), 1.44 g Vitamin Diet Fortification Mixture (ICN #904654), and 1.2 g Salt Mixture XIV (ICN #902850). For the ethanol diet, 11.38 ml of 95% ethanol is added to the fortified Sustacal formula, and water added to make 300 ml of diet with 1 Cal/ml (ethanol 3.6% v/v). For the isocaloric control diet, 15.15 g Maltose Dextrin is added to the fortified Sustacal formula, and water is added to bring it to 1 Cal/ml.

For free-choice drinking, the liquid diets will be given beginning on E7 (168 hours). Each day between 1800-1900 hr, the dams will be weighed, the volume of liquid diet consumed during the previous 24 hours recorded from 30-ml graduated screw-cap tubes, and freshly prepared diet provided. The Ethanol subjects will have free access to excess volumes liquid diet each day. On E7, the ALC dams will be adapted to the liquid diet by giving the control diet (without ethanol) for 1 day as their sole source of calories. From E7 until sacrifice (or until E18 for postnatal studies), the ALC dams will be given excess volumes of the 25% EDC diet each day. For the Pair-Fed (PF) group, each dam is provided with only the volume of control diet its matched ALC counterpart had consumed for that gestational day. Each PF dam is weight-matched to an ALC dam after pregnancy is detected; pregnant dams assigned to the PF group typically are identified as plug-positive 2-3 days after a pregnant dam is assigned to the ALC group.

For the gestational liquid diet study, cohorts of weight-matched nulliparous dams of both stocks will be assigned randomly to one of the prenatal treatment groups: ALC; Pair-Fed control (PF), Chow-fed ad lib (Chow), or ALC+NAP/SAL (20µg/animal/day, i.p.), or PF+ NAP/SAL (20µg/animal/day, i.p.) alone. Dams designated as Chow controls will be given ad lib access to food and water throughout the experiment and body weights obtained each day. Dams assigned to the liquid diet groups will be adapted to liquid diet for one day at E6 without ethanol.

Neonatal Two-dose Alcohol Model

Pregnant dams will be allowed to give birth - (E19 is usually postnatal day 0). Litters

will be culled to eight pups (equal numbers of males and females, when possible) to control for nutritional differences due to competition within the litter. Litters will then be weighed daily to assure the general health of the litter. On the morning of P7 between 10:00 and 12:00, pups will be treated subcutaneously with either saline, ethanol (2.5 g/kg 0 hr and 2 hr each), ADNP (NAP or SAL, 3μg/pup 0 hr and 2 hr each, s.c.), or ethanol+ NAP / SAL (same manner as above respectively). Ethanol is prepared as a 15% w/v solution in sterile saline. For neurogenesis labeling, pups will be injected with BrdU (50mk/kg, i.p.) at 0 hr and 4 hr after first ethanol /saline treatment. The half life of BrdU in the circulation is approximately 270 minutes (Bocknell, 1994). The pups from each litter will be divided randomly into one of the seven groups allowing for two animals per treatment group. They will be sacrificed at 6 hrs following the last treatment or at P45. At the termination of the study, mice will be anesthetized with ketamine cocktail (1 ml/kg) and decapitated for brain tissue or perfused transcardially with 0.4% picric acid and 4% paraformaldehyde for immunostaining or TUNEL staining.

Maternal /Pup Blood Alcohol and Fetal /Pup Brain Alcohol

For Specific Aim 1, we have preliminary data characterizing blood alcohol profiles in cohort of dams on E8, E11, and E14 with free choice liquid alcohol diet, In brief, we will randomly select 8 ALC dams or pups and assign them to 2 groups (n=4 per group) to characterize alcohol concentrations in maternal blood (BACs) and in fetal brain alcohol (fBrAC). For Group 1, tail nick blood samples (50 ul) will be obtained at 2100 hrs on E8, E10, and E11 for dams. Dams on E12 will be tested for BAC at 2100 hrs; the dam will be deeply anesthetized, blood samples obtained, then the fetuses removed, fetal brains extracted and homogenized, and brain alcohol levels determined. For Group 2, the tail blood samples will be obtained at 2100 hrs on E8, E10, and E12, and the dams will be sacrificed for maternal BACs and BrACs at 2100 hrs on E14.

For Specific Aim 2, neonatal model animals will be treated with alcohol at 0 and 2 hours as described in the above section, and the cardiac blood and brain tissue will be taken from groups sacrificed at 2, 4, and 6 hrs after the last alcohol treatment.

BACs and BrAC analysis.

The blood samples will be collected in heparinized capillary tubes, centrifuged, and 5 ul plasma
samples will be analyzed for alcohol concentration using the Analox Alcohol Analyzer, calibrated using a 50 mg/dl ethanol standard and verified with linear controls from 10-200 mg/dl. The brain alcohol concentrations will be analyzed using a gas chromatograph following the procedure of Silveri and Spear (Silveri and Spear 1998).

NAP / SAL Preparation

For preparation of stock NAP or SAL solution, a 25x concentration of working solution of SAL will be dissolved directly into sterile saline, while NAP will be dissolved in DMSO prior to dilution in sterile saline for stocking solution. NAP will be stocked at -20^{o}C while SAL will be used fresh each day. The stock solution will be further diluted into working solution with sterile saline hours before use. The two-step dilution assures that the DMSO is sufficiently diluted before use. The peptides (saline for control groups) will be administered to dam (20ug/animal/day, i.p.) or pups (3 ug/0.2ml/pup, s.c., twice per day along the same time course of alcohol treatment).

Brain Weight

Brain weight serves as an excellent indicator of total development of brain structure. Particularly in early to mid gestation prenatal stages, the majority of the brain mass is constituted by neural precursors prior to the formation of neuropil. Brain weight closely mirrors cell number or alternatively, the degree of apoptosis. We have found a significantly decreased brain weight in the E15 ALC group compared with the PF and Chow groups.

The fetus will be removed from the uterus on E15 and E18. The brain will be removed from the fetuses by carefully peeling the cartilage (Sari, Powrozek et al. 2001; Zhou, Sari et al. 2001)}, and then trimmed between the rostral end of the cortical vesicles to the caudal end of the metencephalic flexure. The brain will then be fixed by immersing in a 4% formaldehyde phosphate buffer for 24 hrs. Prior to weighing, the brain will be rolled over a piece of dry absorbent paper to remove excess fixative buffer. The postnatal brain will be obtained by perfusion of the mice and dissection out of the skull. A similar extent from the rostral end of the cortical vesicles to the caudal end of the metencephalic flexure will be trimmed for weighing in the procedure described above.

Caspase-3 Protein and Enzymatic Activity Assays

For tissue extracts, Chow, PF and ALC group's whole hippocampus tissue or other dissected brain regions frozen with liquid nitrogen will be ground to a powder with a pestle. The powdered tissue will be mixed with TNE buffer (10mM Tris pH7.4, 0.15M NaCl, 1mM EDTA) supplemented with protease inhibitor cocktail (Sigma). The mixture will continue to be ground with the pestle until the suspension is homogeneous. The suspension will be centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant collected, the tissue extract will be frozen at -80°C and assayed at a later date for either protein and/or caspase-3 activity detection.

Before determining the concentration of caspase-3 activity, the total protein estimation in each sample will be evaluated using a Bio-Rad protein assay. The concentrations of caspase-3 activity will be determined using caspase-3 colorimetric assay kit (Assay Designs, Inc. Ann Arbor, MI). The kit involves the conversion of a specific chromogenic substrate for caspase-3 followed by the colorimetric detection of colored product of the reaction that absorbs visible light at 405 nm. The samples in variant dilutions, standards, p-nitroaniline calibrator (pNA) and blank (prepared as described in the protocol shipped with the assay kit) will be plated in duplicate in 96 micro-plates. The blank will be a mixture of active caspase-3 reaction buffer and caspase substrate, which will be served as control. The conversion of substrate into the colored product will be measured after a 3 hours incubation at 37°C. The reaction will be stopped by a 1N solution of hydrochloride acid and multiple samples, standards, pNA and blanks will be read rapidly by an absorbance reader (SUNRISE, Phenix Research Products).

The average net optical density for each standard and samples will be calculated by substracting the average Blank OD from the average OD for each standard and sample. The activity measurements can be quantitated by comparisons of the optical densities obtained with standards or with the pNA. Using graphing software (Prizm), the concentration of active caspase-3 in the samples can be determined by interpolation of the average Net OD for each

standard versus actual concentration of active capasae-3 substrate for the standards. The concentration of active caspase-3 in the samples for all groups will be expressed as Unit/ml.

Immucocytochemistry of 5-HT, Caspase-3, NeuN, Glutamate Transporter, and BrdU

Fetal brains obtained from the pregnant dams will be immersed in formaldehyde freshly prepared from 4% paraformaldehyde and 0.4% picric acid in phosphate buffer, pH 7.4 overnight. The adult brains will be fixed by intracardiac perfusion with the same fixatives. In our immunocytochemistry analysis for comparing fetal brain among treatment groups, a parallel processing system will be adopted in which the ALC and PF, or ALC and ALC+NAP fetal brains are embedded in a single gelatin block with careful rostrocaudal and dorsoventral alignments. Thus, both brains will be cut from the same block into a serial 40μm section using a sliding vibratome. From here on the ALC-PF or ALC- ALC+NAP two-brain-sample sections will be processed in the same vials and treated identically throughout the immunocytochemical processes. This practice eliminates major biases such as amount of primary, secondary and tertiary antibodies, chromogens, and time of color reaction between experimental and control groups. This arrangement has the additional advantage of comparison of the sections of two experimental groups at similar levels side-by-side on the same slide. For BrdU staining, the tissue will be additionally treated with 2N HCl at 37^{o}C for 30-40 min, following by 0.1M borate buffer at 37^{o}C for 10 min, and 3 washes with PBS buffer for 5 min each at room temperature. Free floating sections will be incubated with anti-serotonin antibodies (from Zhou's lab,1;1000), anti-Caspase-3 (rabbit, 1:3000, Cell Signaling, Beverly, MA), anti-NeuN (Chemicon, Temecule, CA) or vesicular glutamate transporter (Chemicon), anti-BrdU antibody (rabbit, 1:500, R&D, Minneapolis, MN) overnight. All antibodies will be diluted in PBS buffer with 0.3% triton-X100 and 2% normal serum from species of the secondary antibody. The Sternberger peroxidase-anti-peroxidase (PAP) indirect-enzyme method will be used for staining. Endogenous peroxidase will be suppressed by pretreatment of tissue with 10% H_2O_2 prior to the immunocytochemical procedure. The PAP reaction will be performed with 0.003% H_2O_2 and 0.05% 3'3-diaminobenzidine. The primary, secondary and marker antibodies will be diluted in PBS containing 0.3 % Triton X-100 and 1.5% normal sheep-serum. The primary antibody will be incubated overnight; the second and third, for one hour each. Between antibody incubations, the sections will be washed with three 5-minute rinses in PBS. All sections will be Nissl-counterstained with methyl green to reveal background cells and profile brain structures.

TUNEL Staining and Double-labeling with caspase-3

The percentage of cells undergoing apoptosis will be determined using dUTP-fluorescein labeled TUNEL(TdT-mediated dUTP Nick End Labeling) reaction following the manufacturer instructions (Roche, Indianapolis, IN) which is a routine protocol in our laboratory and published previously (Zhou, Kelley et al. 2000). In brief, sections will be treated with Proteinase K (10-20 μg/ml) for 5 min at 37^{o}C, rinsed with phosphate-buffered saline (PBS) 3 times for 5 min and then incubated with 3% H_2O_2 in methanol for 10 min at room temperature. The sections will be rinsed with PBS 3 times for 5 min and incubated in permeabilization solution (0.1% TX-100 in 0.1% sodium citrate) for 2 min on ice (4^{o}C). After the sections are rinsed twice in PBS for 5 min, they will be incubated with TUNEL reaction mixture (50 μl from bottle 1 and 450 μl from bottle 2, Roche Pharmaceuticals, Inc, IN) for 1h at 37^{o}C, the control will be used by incubation in solution from bottle 2. Sections will be rinsed 3 times for 5 min with PBS and incubated in converter-POD for 30 min at 37^{o}C. After the sections are rinsed with tris-buffered saline (TBS), they will be incubated in 0.05% 3'-3'-

diaminobenzidine tetrahydrochloride and 0.003% H_2O_2 in TBS to reveal the peroxidase activity. Random fields of cells will be photographed under phase and fluorescent Leitz OrthoPlan II microscopy.

A subset of brain slides will be used to determine whether the caspase-3 positive cells are also having DNA breakage while undergoing apoptosis, we will first assay with a rhodamine labeled goat anti-rabbit antibody for caspase-3 and then perform a TUNEL assay, which labels cells undergoing apoptosis with fluorescein (green). The staining will be observed and photographed under Leitz OrthoPlan II fluorescent microscope. Double exposure photography with fluorescein and then rhodamine filters will be done for simultaneous observation of TUNEL and caspase-3. The double staining procedure is routine in our laboratory (Zhou and Chiang 1998; Zhou, Lesch et al. 2002).

Stereology

The BrdU-, NeuN-, caspase-3-, or 5-HT-im cells will be counted using the optical fractionator technique by an investigator blind to experimental groups. The counting will be performed under a high power objective lens (60-100X) using a microscope (Leica DMLB) connected to motorized stage controller (MAC5000, LUDL Electronic Products) that can precisely move and record movement in the X, Y, Z planes. Optical images will be captured by a camera (SPOT II) fastened to the microscope and projected onto a high resolution monitor (WACOM) for counting with the assistance of stereological design based software (Stereoinvestigator, MicroBrightField, Inc). This system currently used to study the NIH funded project on chronic alcohol on neuroadaptation will be shared for this proposed study. We are also currently collaborating with Dr. Goodlett on stereological counting of NeuN+ neurons on cortical barrel neurons. The optical stereological counting of neurons in the hippocampus is detailed in Dr. Goodlett's Methods section.

Briefly, the optical fractionator method will be employed to achieve systematic random sampling of the hippocampus (or other region specified in the Aims). The hippocampus will be sliced into numbered 40 μm sections throughout the entire structure. Every nth section will be selected for sampling beginning at a random section number less than n. For example, if a nucleus was cut into 50 sections and every 5^{th} tissue section was to be used for counting, we would begin at a random number between 1-5 and use every 5^{th} section from the starting point until between the $45\text{-}50^{th}$ sections. In each section, the entire region containing nucleus hippocampus will be outlined at low power. The penetration of the immunostaining in sections will be determined. If full-penetration is not achieved, the optical counting will be confined 5 μm from the edge of penetration. The labeled cells will be counted within a defined counting frame in the X, Y and Z axes within the defined contour at a defined number of locations in the X-Y plane. The sampling locations within the contour will be selected randomly by the computer software and the stage controller will automatically position the section so that it will be precisely aligned at a sites in the X-Y plane within the drawn contour at the higher power objective. From there, the investigator will manually focus through the section for predetermined depth (through the Z plane) and count the number of labeled cells as they come into focus. This method ensures that every BrdU-, NeuN-, or caspase-3-im cells within the hippocampus or 5-HT-im neurons in raphe has an equal chance of being counted. The total number of labeled cells in the sampling fractions will be multiplied by the total volume of hippocampus that was sampled to obtain an estimate of the total number of labeled cells in each hippocampus or raphe.

	Yr 1	Yr 2	Yr 3
Aim 1. Prenatal assay	x	x	x
Aim 2. Neonatal assay	x	x	x