DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

The long-term goals of this component are to use rodent models of binge alcohol exposure during the 3rd trimester equivalent to screen and identify molecular agents that may be effective in preventing prenatal alcohol-induced brain damage and neurodevelopmental disorders. These studies will use two neonatal rodent models. The first is the well-studied model involving binge alcohol exposure on postnatal days (PD) 4-9 in outbred rats, which is known to produce structural and functional damage to cerebellar systems. The second is a model involving a single binge alcohol exposure on PD 7 in the C57BL/6 inbred mouse strain (the focus of Zhou's mouse component), known to induce extensive forebrain neuronal cell death. These studies will assess the effectiveness of candidate therapeutic agents by evaluating alcohol-induced damage to cerebellar (rat) and hippocampal (mouse) circuits that are known to mediate specific variants of Pavlovian eyeblink classical conditioning (ECC). In keeping with the emphasis of the Core, L-NAP, a 9-amino acid derivative of activity-dependent neuroprotective protein, will be the first candidate agent tested, because of its demonstrated effectiveness in several models of cell death and in a mouse model alcohol-induced teratogenesis. If L-NAP is found to be effective, additional studies of two structural derivatives of L-NAP for which the capacity to protect against neuronal death vs. ethanol teratogenesis has been dissociated, will also be evaluated. Specific Aim 1 tests the hypothesis that the candidate agent will protect against alcohol-induced caspase-3 activation on PD 4 in Purkinje cells, an indicator of acute Purkinje cell death. Specific Aim 2 tests the hypothesis that the candidate agent will protect against functional and structural damage to cerebellar systems mediating eyeblink conditioning induced by binge alcohol exposure on PD 4-9. Specific Aim 3 tests the hypothesis that binge alcohol exposure on PD 7 in C57BL/6 mice will damage hippocampal circuits necessary for eyeblink discrimination reversal learning, and that the candidate agent will protect against this hippocampal damage. Because the neural substrates and procedures for ECC are similar across species, the outcomes from these animal models—including those of the sheep model component of this Consortium—can be translated directly to humans to guide and inform future studies of therapeutic prevention of FASD. A key advantage of integrated approaches across the Consortium is that as promising candidate molecular agents emerge, these animal models can provide *in vivo* tests of their therapeutic effectiveness.

PERFORMANCE SITE(S) *(organization, city, state)*

Indiana University-Purdue University Indianapolis (IUPUI), Indianapolis, IN
University of Delaware, Newark, DE

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
Goodlett, Charles R., Ph.D.	Indiana University (IUPUI)	PI
Stanton, Mark E., Ph.D.	University of Delaware	Co-Investigator
Zhou, Feng, Ph.D.	Indiana University School of Medicine	Collaborating Investigator

a. SPECIFIC AIMS

In keeping with the long-term goals of the multidisciplinary, collaborative Basic Science Core of the Consortium, this component will use two neonatal rodent models of binge alcohol exposure during the "3rd-trimester equivalent" to evaluate the therapeutic potential of the neuroprotective peptide L-NAP and other candidate therapeutic agents. The well-studied neonatal rat model involving daily binge exposure on postnatal days (PD) 4-9, the period of brain development comparable to that of the human 3rd trimester, will be used to screen the effectiveness of candidate agents in protecting against cerebellar damage. The primary measures of neuroprotection will be prevention of alcohol-induced death of cerebellar neurons and functional impairment of Pavlovian eyeblink classical conditioning (ECC) mediated by cerebellar neural circuits, effects of binge alcohol exposure that are now well established in this neonatal rat model. The second approach, undertaken in collaboration with the mouse model component of this Consortium (Zhou, PI), will use an ECC task that depends on hippocampal interactions with the cerebellum to explore effects of (and protection against) the extensive apoptotic cell death in the hippocampal formation (and other forebrain populations) recently shown to result from acute binge exposure on PD 7 in C57BL/6 mice.

Experimental animal model studies of structural and functional damage to cerebellar and hippocampal neural systems mediating ECC provide several key advantages for meeting the overall goals of this Corsortium. First, structural damage to cerebellum is a known consequence of prenatal alcohol exposure in humans with fetal alcohol spectrum disorder (FASD). Second, the functional status of multiple memory systems can be probed using different procedural variants of ECC. Cerebellar-brain stem circuits are essential for all ECC, but "higher-order" conditioning procedures such as trace conditioning and discrimination reversal additionally require the functional integrity of hippocampal circuits. Third, the experimental analyses and conclusions of these animal model studies can be translated relatively directly to humans. The behavioral procedures and neural circuits underlying ECC in humans and non-human experimental animals are similar, so the animal model outcomes can guide, inform, and even be replicated in future studies of therapeutic efficacy of candidate compounds in human populations at risk for FASD. Fourth, ECC is well suited for early detection of the functional status of cerebellar and hippocampal circuits (including in human infants), so translation of therapeutic efficacy to human studies is facilitated by early developmental assessment.

Specific Aim 1 tests the hypothesis that the candidate therapeutic agents will protect against activation of caspase-3 in cerebellar Purkinje cells induced by alcohol exposure on postnatal day (PD) 4, an event mediating apoptotic cell death of Purkinje cells. Using a factorial design, groups of 4-day-old rats will be given either saline or the candidate agent (L-NAP or its derivatives) then treated either with alcohol or control intubations and perfused for active caspase-3 immunocytochemistry 8 hours after the first intubation.

Specific Aim 2 tests the hypothesis that the candidate therapeutic agents will protect against eyeblink classical conditioning deficits associated with the cerebellar cell loss induced by alcohol exposure on PD 4-9. Using a factorial design, groups of rats will be treated daily on PD 4-9 with L-NAP or vehicle 30 minutes before alcohol or control intubations, and tested as juveniles on delay ECC. Purkinje cells and deep interpositus nucleus neurons will be counted stereologically. Effectiveness of candidate agents will be determined by the extent of protection against conditioning deficits and loss of cerebellar neurons, with follow-up confirmation of neuroprotection by evaluation of separate groups of adult subjects.

Specific Aim 3, developed in collaboration with the mouse component (Zhou, PI), will test the hypothesis that the cell death induced in hippocampal circuits by binge alcohol exposure on PD 7 in C57BL/6 mice (Aim 2 of Zhou's mouse component), will result in deficits in hippocampal-dependent ECC learning tasks. These studies will build on the structural and cellular analyses of the Zhou component, extending the analysis of the extensive alcohol-induced cell death recently reported for hippocampus and other forebrain populations (but not cerebellum) in this very vulnerable inbred strain. We will test the predictions that the PD 7 exposure will produce deficits on discrimination reversal learning. Follow-up studies will test the effectiveness of candidate agents to prevent the disruption of this hippocampal-dependent variant of ECC.

Although the proposed studies are written specifically for studies of L-NAP and its derivatives, the Aims reflect a general strategy that can be applied to any compound determined by the Basic Science Core to have promise as a potential therapeutic agent. A candidate can be screened relatively quickly for its ability to protect against acute alcohol-induced activation of caspase-3 (in Purkinje cells on PD 4 in this component or forebrain neurons on PD 7 in Zhou's component). Additional studies of promising candidate agents can determine whether they afford neuroprotection against binge alcohol exposure over more extended portions of the 3rd-trimester equivalent.

b. BACKGROUND and SIGNIFICANCE

Exposure to Alcohol Induces a Spectrum of Enduring Brain Damage and Behavioral Disorders

Since the first scientific report of adverse outcomes from alcohol abuse during pregnancy (Lemoine et al., 1968) and the formal identification of Fetal Alcohol Syndrome [FAS] (Jones & Smith, 1973), a host of clinical, epidemiological, neuropathological, neuropsychological, and neuroimaging studies have demonstrated the harmful effects of prenatal exposure to alcohol on the developing central nervous system [CNS] in humans (Stratton et al., 1996). Life-long cognitive, motor, social and emotional disabilities are evident (Streissguth et al., 1991; Streissguth et al., 1996; Jacobson et al., 1998; Mattson & Riley, 1998; Roebuck et al., 1998a; Streissguth et al., 1998), and functional deficits occur both in children diagnosed with FAS (Jones et al., 1973) and in children exposed to alcohol *in utero* who do not meet all diagnostic features of FAS, termed alcohol-related neurodevelopmental disorder [ARND] (Stratton et al., 1996; Mattson et al., 1998). Wide variability in the extent of brain damage and functional disorders following prenatal alcohol exposure (regardless of diagnostic status) suggests that these are best considered as developmental spectrum disorders (Day & Richardson, 1991; Streissguth et al., 1994; Abel, 1995; Abel & Hannigan, 1995; Jacobson et al., 1998; Mattson & Riley, 1998; Streissguth et al., 1998; Sampson et al., 2000), leading to the current appellation of *fetal alcohol spectrum disorders* [FASD]. This wide phenotypic variation is, in part, a function of the pattern (especially binge drinking), duration and timing of the prenatal alcohol exposure (West et al., 1989; Streissguth et al., 1994; Jacobson et al., 1998). Alcohol produces many effects on the developing brain, likely mediated by multiple mechanisms of pathogenesis that depend on these exposure factors (Schenker et al., 1990; Goodlett & Horn, 2001). Binge drinking, particularly when it extends into the third trimester, increases the risk for cognitive disorders (Smith et al., 1986a; Smith et al., 1986b). Demonstrations that teratogenic effects of alcohol vary with dose, pattern, and developmental timing of alcohol exposure indicate that any intervention in human pregnancy must be concerned with potential exposure over all of gestation. A key goal of this Consortium Basic Science Core is to develop therapeutic agents that can protect against specific neuroteratogenic effects at distinct critical periods of vulnerability, which likely involve different mechanisms of damage at different developmental periods.

The Cerebellum and Hippocampus Are Targets of Damage in Fetal Alcohol Spectrum Disorders

Damage and functional disruption of cerebellar and hippocampal systems appear to be important neuropathological features of FASD. Evidence of cerebellar damage comes from autopsy of infant FAS cases (Clarren, 1986), neurobehavioral evidence of cerebellar dysfunction in FASD (Jones et al., 1974; Olegard et al., 1979; Streissguth et al., 1980; Kyllerman et al., 1985; Marcus, 1987; Jacobson et al., 1998; Roebuck et al., 1998b; Roebuck et al., 1998c), and MRI neuroimaging studies in individuals with FASD (Sowell et al., 1996; Roebuck et al., 1998a). Likewise, hippocampal dysfunction may be an important component of deficits in learning and cognition in FASD, based on neuropsychological evidence of deficits on hippocampal-dependent learning tasks. For example, FAS children were found to have deficits in tasks known to be sensitive to hippocampal damage including the virtual Morris-maze task measuring spatial navigation (Hamilton et al., in press), spatial learning in the stepping stone maze (Streissguth et al., 1994; Uecker & Nadel, 1996; Olson et al., 1998; Uecker & Nadel, 1998), and deficits in spatial memory for objects, impaired delayed (but not immediate) object recall and distorted recall of spatial arrays (Streissguth et al., 1994; Uecker & Nadel, 1996; Olson et al., 1998; Uecker & Nadel, 1998). Children exposed prenatally to alcohol have been characterized as having difficulty encoding information on tests of verbal learning, and show delay-dependent impairments in recall, increased intrusive and perseverative errors, impaired recognition memory, and susceptibility to distracters (Mattson et al., 1996; Mattson & Riley, 1998; Mattson et al., 1998). Although quantitative structural MRI analysis has not indicated that the hippocampal formation is disproportionately affected (relative to the overall small brain size) in FASD (Roebuck et al., 1998a; Archibald et al., 2001), a recent study (Bhatara et al., 2002) using single-photon emission computed tomography in 3 children with FAS suggests that temporal lobe dysfunction might be a prominent feature of FAS.

Cerebellar and Hippocampal Damage Induced by Alcohol Exposure During the 3rd-Trimester Equivalent

In rat models, dose-dependent reductions in brain weight and loss of neurons in several brain regions are induced by binge-like alcohol exposure during the neonatal period (West et al., 1989; Goodlett & Johnson, 1999). This period in rats has been referred to as the "third-trimester-equivalent", because it includes brain

developmental events similar to that of the human third trimester (Dobbing & Sands, 1979; West, 1987; Bayer et al., 1993). Binge-like exposure to alcohol on postnatal days [PD] 4-9 produces loss of Purkinje and granule cells in the cerebellar cortex that increases with increasing peak blood alcohol concentrations (BACs). PD4-6 appears to be the period of greatest vulnerability (Hamre & West, 1993; Marcussen et al., 1994; Goodlett & Lundahl, 1996) and cell loss varies directly with dose and peak BAC (Goodlett et al., 1998). Proportional cell loss in other neuronal populations directly linked by afferent or efferent projections to the cerebellar cortex, e.g., the inferior olive (Napper & West, 1995; Tran et al., 2000) and the deep cerebellar nuclei (Green et al., 2002b). Heavy neonatal binge alcohol exposure produces long-lasting deficits in behaviors that are known to depend on cerebellar function, including gait, coordinated locomotor performance (Meyer et al., 1990a, b; Goodlett et al., 1991; Goodlett & Lundahl, 1996; Thomas et al., 1996; Thomas et al., 1998) and, most important for this proposal, eyeblink classical conditioning (Stanton & Goodlett, 1998; Green et al., 2000). As discussed below (on p. 25) and shown in **Fig. 1**, the cerebellar cortex, deep cerebellar nuclei, and inferior olive are all components of the neural circuit essential for eyeblink conditioning (Kim & Thompson, 1997). Overall, there is compelling evidence that binge alcohol exposure in the 3rd trimester equivalent produces structural damage to the cerebellum accompanied by long-lasting deficits in cerebellar-dependent behavior.

Neonatal binge exposure in rats and mice has also been shown to induce cell loss and apoptosis of neurons in forebrain limbic and cortical regions, including the hippocampal formation. Cell loss has been reported in several regions of the hippocampal formation, but the most consistent effect is loss of pyramidal neurons in the CA1 subfield when BAC levels exceed 200 mg/dl (Pierce et al., 1989; Bonthius & West, 1990, 1991; Miller, 1995; Bonthius et al., 2001). In a highly publicized report in *Science* (Ikonomidou et al., 2000), massive neuronal degeneration and apoptotic cell death was reported in the forebrain of rats given two subcutaneous doses of 2.5 g/kg of alcohol, 2 hours apart, on PD 7, in which mean BACs were reported to be above 500 mg/dl. The neurodegeneration was especially prominent in layers II and IV of cingulate, parietal, frontal and occipital cortex, and in hippocampus and thalamus.

More recently, Olney and colleagues have replicated and extended these findings in C57BL/6 mice, again using 2.5 g/kg subcutaneous injections two hours apart on PD 7 (Dikranian et al., 2001; Olney et al., 2002a; Olney et al., 2002b). The extensive neuronal degeneration in cortex, thalamus, and hippocampus was correlated with immunocytochemical detection of the active subunit of caspase-3, indicative of apoptotic cell death in these forebrain regions. These effects in B6 mice, which are the focus of the mouse model component (Zhou, PI), are also of particular interest for this component, because neurodegeneration and cell death was especially heavy in hippocampal field CA1, in subiculum, and in retrosplenial cortex. As discussed on p. 25 and shown in **Fig. 1**, these brain regions are components of the neural circuit necessary for higher-order learning in variants of eyeblink classical conditioning that require hippocampal-cerebellar interactions. Aim 3 proposes to use this PD 7 mouse model to evaluate effects of alcohol-induced damage to this hippocampal circuit on higher-order conditioning, and the potential of candidate agents to protect against .

Behavioral studies have also shown that binge-like exposure during the 3rd trimester equivalent in rats produces developmental deficits in hippocampal-dependent learning and memory (Goodlett et al., 1987; Greene et al., 1992; Thomas et al., 1996). Seven of eight published studies, using different variants of the neonatal rat model of binge alcohol exposure, have reported alcohol-induced deficits in place learning (but not cued learning) in one or both sexes using the Morris water maze when blood alcohol concentrations exceeded 230 mg/dl (Goodlett et al., 1987; Kelly et al., 1988; Goodlett & Peterson, 1995; Pauli et al., 1995b; Goodlett & Johnson, 1997; Tomlinson et al., 1998; Cronise et al., 2001; Johnson & Goodlett, 2002), and PD 7-9 appears to be a temporal window of vulnerability to this effect (Goodlett & Johnson, 1997; Johnson & Goodlett, 2002). Two other studies (Girard et al., 2000; Girard et al., 2001) reported deficits on a spatial working memory version of the water maze task in adult male rats, and deficits in swim-escape spatial delayed alternation have also been reported (Thomas et al., 1996). Despite this evidence of spatial learning deficits, questions still remain whether these deficits reflect impaired processing specific to the hippocampal formation, because other cortical and cerebellar systems are important for learning and performance in these tasks (Kolb et al., 1983; Goodlett et al., 1992). The eyeblink conditioning studies proposed with the mouse model in Aim 3 of this application are capable of determining whether the binge exposure on PD 7 selectively impairs learning that depends on hippocampal interactions with cerebellar function, while sparing learning and performance that depends on the essential cerebellar-brain stem circuits *per se* (see below).

Animal Model Studies of Eyeblink Classical Conditioning Can Help Develop Treatments for FASD

Experimental animal model studies have been necessary to answer many essential questions about the nature and risk for alcohol-induced damage to the developing brain, i.e., documenting that alcohol *per se* causes neuroteratogenic effects and identifying many important risk factors (dose, pattern, and developmental timing of exposure; genetic and maternal factors) that contribute to phenotypic variation in the extent of alcohol-induced damage (Becker et al., 1989; Driscoll et al., 1990; Goodlett & West, 1992; Hannigan, 1996; Maier et al., 1996; Boehm et al., 1997; Goodlett & Johnson, 1999).

We have contended (Goodlett, 1999) that ***the next generation of animal model research should focus on three key areas relevant to human FASD***: **1)** identifying mechanisms of damage that can inform development of interventions to protect the conceptus; **2)** identifying therapeutic interventions or treatments, administered as early in development as feasible, to prevent or ameliorate the effects of prenatal alcohol-induced brain damage; and **3)** identifying sensitive indices of fetal alcohol exposure or damage to permit early detection and guide treatment. Progress toward these goals demands conceptual integration across different levels of analysis of the neurosciences spanning basic and clinical disciplines, as represented in this multidisciplinary consortium. For animal model studies to be most effective in identifying therapeutic agents to treat or prevent FASD, neurobehavioral endpoints must be chosen that can be directly related to similar endpoints in humans. The neural systems mediating the behavior must be relatively well defined and should be essential to the behavior both in the animal model and in humans. The neurobehavioral functions should also be suited for experimental assessment in animals and humans, and the neural systems and behaviors should be known to be a target of fetal alcohol exposure in humans.

Following these principles, we began using eyeblink classical conditioning (ECC) as a model system for studying the neuroteratogenic effects of alcohol (Goodlett et al., 2000), focusing on the cerebellar-brain stem circuit that is essential for this learning (Kim & Thompson, 1997; Steinmetz, 2000). That continuing work, funded by NIAAA grant AA11945 (Goodlett, PI), has conclusively established that heavy binge alcohol exposure in the neonatal rat (during the period of brain development comparable to that of the human third trimester) damages the cerebellum and, at high doses, severely impairs ECC (Stanton & Goodlett, 1998; Green et al., 2000). We also have established that this learning deficit is correlated with extensive loss of cerebellar neurons and with impaired learning-related plasticity of cerebellar neurons (Green et al., 2002a; Green et al., 2002b).

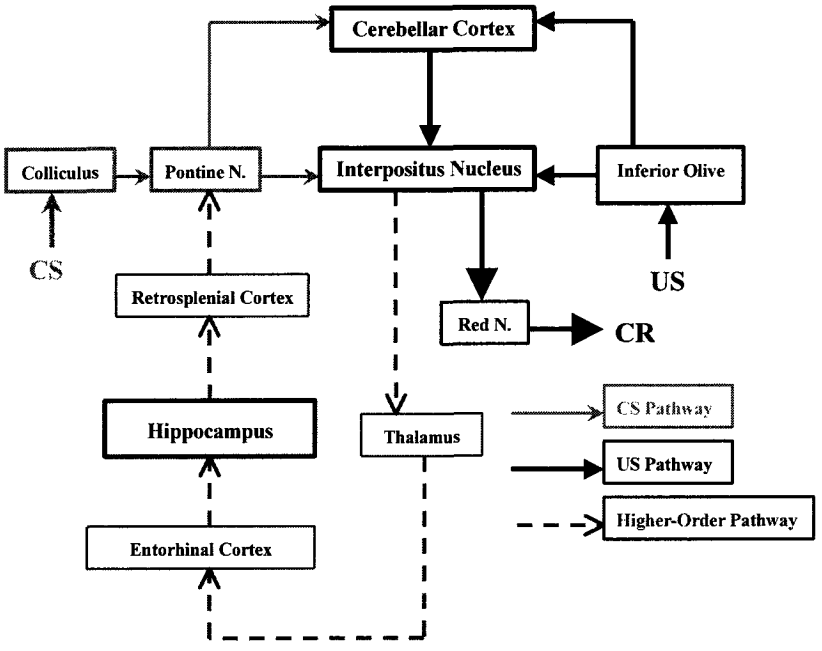


Fig. 1: Cerebellum, Hippocampus and Eyeblink Conditioning (Adapted from Schmajuk & Dicarlo, 1991). CS: Conditioned Stimulus; US: Unconditioned Stimulus; CR: Conditioned Response

Our success with this analysis of ECC deficits in the neonatal rat model creates an opportunity to extend this work to test the neuroprotective potential of candidate molecular therapies, selected on the basis of their known or suspected ability to prevent or limit alcohol-induced neuropathogenesis. Our ongoing work has identified variants of ECC that provide four key advantages in the search for therapeutic agents.

1) ECC provides a means to assess the functional status of the cerebellar-brain stem circuit essential for this basic form of associative learning.

2) By using straightforward manipulations of the Pavlovian conditioning procedures, variants of ECC can also assess multiple memory systems, including learning and memory functions that depend on interactions between the cerebellum and the hippocampal formation.

3) ECC can be applied relatively early in development as a sensitive functional index for *early detection* of alcohol-related neurodevelopmental disorders.

4) ECC procedures can be directly translated for use in humans to assess the extent of alcohol-induced functional impairment and potential therapeutic effectiveness of candidate agents reaching clinical trials.

Using Eyeblink Conditioning To Study Learning and Memory Functions of Defined Neural Systems

Classical (Pavlovian) eyeblink conditioning is a simple nonverbal learning task that involves paired presentations of a neutral conditioned stimulus (CS, e.g., a tone that initially does not elicit a consistent eyeblink), with a biologically significant unconditioned stimulus (US, e.g., a mild airpuff for humans, mild periocular shock for rodents, that consistently elicits a reflexive eyeblink—the Unconditioned Response, UR). With repeated paired presentations of the CS and US, the CS comes to elicit an eyeblink with an onset before the US is presented, and the peak amplitude of this conditioned eyeblink response (CR) comes to approximate the time of the US onset (see **Fig. 2, middle panel**). In "delay conditioning", the CS precedes, overlaps and co-terminates with US. The interval between CS and US onset is referred to as the "delay interval" or *interstimulus interval* (ISI). Classical conditioning of eyeblink responses is perhaps the best-understood model of mammalian associative learning in neuroscience (Anderson & Steinmetz, 1994; Kim & Thompson, 1997), and has a long history of application in human studies (Woodruff-Pak & Steinmetz, 2000a). Twenty years of converging evidence using lesion, reversible inactivation, neural recording, tract-tracing and human neuroimaging methods has generated a large body of research demonstrating the critical involvement of a cerebellar-brain stem circuit in the acquisition and performance of classically conditioned eyeblink responses

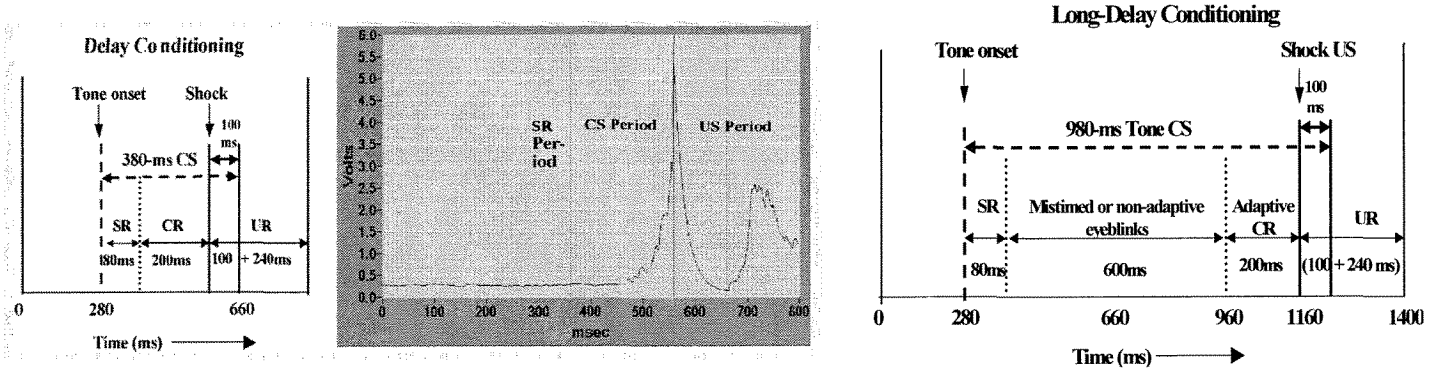


Fig. 2: Trial Epochs for Short-Delay (left) and Long-Delay (right) Conditioning. The middle panel is a digitized EMG response from a rat, showing a clear, well-timed CR in paired trial of a well trained rat given short-delay training. Note that the EMG signal is shunted during the 100-msec shock US presentation, to eliminate stimulus artifact.

(McCormick & Thompson, 1984a; Thompson, 1986; Lavond et al., 1993; Logan & Grafton, 1995; Kim & Thompson, 1997). This research shows that a neural circuit in the brain stem and cerebellum is necessary and sufficient for simple delay conditioning (McCormick & Thompson, 1984a; Thompson, 1986; Lavond et al., 1993; Logan & Grafton, 1995; Kim & Thompson, 1997).

The pathways responsible for projecting the conditioning stimuli from the periphery to the cerebellum have been defined (Thompson, 1986; Krupa et al., 1993; Kim & Thompson, 1997) and are shown in the upper part of **Fig. 1**. The CS is projected via the cochlear nuclei to discrete portions of the pontine nuclei, and the mossy fiber axons from these pontine neurons project the CS to discrete regions of cerebellar cortex and to the anterior interpositus nucleus [IP]. The US is projected via the trigeminal nucleus to the dorsal accessory inferior olive, then via the climbing fibers from the olivary neurons to discrete regions of cerebellar cortex {Larsell's Lobule HVI & anterior lobules (HI-HV)} and to the IP. Training-related plasticity in neurons in these two sites—IP neurons and cerebellar Purkinje cells—encode the conditioning procedure and mediate the associative learning (McCormick & Thompson, 1984b; Berthier & Moore, 1986; Hardiman & Yeo, 1992; Perrett et al., 1993; Gould & Steinmetz, 1996; Katz & Steinmetz, 1997; Garcia & Mauk, 1998; Attwell et al., 2001; Ohyama & Mauk, 2001). Neuroplasticity arising from converging CS and US input projections at IP neurons is essential for CR acquisition and performance. Neuroplasticity at synapses converging on anterior and HVI Purkinje neurons may not be required for acquisition, but does regulate rate of acquisition, amplitude and timing of CRs (Lavond & Steinmetz, 1989; Perrett et al., 1993; Chen et al., 1996; Garcia & Mauk, 1998; Attwell et al., 2001; Ohyama & Mauk, 2001).

One procedural variant used in this proposal is the manipulation of the interstimulus interval by shifting the ISI from one interval to another (Mauk & Ruiz, 1992; Kehoe et al., 1993). The ISI shift reveals cerebellar-dependent learning processes that regulate the precision and plasticity of timing of CRs. For example, when the ISI is shifted, the peak CR amplitude normally shifts to approximate the time of the US onset at the new ISI.

The anterior and HVI lobules are critical for these CR timing functions (Lavond & Steinmetz, 1989; Perrett et al., 1993; Medina et al., 2000).

Although cerebellar circuitry is sufficient for acquisition and regulation of CR timing of delay ECC, there are other "higher order" conditioning phenomena that additionally engage the necessary involvement of the hippocampus (**lower part of Fig. 1**). One well-known example is *trace conditioning*, involving paired presentations of a CS and US arranged so that the CS terminates before the onset of the US thus leaving a temporal gap (trace interval, e.g., 500 msec) between the CS and US. Hippocampal lesions (including in humans) prevent acquisition of trace conditioning (Port et al., 1986; Solomon et al., 1986; Moyer et al., 1990; Kim et al., 1995; McGlinchey-Berroth et al., 1997; Weiss et al., 1999; Ivkovich & Stanton, 2001). Trace conditioning engages hippocampal plasticity in unit activity (Moyer et al., 1996; Weiss et al., 1996; McEchron & Disterhoft, 1997; Power et al., 1997), induces changes in PKC expression (Van der Zee et al., 1997), induces increases in the number of multiple-synapse boutons (Geinisman et al., 2001), and increases survival of newly generated granule cells in adult rats (Gould et al., 1999; Shors et al., 2001).

The procedural variants of eyeblink conditioning in the proposed rat model studies (binge exposure on PD4-9) will include acquisition of standard delay conditioning and shifts in the ISI after acquisition, to assess neuroprotection of cerebellar-brain stem function. In the mouse model studies (binge exposure on PD 7), in which forebrain neuronal death is much more prominent than cerebellar cell death, we will use discrimination reversal learning to evaluate an ECC variant that depends on functional interactions of cerebellar system with the hippocampal formation (Berger & Orr, 1983). In addition, these rodent model studies will precede and inform a similar, novel analysis of ECC in the sheep model component (Cudd, PI). The unique convergence of expertise and resources across this Consortium, the power of ECC to assess the functional status of hippocampal and cerebellar systems underlying specific forms of learning and memory across these three animal models and the potential extension of ECC assessments to humans, are compelling advantages of using this approach to assess neuroprotective therapies for FASD.

Therapeutic Agents Can Prevent Alcohol-Induced Developmental Damage

The goal of this Basic Science Core is to help develop effective, practical treatments that prevent or limit alcohol-induced damage to the developing fetus in high-risk pregnancies (Goodlett, 1999; Warren & Foudin, 2001). A rational approach should seek to interrupt either the initiation or the progression of known or suspected cascades of alcohol-induced neuropathogenesis. However, alcohol almost certainly acts through multiple mechanisms to induce developmental abnormalities (e.g., altered neurogenesis, abnormal migration, disrupted cell adhesion or cell-cell interactions, altered regulation of gene expression, impaired cell signaling, induction of oxidative stress or excessive apoptotic cell death), and these different mechanisms of initiation or different pathogenic cascades may operate at different periods of brain development (Schenker et al., 1990; Goodlett & Horn, 2001). Any search for therapeutic intervention should evaluate the candidates using models with exposure at different developmental periods, targeting mechanisms that are responsible for known neuroteratogenic effects during those periods.

Collaborative Approaches to Therapeutic Intervention with L-NAP Across the Consortium. The mission of the Basic Science Core is to use *in vitro* and *in vivo* models to explore and develop a molecular interventions that may limit or prevent the neuroteratogenic effects of alcohol. The collaboration of the four research groups focuses on a class of neuroprotective peptides that have also been found to block ethanol teratogenesis in C57BLB/6 (B6) mouse embryos. Work performed in the Charness laboratory has demonstrated the peptide NAPVSIPQ [NAP] prevents ethanol inhibition of L1 cell adhesion function (ethanol antagonism) and also prevents teratogenic dysmorphogenesis in B6 embryos. The Basic Science Core has chosen to focus its initial efforts on the potential therapeutic effectiveness of NAP, one of two neuroprotective peptides (the other being SALLRSIPA [SAL]) derived from two larger proteins (activity-dependent neuroprotective protein and activity-dependent neurotrophic factor, respectively) released by glia in response to vasoactive intestinal peptide (VIP) (Brenneman & Foster, 1987; Brenneman et al., 2000). NAP and SAL are extremely potent neuroprotective agents in a number of systems (EC50 in the femtomolar range), including cellular models of oxidative stress, tetrodotoxin-induced cell death, and neurodegeneration in apolipoprotein E-deficient mice (Brenneman et al., 1998; Offen et al., 2000; Steingart et al., 2000). Structure activity analysis reveals three residues near the carboxyl terminal (SIP) that are critical for neuroprotective activity (Brenneman et al., 1998). Known cellular actions include increased expression of NF-KB and heat shock protein-60 (hsp-60) (Zamostiano et al., 1999; Glazner et al., 2000), inhibition of oxidative stress, and reduction in reactive oxygen species (Glazner et al., 1999). NAP and SAL recently were shown to prevent ethanol-induced fetal death and growth abnormalities in a mouse model of FAS (Spong et al., 2001). Recent data suggest that the

D-enantiomers of NAP and SAL prevent ethanol-induced embryo toxicity (Brenneman and Spong, unpublished data). The mechanism of action of these peptides is complex and poorly understood.

Although NAP and SAL were first recognized as neuroprotective agents, recent studies by Charness and colleagues have shown that their prevention of ethanol-induced dysmorphology in C57BL/6 (B6) mouse whole embryo culture is correlated with antagonism of ethanol effects on L1-mediated cell adhesion. NAP and SAL, like octanol, completely antagonize the disruptive effect of 100 mM ethanol on L1-mediated cell adhesion *in vitro*, with half-maximal inhibition at ~10^{-14} M for NAP and at ~10^{-11} M for SAL (Wilkemeyer et al., 2002). D-NAP and L-NAP were equipotent, and structure activity analyses with L-NAP also suggested significant structural specificity. The structure activity relation for a series of alanine scanning substitutions of NAP is similar for NAP antagonism and NAP prevention of ethanol-induced growth retardation in mouse embryos, but different from that of NAP protection of cortical neurons against tetrodotoxin. Single amino-acid substitutions of alanine in NAP have been identified that selectively eliminate either the ethanol antagonism / embryoteratogenesis (I6A-NAP) or the neuroprotection against TTX-induced death (P7A-NAP). These two structural derivatives may be useful in the neonatal rodent models to determine whether these neuroprotective peptides can prevent alcohol-induced damage during the 3rd-trimeseter equivalent, with the potential to shed light on whether neuroprotection or ethanol antagonism is more effective as a therapeutic approach to binge drinking later in pregnancy.

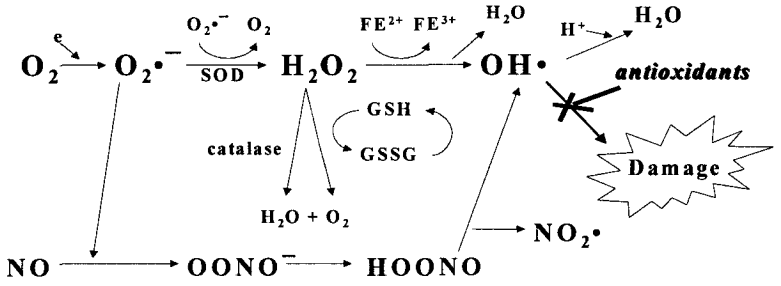


Fig. 3: Formation and degradation of reactive oxygen species (ROS). Oxygen in the presence of electrons can form the free radical superoxide (upper left). SOD can rapidly convert superoxide to hydrogen peroxide, which in turn can generate highly reactive hydroxyl radicals when iron is present. Superoxide can also combine with NO to form hydroxyl and nitrogen dioxide radicals. Macromolecular damage will result if reactive oxygen molecules are not neutralized. Catalase and GSH remove ROS via enzymatic mechanisms that convert hydrogen peroxide to water and O_2. Vitamin E prevents damage by scavenging ROS and lipid peroxyl radicals.

Given that L-NAP can both protect against cellular models of cell death and antagonize ethanol's disruption of L1, it appears that these peptides have multiple pharmacological actions, imparting different protective actions against ethanol-induced damage to the developing brain. One of these actions appears to include protection against **oxidative stress**, an imbalance between the formation and degradation of reactive oxygen species (ROS). Oxidative stress has been implicated in ethanol pathogenesis in several model systems (Henderson & Chen, 1999; Halliwell & Gutteridge, 2000), including the cerebellar damage induced in the neonatal rat model (Heaton et al., 2000). ROS (**Fig. 3**) include O_2-centered radicals (superoxide), hydroxyl and nitrogen dioxide radicals, and highly reactive oxygen non-radicals (hydrogen peroxide, nitric oxide, and peroxynitrite anion). Antioxidants normally neutralize ROS and prevent damage (Woods & Fantel, 1998; Seis, 1999; Halliwell & Gutteridge, 2000). Superoxide dismutase (SOD), catalase, and glutathione peroxidase help remove ROS enzymatically. Vitamins E and C scavenge ROS and lipid peroxyl radicals to prevent damage. Excessive ROS can cause damage to biological macromolecules (Grisham, 1992; Woods & Fantel, 1998; Halliwell & Gutteridge, 2000), and this oxidative damage can result in effects ranging from altered cell function to demise by apoptosis or necrosis.

Alcohol exposure can increase ROS formation in liver (Kurose et al., 1997; Bailey & Cunningham, 1998; Henderson & Chen, 1999), synaptosomes (Montoliu et al., 1994), astrocytes (Montoliu et al., 1995; Gonthier et al., 1997), neural crest cells (Davis et al., 1990; Chen & Sulik, 1996; Chen et al., 1997) and cerebellar granule cells (Huentelman et al., 1999). Alcohol-induced oxidative damage occurs in the liver (Henderson & Chen, 1999), embryo culture (Kotch et al., 1995), fetal brain (Uysal et al., 1989; Henderson et al., 1995; Muresan, 1997), and adult cerebellum (Nordmann et al., 1990), associated either with increased ROS formation or depleted anti-oxidant defenses in fetal brain (Reyes et al., 1993; Addolorato et al., 1997).

Antioxidant supplements intended to limit alcohol-induced oxidative stress and to reduce tissue pathology indicate that boosting antioxidant defenses may provide a useful treatment to prevent alcohol-related developmental defects. Ethanol teratogenesis in mice was diminished by the addition of SOD (Kotch et al.,

1995). Vitamin E supplementation prevented ethanol-induced cell loss in hippocampal cultures (Mitchell et al., 1999) and cerebellar granule cell cultures (Horn et al., 2002). Vitamin E was recently reported to dose-dependently protect cerebellar Purkinje cells in the neonatal rat binge exposure model (Heaton et al., 2000). Studies of Vitamin E represent one line of research exploring neuroprotective agents that is currently being pursued by funded research outside this Consortium, including studies in the Goodlett laboratory (funded by NIAAA grant AA11945). However, protection against oxidative stress clearly may be an important potential neuroprotective mechanism of L-NAP, and anti-oxidant approaches are broadly considered to be an important part of the Core's mission.

Integration across the Consortium. This component will focus on neonatal rats and mice, to provide a means to identify the therapeutic potential of candidate agents to prevent or ameliorate the consequences of binge drinking during the human 3rd trimester. Development of novel molecular neuroprotective agents will be directed by Dr. Charness, and the candidates will be tested in four different animal models: prenatal mouse (Zhou), neonatal rat (Goodlett) and mouse (Zhou; Goodlett) and sheep (Cudd). Each model has distinct advantages. For example, L-NAP has known protective effects in prenatal mice. The neonatal rat model has been well characterized behaviorally and neuroanatomically. Rodent models can screen some effects relatively quickly (in weeks). The sheep model is the only *in utero* model of 3rd-trimester exposure. The four components will be integrated in this mission, but flexible enough to make rapid changes in direction if needed to follow promising new directions. In that regard, tests of protection in 3rd-trimester equivalent models will proceed first in rats, then mice, then sheep. Findings from the molecular studies of Charness (or elsewhere) may demand that new candidates be tested relatively quickly, facilitated by developing these three animal models under the Consortium umbrella. Finally, the structural and ECC behavioral studies of the rat, mouse, and sheep can be directly translated to human FASD populations represented in the rest of the Consortium.

Eyeblink Conditioning and Early Neurobehavioral Assessment and Intervention in Humans. An important advantage of including the animal model studies of eyeblink conditioning is that the same procedure can be used with both human and animal subjects (Woodruff-Pak & Steinmetz, 2000a, b). Indeed the procedure is able to distinguish human populations that differ along important cognitive and neurological dimensions. These include children and adults with dyslexia (Coffin & Boegle, 2000), mental retardation (Ohlrich & Ross, 1968), temporal lobe amnesia (McGlinchey-Berroth et al., 1997; Clark & Squire, 1998), and autism (Sears et al., 1994; Sears & Steinmetz, 2000). Dr. Stanton is engaged in studies of eyeblink conditioning in human infants as young as 5-6 months of age (Ivkovich et al., 2000)) and has found differences between high-risk, very-low-birth-weight infants and normal infants at this age with this procedure (Herbert et al., (submitted, pending EPA internal review)). As our animal-model research progresses and intervention strategies are more fully developed, there is a very real possibility that these strategies could be tried on infants and children at risk for FASD (see Appendix Item 10).

c. PRELIMINARY STUDIES

The neonatal rat studies proposed to test the therapeutic efficacy of L-NAP and its structural derivatives (or any other candidate agent) take advantage of the work that we have pursued over the last 5 years in characterizing effects of binge alcohol exposure on eyeblink classical conditioning (ECC) and its links to structural and functional damage in the cerebellar circuits mediating ECC. This ongoing work, including new studies on the potential for Vitamin E supplements to prevent these effects, is supported by NIAAA grant AA11945 (Goodlett, PI). In this section, we will briefly summarize and present some essential data that demonstrate the appropriateness for using this model and this approach as part of the Consortium's mission to develop therapeutic agents.

Heavy Binge Alcohol Exposure on PD 4-9 Induces Permanent Deficits in Delay ECC

Over the last 5 years, we have completed six independent replications, across 3 different laboratories, showing that the neonatal binge alcohol exposure induces significant, permanent deficits in acquisition and terminal performance of conditioned eyeblink responses. Our initial study, which served as the foundation for the original proposal (testing done in the Stanton lab while he was at the US EPA), found that juvenile rats exposed to 5.25 g/kg of ethanol on postnatal days (PD) 4-9 (via artificial rearing), failed to acquire standard short-delay eyeblink conditioning when tested on PD24 (Stanton & Goodlett, 1998). Since then, we have found that both the dose (i.e., peak BAC) and developmental timing critically determine the extent of these learning deficits in rats. In these subsequent studies, pups were given alcohol via intragastric intubations, and deficits were still evident even when training was extended to 600 trials (**Fig. 4**). The alcohol-treated rats did

acquire some conditioning in the later sessions, but never reached the same level of performance as controls. In what has become one of our most important observations, it was also determined that the dose-response curve for effects on short-delay training was quite steep (**Fig. 5**). The high dose (5.25 g/kg/day) induced the previously observed deficit, but neither a 3.3 g/kg/day nor a 4 g/kg/day dose produced significant group differences. Part of our current effort is now focused on developing procedural variants of eyeblink conditioning (including the ISI manipulation proposed in this application) that are more sensitive to (the known) cerebellar cortical damage produced by the lower alcohol doses. Our recent studies have confirmed the significant alcohol-induced deficit in acquisition of short-delay (280msec ISI) eyeblink conditioning in juvenile rats (PD31-34), and further demonstrated that the impairment relative to controls was comparable when long-delay (880msec ISI) training procedures were used (**see Fig. 6**). With paired training, the EtOH-treated rats had significant, and comparably large, impairments in their CR frequencies (and amplitudes, not shown), relative to the two control groups. At both ISIs, the mean terminal performance by session 6 of the alcohol groups never surpassed 51% compared to >70% for both control groups. The UR performance measures (frequency and amplitude) did not differ significantly among groups in either delay procedure, and CR frequencies of unpaired groups of all treatments never surpassed 20% in any session. With doses at or above 5 g/kg, it is clear that the conditioning deficits generalize across labs and to longer ISIs, making it certain that alcohol effect on delay conditioning in the rat studies of neuroprotection experiments is consistent and reliable. Likewise, we have also recently documented that a 5.0 g/kg dose on PD 4-9 also induces profound deficits in acquisition of trace conditioning (**see Fig. 7**), perhaps the best studied form of ECC that depends on hippocampal-cerebellar interactions.

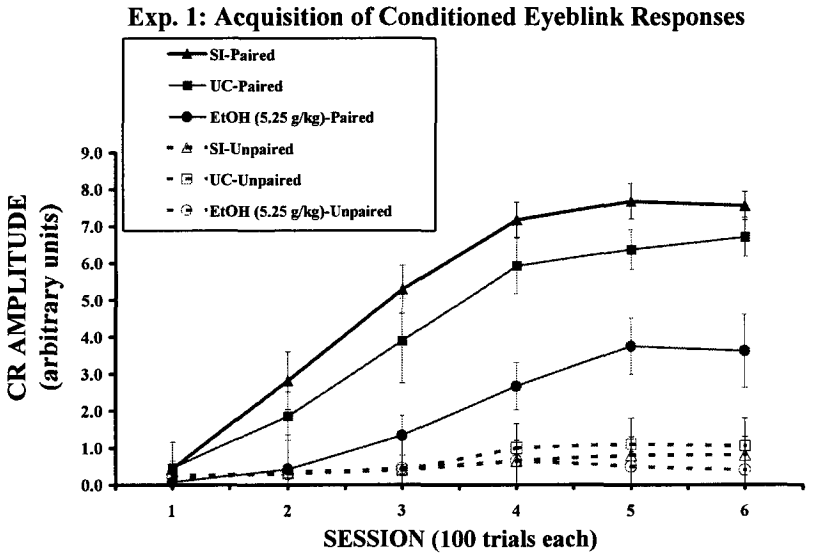


Fig. 4: Binge alcohol exposure on PD 4-9 (5.25 g/kg/day) induces severe deficits in eyeblink classical conditioning in juvenile rats. **Solid lines: groups given Paired training; Dashed lines: groups given Unpaired training. SI: Sham Intubated (0 g/kg) Control; UC: Untreated controls; EtOH 5.25: Alcohol intubated, 5.25 g/kg/day).** The EtOH group given Paired training had impaired acquisition and terminal performance compared to controls. Note that the Unpaired groups showed no increases in responding to the tone, confirming that the conditioned responding seen in the Paired groups reflects associative learning about the CS-US pairing.

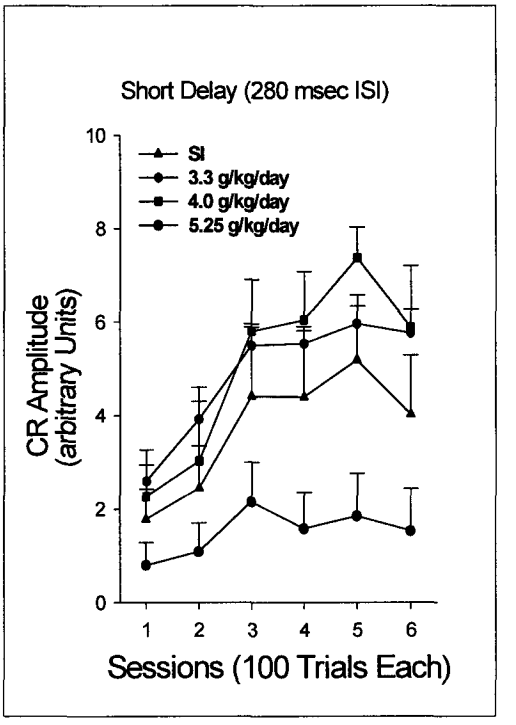


Fig. 5. Dose-response of PD 4-9 alcohol exposure on juvenile ECC. **SI: Sham Intubated (0 g/kgday); 3.3, 4.0, 5.25 g/kg/day: Alcohol dose groups** (peak BACs of 140, 220, and 331 mg/dl, respectively). Only the high dose of alcohol on PD4-9 significantly impaired CR acquisition.

In addition to the above studies in juvenile rats, we have now reported in two studies that deficits in delay eyeblink conditioning persist into adulthood, and that the effects found in adults are comparable to and consistent with the effects found in juveniles (Green et al., 2000; Green et al., 2002b). Our studies in adults have also demonstrated that exposure over just 3 days of the neonatal period (either PD 4-6 or PD 7-9) is not sufficient to produced significant acquisition deficits, even though the PD 4-6 treatment produces about 25-30% loss of cerebellar Purkinje cells. Based on the studies of dose-response and of developmental timing of exposure, it appears that alcohol-induced acquisition deficits in basic delay ECC are not evident until cerebellar

cell loss approaches ~40% (see below). However, our currently funded studies using manipulations of the interstimulus interval (ISI)—variants of the ECC procedures that are known to be more sensitive than basic acquisition to cerebellar dysfunction because they challenge cerebellar plasticity of the timing of conditioned responses—are pursuing functional correlates of the less severe cerebellar damage induced by the lower alcohol doses. These ISI manipulations are also proposed in Aim 2, Exp. 2.3 of the current proposal.

The Alcohol-induced ECC Deficits are Associated with Cell Loss and Impaired Learning-Related Neural Plasticity in the Essential Cerebellar Circuit. We and others have published a series of studies demonstrating that alcohol exposure on PD 4-9 induces a dose-dependent loss of cerebellar Purkinje cells that varies directly with the peak BAC achieved (Goodlett & Johnson, 1999). PD 4-6 constitutes a temporal window of vulnerability (Hamre & West, 1993; Goodlett & Lundahl, 1996; Goodlett

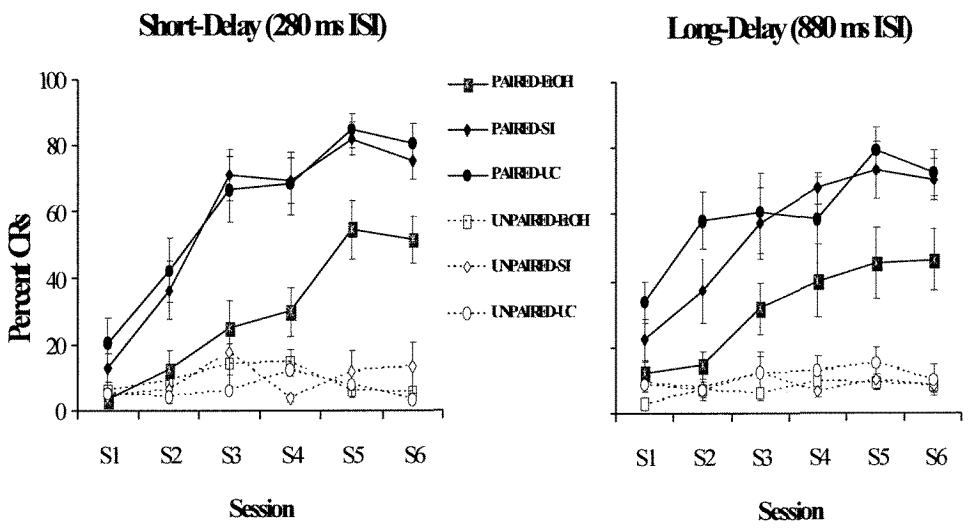


Fig. 6. PD4-9 exposure to 5.25 g/kg/day alcohol impairs both short-delay and long-delay eyeblink conditioning in juvenile rats. **Paired**: Paired training groups; **Unpaired:** Unpaired Training Control groups; **EtOH:** 5.25 g/kg alcohol on PD 4-9; **SI:** Sham Intubated (0 g/kg) on PD 4-9; **UC:** untreated control

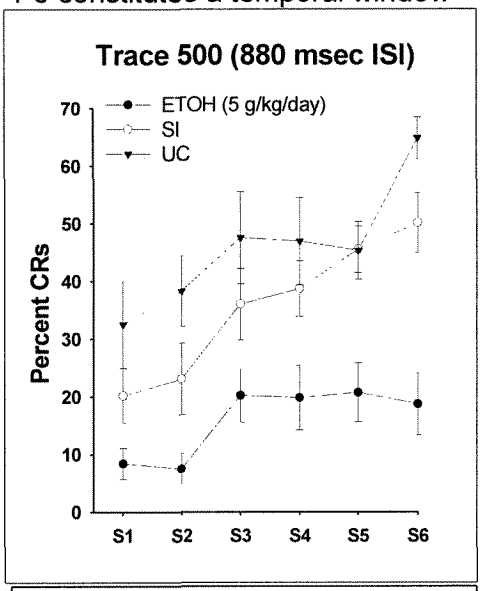


Figure 7. Rats given 5 g/kg/day of alcohol on PD 4-9 fail to acquire trace conditioning as juveniles (n's =7-9 per group). **EtOH:** Alcohol-treated group; **SI**: Sham Intubated group (0 g/kg/day); **UC:** Untreated Control group.

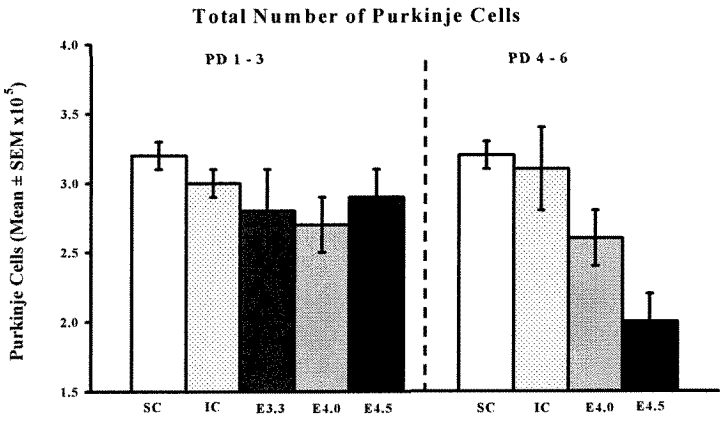


Fig. 8: Alcohol on PD 4-6 induced dose-dependent cell loss; comparable exposure on PD1-3 did not (Purkinje cell counts on PD10). **SC:** Suckle Control; **IC:** Sham Intubated Control; **E3.3, E4.0, E4.5:** Alcohol intubations of 3.3 g/kg/day, 4.0 g/kg/day, or 4.5 g/kg/day on either PD 1-3 (left panel) or PD 4-6 (right panel).

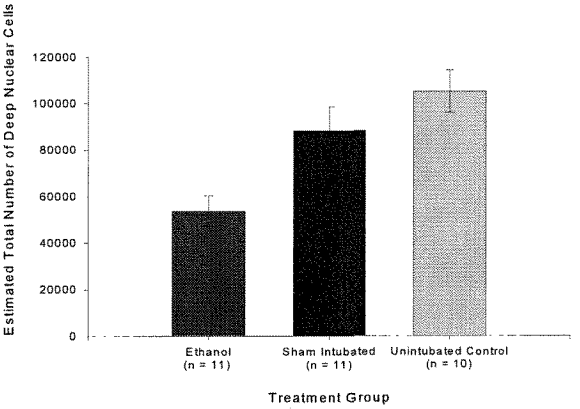


Fig. 9: Alcohol-induced loss of neurons in the deep cerebellar nuclei, counted using the optical fractionator in adult rats given ECC training (Green et al., 2002b). The ethanol group (5.25 g/kg/day, PD 4-9) had significantly fewer neurons then either control group.

& Eilers, 1997), in that exposure during this period, but not before (e.g., **see Fig. 8**) or after, produces dose-dependent loss of Purkinje cells. More recently, cell counts have been completed in both juvenile and adults rats given 5.25 g/kg/day of alcohol on PD4-9 and trained on delay ECC. In the juvenile rats contributing to the data of Fig. 4 above, total cerebellar Purkinje and granule cells, and total cells in the inferior olive were counted using the optical fractionator (see Part IIi for description). Compared to unintubated and sham controls, the alcohol-treated group had significant reductions in all three neuronal populations, consistent with the severe deficit in eyeblink conditioning. Unbiased stereological cell counts of the deep nuclei of rats from our initial adult behavioral study also revealed markedly fewer cells in the deep nuclei of the alcohol-treated rats compared to the two control groups (see **Fig. 9)** (Green et al., 2002b). Moreover, the slopes of individual regression line of mean amplitudes on sessions 1-4, a measure of acquisition in eyeblink conditioning, was strongly correlated (+0.80) with deep nuclear cell number, supporting the view that loss of deep nuclear cells is likely to be at least one of the causes of impaired learning of eyeblink conditioning in Group EtOH. In these same animals, alcohol also significantly reduced the number of Purkinje cells in the cerebellar cortex (data not shown). Studies funded under Dr. Goodlett's grant AA11945 have also shown that neonatal binge exposure induces functional impairments in neurons of the interpositus nucleus (IP). Single unit activity was recorded in awake rats undergoing delay eyeblink conditioning, from a pair of microelectrodes implanted in the IP ipsilateral to the eyelid that received the US. Behavioral data from rats that underwent paired conditioning and rats that underwent unpaired training closely replicated other studies. In rats that underwent paired conditioning, all groups had low levels of activation of neurons during the CS-US interval in the beginning of training (Session 1) and all groups had high activity during the CS-US interval at the end of acquisition (Session 10). However, the alcohol group showed significantly less activation in the middle sessions of acquisition (e.g., Session 5), compared to both control groups, which both showed a strong "neural model" of the CR by the 4th and 5th session (Green et al., 2002a). These studies indicate that in addition to the significant depletion of neurons in the cerebellar regions essential for ECC, the neurons that are present have a significant deficit in learning-related neural plasticity that correlates with (and likely mediates) behavioral expression of learning.

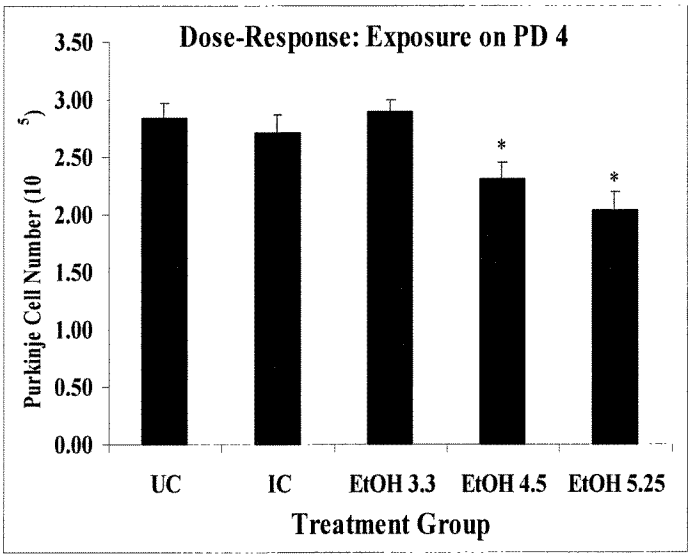


Fig. 10: A single binge exposure to alcohol on PD 4 (2 intubations of alcohol, 2 hours apart) induces dose-dependent loss of Purkinje cells (counted using optical fractionator on PD 10). **UC:** untreated control; **IC:** intubated control (0 g/kg); **EtOH 3.3, 4.5, 5.25:** Groups given alcohol doses of 3.3., 4.5, or 5.25 g/kg per day on PD 4; mean peak BACs were 140, 220, and 290 mg/dl, respectively.

PD 4 Binge Alcohol Exposure Induces Dose-Dependent Purkinje Cell Death and Caspase-3 Activation

We and others have shown that cerebellar Purkinje cells pass through a period of enhanced vulnerability to alcohol-induced cell death (Hamre & West, 1993; Pauli et al., 1995a; Goodlett & Eilers, 1997; Goodlett & Johnson, 1999), with PD 4-6 being the time of greatest susceptibility (e.g., see **Fig. 7**). In a recent study, we have shown that the Purkinje cell loss induced by a single binge episode on PD 4 (2 intubations of alcohol, 2 hours apart) is a linear function of dose (and BAC), as shown in **Fig. 10.**

In an important recent study (Light et al., 2002a), Light and colleagues demonstrated that intubations of a single dose of 6 g/kg of alcohol on PD 4 (mean peak BACs of 501 mg/dl) induced the predicted lobule-specific reduction in Purkinje neurons (labeled by calbindin D28k-immunocytochemistry) 24 hours later. More importantly, the Purkinje cell loss was consistent with induction of apoptosis, demonstrated by fluorescence double-labeling of Purkinje cells with either active caspase-3 immunocytochemistry or with TUNEL labeling. The activation of caspase-3 in Purkinje cells peaked around 8 hours after treatment, and the lobular pattern of activation at 8 hours matched the pattern of Purkinje cell loss 24 hours later. Caspase-3 is a cysteine protease that is considered to play a key role in the cellular execution phase of apoptosis (Slee et al., 199; Porter & Janicke, 1999; D'Mello et al., 2000). This work by Light and colleagues not only demonstrated that high doses of alcohol on PD 4 induced caspase-3-dependent Purkinje cell death, but also showed that similar alcohol treatments on PD 9 did not activate

caspase-3 eight hours later, confirming that PD 4 is part of a temporal window of enhanced vulnerability to

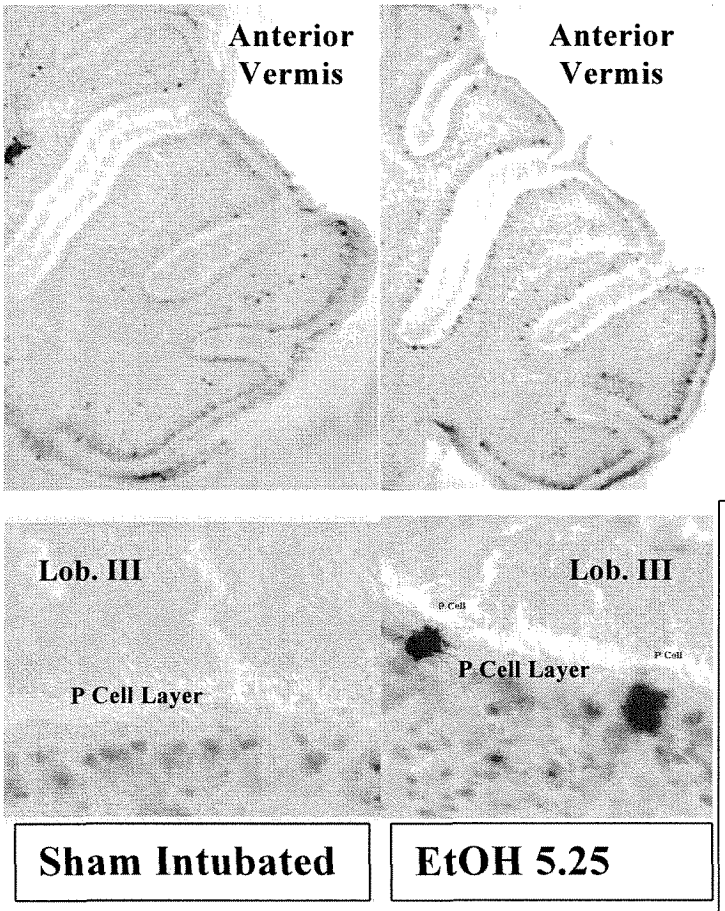


Fig. 11: immunocytochemistry for active caspase-3 (see Appendix Item 1 for enlargement)

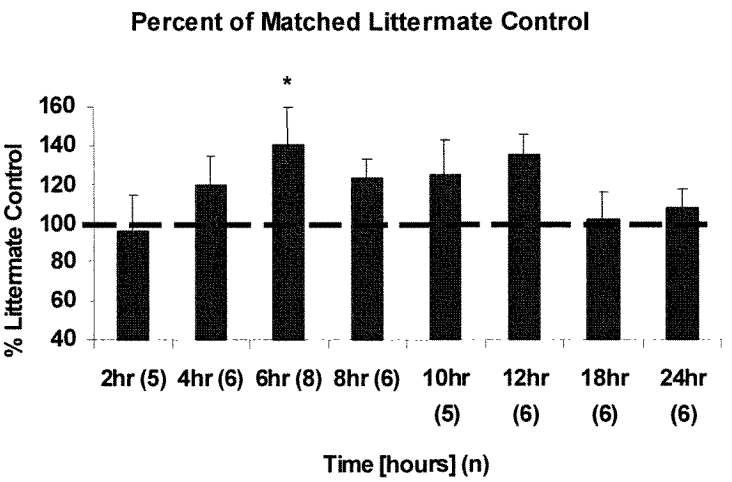


Fig. 11 (left). Immunocytochemical labeling of active caspase-3 in the vermis 8 hours after treatment on PD 4 (Sham: left panels; Alcohol: right panels). Note the high linear density of immunopositive Purkinje neurons in the alcohol-treated pup (dark, large cells in the P cell layer, against the background of some immuopositive granule cells in the superficial portion of the granular layer (just below the P cell layer). The higher magnification (600X) lower panels show that the labeled cells in the Purkinje layer have the morphology of Purkinje cells on PD 4 (note perisomatic processes on the Purkinje cell in the plane of focus EtOH pup (lower right panel of Fi.g. 11.

Fig. 12 (above): Time-dependent activation of caspase-3 activity by 5.25 g/kg of alcohol, administer in 2 intubations, 2 hours apart. The X-axis shows hours after the 1st intubation (and group n). Caspase-3 activity was measured by fluorescence emitted by cleaved substrate protein Ac-DEVD-AMC after 1-hour incubation with protein isolated from cerebellum. Data are expressed as activity of alcohol pup as per cent of littermate control matched by sex and time.

alcohol-induced Purkinje cell apoptosis. This window of vulnerability has now been demonstrated by cell counts (Hamre & West, 1993; Goodlett & Eilers, 1997), by markers of apoptosis (Light et al., 2002a), and by loss of neurotrophin receptors (Light et al., 2002b).

Using a polyclonal antibody raised against the cleaved (active) subunit of caspase-3 (#9661; Cell Signaling Technology, Beverly, MA), we have confirmed the alcohol induces immunolabelling of activated caspase-3 in Purkinje cells 8 hours after the initial alcohol intubation in our 2-intubation model (total dose of 5.25 g/kg; peak BACs between 300-380 mg/dl) (see **Fig. 11**). We also used a fluorescence assay of caspase-3 activity measuring cleavage of 50 μM of caspase substrate peptide, obtained from Bachem). This assay indicated that 5.25 g/kg ethanol treatment induced a significant, time-dependent elevation in substrate cleavage (that could be blocked by inhibitors of caspase-3), and the alcohol-induced increase was significantly above controls at 6 hours post-intubation (see **Fig. 12**). Given the evidence from Light *et al.*, confirmed and extended by our preliminary data, this application proposes to use semi-quantitative analyses of the relative density of Purkinje cells in the cerebellar vermis exhibiting alcohol-induced immunolocalization of active caspase-3, in the presence or absence of candidate therapeutic agents, to serve as an initial screen for the neuroprotective potential of the agent on alcohol-induced cerebellar cell death.

Eyeblink discrimination reversal training in C57BL/6 (B6) mice

As discussed in Section b, higher-order conditioning phenomena are known to require the functional integrity of hippocampal circuits in addition to the cerebellar-brain stem circuits that are essential for all eyeblink conditioning. Experimental lesions of the hippocampus in rabbits and rats selectively impair both trace conditioning and discrimination reversal learning (Berger & Orr, 1983; Port et al., 1986; Solomon et al., 1986; Moyer et al., 1990; Kim et al., 1995; McGlinchey-Berroth et al., 1997; Weiss et al., 1999; Ivkovich & Stanton, 2001). As part of an ongoing project evaluating behavioral correlates of genetic modifications in mice that affect the hippocampal formation, the Stanton lab has developed experimental procedures to test discrimination reversal in B6 mice (see **Fig. 13**, showing acquisition and reversal for B6 mice, the wildtype

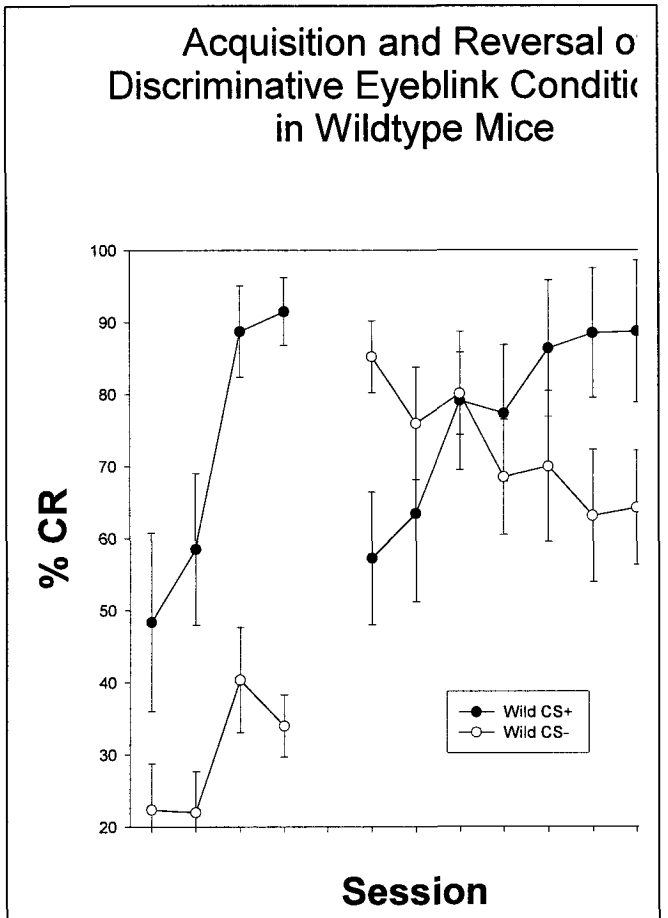


Fig. 13. Discriminative eyeblink conditioning and reversal in normal **adult C57BL/6 mice** (n=8), performed in the Stanton laboratory. Acquisition: 1st four sessions (left); Reversal: Sessions 5-12 (right).

controls in these studies). Mice were trained on a tone-light discrimination according to general procedures described for developing rats by Paczkowski, Ivkovich & Stanton (1999, Exp. 2), except for the following parametric adjustments: 2 sessions/day, tone-CS was 70 dB, periocular-shock US was set at 0.75 mA. During acquisition (left) one stimulus (tone or light, counterbalanced across half the mice, n=4) was paired with the US (CS+, closed symbols) and the alternate stimulus was presented alone (CS-, open symbols). Differential conditioning appeared as clear differences in percentage eyeblink CRs between CS+ and CS- across the four acquisition sessions. During reversal training (right) reinforcement contingencies were reversed, such that the original CS+ was presented alone and the original CS- was paired with the US. Discrimination reversal appeared as an increase in eyeblink CRs to the new CS+ and a decline in eyeblink CRs to the new CS-. These trends were reflected in significant differences in responding to CS+ vs. CS- at the beginning and end of reversal (stimulus x sessions interaction, $p < .01$; Stanton, Caron & Wetsel, 2002).

In those ongoing studies, the genetic modification affecting hippocampal function resulted in normal acquisition of discriminative responding, but (unlike wildtype B6 mice) failed to extinguish responses to the previous CS+ when the contingencies were reversed (data not shown). These procedures developed in B6 mice by Dr. Stanton will be suitable to test our prediction in Aim 3 that the damage to the "higher order" hippocampal circuit (in the absence of damage to the cerebellum) induced by binge exposure on PD 7 in B6 mice will produce selective deficits on ECC reversal learning.

d. RESEARCH DESIGN AND METHODS

Section d is divided into two parts. **Part I** contains the descriptions of each proposed experiment in order by Specific Aim followed by a Potential Pitfalls and Time Requirements for each Specific Aim. **Part II** contains details about methods or procedures described for each experiment of part I. Subjects will be Long-Evans rats and C57BL/6 mice, offspring of timed-pregnant dams purchased from Harlan Labs (see IIa,b). The proposed neonatal binge alcohol treatment in rats (5 g/kg per day on PD 4-9) will be administered using intragastric intubation. For the B6 mice, the binge alcohol treatment (5 g/kg on PD 7) will be given by subcutaneous injection (see IIc). The anticipated **Timetable** showing the timing and sequence of experiments over the five-year period is provided at the end of Part II.

PART I. DESCRIPTION AND DESIGN OF INDIVIDUAL EXPERIMENTS

SPECIFIC AIM 1: EXPERIMENTS TESTING THE HYPOTHESIS THAT THE CANDIDATE THERAPEUTIC AGENTS WILL PROTECT AGAINST ACTIVATION OF CASPASE-3 IN CEREBELLAR PURKINJE CELLS BY ALCOHOL EXPOSURE ON POSTNATAL DAY (PD) 4, A KEY EVENT MEDIATING APOPTOTIC CELL DEATH OF PURKINJE CELLS.

Exp. 1.1: Does L-NAP protect against alcohol-induced activation of caspase-3 in PD 4 Purkinje cells?

Rationale. The work by Light *et al.* (2002), confirmed in our Preliminary Studies, demonstrates that binge alcohol exposure on PD 4 induces active caspase-3 in Purkinje cells that can be detected immunocytochemically and that correlates well with patterns of Purkinje cell loss. This reliable (and dose-related) activation of caspase-3 provides a useful screening tool to assess the potential of candidate therapeutic agents to protect against acute alcohol-induced apoptotic cell death of cerebellar Purkinje cells on

PD 4. Using semi-quantitative analyses of cerebellar Purkinje cells in vermal sections labeled using caspase-3 immunocytochemistry, we can relatively quickly (~4-5 weeks) determine whether L-NAP (or other candidate agent), administered before an acute episode of binge alcohol exposure, reduces the number of Purkinje cells immunopositive for active caspase-3 death, an indicator of Purkinje cells likely destined for apoptotic cell death that is strongly correlated with patterns of Purkinje cell loss in the cerebellar vermis (Light *et al.*, 2002)

Experimental Design & Protocol. A minimum of six litters will be evaluated, testing the protective effects of L-NAP against our 5 g/kg alcohol treatment (see Part IIc). On PD 4, pups will be randomly assigned within litter to one of the following six treatments: Control (Vehicle Injection/Sham Intubation); Alcohol (Vehicle/Alcohol); L-NAP alone (L-NAP High Dose/Sham), and Alcohol combined with one of 3 doses of L-NAP (L-NAP-Low/Alcohol; L-NAP Intermediate/Alcohol; L-NAP High/Alcohol). Approximately equal numbers of males and females will be assigned to each condition, and sexes will be combined because no differences between males and females in the extent of neonatal alcohol-induced Purkinje cell loss have been reported in any of the published studies. The doses of L-NAP we will explore first will be 10ug, 25ug, and 50ug per pup (divided into two injections, each given 30 min before the alcohol), a range comparable (on a g body weight basis) to that found to be effective in protecting against teratogenesis in B6 mice on gestational day 8 (20-40ug per dam). As described in Part IIc, the pups will be first injected subcutaneously with L-NAP (half of the daily dose) or vehicle, then 30 minutes later given the first (of two) intubations (2.5 g/kg alcohol or sham, each intubation). The sequence will be repeated a second time 2 hours later. All pups will be perfused 8-9 hours after the first intubation, and a minimum of six sagittal frozen sections through the vermis will be processed for immunodetection of the active subunit of caspase-3 using streptavidin-biotin perixoidase immunohistochemistry as describe in Part IIe. Additional sections will be processed for double-labeling with fluorescent secondary antibodies to active caspase-3 and calbindin D28k for double-labeling confirmation of the Purkinje cell-specific activation of caspase-3. Using the peroxidase-labeled vermal sections, the number of caspase-3 immunolabeled cells in the Purkinje cell layer (nearly all will be apoptotic Purkinje neurons) will be counted using the 100X-oil objective in the anterior lobules (I-V), and the posterior lobules (VIII-X), the vermal regions of greatest PD 4 alcohol-induced Purkinje cell loss (Goodlett et al., 1990; Pierce et al., 1993; Light et al., 2002a). For each section, counts will be converted into linear densities (within each regions) by dividing the number of positive cells by the length of the Purkinje cell line for each respective region (anterior; posterior).

Analysis and Interpretation. For each subject, the linear densities will be averaged within region across all 6 sections per subject, and the two regions will be analyzed separately with one-way analyses of variance (ANOVA). Significant, large increases in linear densities of labeled Purkinje cells in the group given alcohol (vs. controls) are expected. Neuroprotective effects of L-NAP will be determined by planned comparisons between each L-NAP-alcohol dose group and the alcohol-alone group, with protection indicated by a significant reduction in the linear density of labeled Purkinje cells in either (or both) vermal regions. Differences between L-NAP alone and controls will also be assessed.

Exp. 1.2: Do structural derivatives of L-NAP that differ in their protective profiles in other models differentially protect against alcohol-induced activation of caspase-3 in PD 4 Purkinje cells?

Recent site-directed mutagenesis work by Charness and colleagues indicates that the I6A-NAP peptide fails to show antagonism of ethanol's disruption of L1 or of mouse teratogenesis, but does retain its neuroprotection against TTX-induced cell death. In contrast, the P7A-NAP peptide retains most of its ethanol antagonism capacity but loses its neuroprotective effect on TTX cell death. These two structural derivatives of L-NAP could be very informative about the mechanism of neuroprotection in a variety of models, in the former case showing correlation with effects mediated by L1 and in the latter showing correlation with neuroprotection or reduction of oxidative stress. These two compounds will also be tested in our PD 4 Purkinje cell caspase-3 activation model, to determine whether either is effective in limiting alcohol-induced Purkinje cell apoptosis.

Experiment 1.3: Do other novel candidate compounds protect against alcohol-induced activation of caspase-3 in PD 4 Purkinje cells?

Although the focus of the proposed studies in this component and across the entire Basic Science Core is on the neuroprotective peptides (mainly L-NAP), we expect that other candidate compounds will emerge over time as this Consortium project moves forward. Some of these may come from work within the Consortium, others may come from research from other investigators or from neuroprotection studies in other models of neuronal death. On the assumption that other candidates will continue to emerge, we include this

experiment to indicate that some promising candidates will be screened for their capacity to limit or prevent alcohol-induced activation of caspase-3 in Purkinje cells on PD 4. These studies will proceed as described for Exp. 1.1. We expect to assess at least one novel compound in Year 02 and others in Year 04 of the project (see Timeline at the end of Part II).

Potential Pitfalls: We recognize that the semi-quantitative measures of linear density of putative Purkinje cells immunolabeled for caspase-3-like immunoreactivity will not provide definitive data about the extent or mechanism of action of protection against alcohol-induced Purkinje cell death. For example, we will not be able to ascertain whether caspase-3 positive cells reflect quantitatively the size of the population of Purkinje cells destined to die, nor can we know how much of the Purkinje cell population is actually lost or what proportion is protected. We also do not make any assumptions about cell loss that may be delayed. This Aim is intended only as a means to screen candidate agents in this acute *in vivo* model for potential effectiveness in protecting the cerebellum. If no protection is observed with the dose and schedule of L-NAP treatment, we will pursue additional doses, schedules or structural variants of activity-dependent neuroprotective peptides (ADNP/ADNF), using the caspase-3 activity screen to try to identify more effective regimens. Positive results will mean that the agent will be moved on to Aims 2 and 3. Negative results won't necessarily preclude further study (as in the case of L-NAP), because the candidate agents may have protective potential that extends beyond what we can detect with these measures of caspase-3 activation in the PD 4 rat cerebellum. These studies are relatively efficient and require relatively little investment of time and resources (compared to the eyeblink studies, for example) and for that reason will be used to screen various candidate agents before deciding to move them forward in the more labor-intensive studies in neonatal rodents.

Time Requirements for Aim #1. The initial study (Exp. 1.1, n=36) screening L-NAP effects on activation of caspase-3 in Purkinje cells on PD 4 will require about 2 months to complete, as will the subsequent screening study of neuroprotection candidates (see Timeline at the end of Part II). L-NAP and its derivatives will be tested in the first year; other novel agents can be tested in the last half of Years 02 and 04.

SPECIFIC AIM 2: EXPERIMENTS TESTING THE HYPOTHESIS THAT THE CANDIDATE THERAPEUTIC AGENTS WILL PROTECT AGAINST CEREBELLAR CELL LOSS AND ASSOCIATED EYEBLINK CLASSICAL CONDITIONING DEFICITS INDUCED BY ALCOHOL EXPOSURE ON PD 4-9.

Exp. 2.1: Does L-NAP protect against deficits in delay eyeblink classical conditioning and associated cerebellar damage induced by binge alcohol exposure on PD 4-9 in neonatal rats?

Rationale. Our ongoing studies using the neonatal rat model have documented that relatively high levels of alcohol exposure on PD4-9 cause deficits in eyeblink classical conditioning that are consistently evident in weanling rats (see Preliminary Studies) and in adults (Green et al., 2000), and are associated with loss of cerebellar neuronal populations and deficits in conditioning-related neuronal unit activity in the interpositus nucleus (Green et al., 2002a; Green et al., 2002b). Given the critical involvement of the cerebellum in the acquisition and performance of eyeblink CRs, these ECC deficits produced in the neonatal rat model provide an ideal means to test the potential of L-NAP and its structural derivatives and other candidate therapeutic agents (see Background & Significance), to protect against neonatal binge alcohol-induced damage to cerebellar systems and resulting deficits in acquisition of eyeblink conditioning.

Experimental Design & Protocol. Exp. 2.1 will use a 2 X 2 factorial, split-litter design with PD 4-9 L-NAP Treatment [L-NAP injection; Vehicle Injection] and PD 4-9 Alcohol Treatment [Alcohol Intubation (5 g/kg/day); Sham Intubation] as factors. Littermates (4 male; 4 females) will be randomly assigned by sex to each of the 4 treatment combinations (n=12 per group, minimum of 12 litters). The dose of L-NAP will be chosen after the results of Exp. 1.1 are known, but likely will range between 10-50 ug per pup per day. L-NAP or vehicle will be administered in two subcutaneous injections, given 30 minutes before the each intubation each day. The alcohol will be administered by gavage (in milk formula) on PND 4-9 (see IIc), and produces mean peak blood alcohol concentrations (BACs) around 300-350 mg/dl. Peak BACs will be estimated from a tail blood sample taken 2 hours after the second alcohol intubation on PND 4 (see IId). An additional Normal Control (NC) group, derived from 12 other litters (1 male, 1 female per litter) that are reared normally and are neither treated nor handled on PD 4-9, will provide a comparison group for the effects of the intubation/injection treatments.

The rats will be weaned on PD 21, undergo surgery to implant eyelid recording and periorbital stimulating electrodes on PD 24 (see Part IIf), and begin ECC training on short-delay conditioning (280 msec ISI) on PD

26 (see Part IIg). Sessions will be given three consecutive days, twice a day approximately 5 hours apart. Each session will have 100 trials, 90 paired presentations of the CS (tone) and US (mild periorbital shock), and 10 CS-alone trials. At the end of training, all rats will be perfused and the cerebella collected for subsequent counts of Purkinje cells and interpositus nucleus [IP] neurons described in Exp. 2.2 below.

Analysis and Interpretation. Several measures of learning (CR percentage; amplitude, onset latency, peak latency) and performance (UR latency and amplitude) will be subjected to mixed design ANOVAs. Data will be averaged within session (the trial-blocks factor will also be examined preliminarily but we expect to pool data across this factor). UR measures are expected to be unaffected by alcohol. Based on previous findings, CR acquisition will be impaired by alcohol. The effectiveness of the candidate agent will be determined in a 2-step process. First, measures of learning and performance of the NC and the Vehicle/Sham (V/S) control groups will be compared, with Group (NC or V/S) and Sex as grouping factors and Session as a repeated measure. If the two groups do not differ significantly, they will be combined into a single Control group; if they do differ, then only the V/S group will enter into the analyses of alcohol and L-NAP effects, and any differences between NC and V/S controls will be interpreted with appropriate caution. The treatment effects will be analyzed with 2 (L-NAP; Vehicle) X 2 (Alcohol; Control) X 2 (Sex) X 6 (Session) mixed ANOVAs. A significant main effect of Alcohol and an Alcohol X Session interaction are expected, as reported in our previous studies. If L-NAP imparts protection against ECC deficits, the acquisition curve of the L-NAP/Alcohol group would approach the controls, and should be faster and reach higher terminal performance than the Vehicle/Alcohol group, yielding an Alcohol X L-NAP interaction (perhaps with Session, as well). Any deleterious effects of neonatal L-NAP treatment alone on ECC will be assessed by comparison of the L-NAP/Sham group with Controls. One advantage of using ECC to evaluate functional neuroprotection of cerebellar neural systems it that even a relatively modest protection against cerebellar cell loss (e.g., from ~40% loss to 30% loss) should be sufficient to normalize acquisition of standard delay conditioning, given our previous dose-response findings that alcohol treatments that produce 25-30% Purkinje cell loss do not produce significant acquisition deficits in ECC (see **Fig. 5**).

Exp. 2.2: Does L-NAP protect against cerebellar cell loss induced by neonatal binge alcohol exposure? Does cerebellar structural neuroprotection correlate with eyeblink conditioning?

Rationale. As shown in the Preliminary Studies, heavy binge alcohol exposure on PD 4-9 caused deficits in eyeblink classical conditioning that were associated with neuronal loss in the cerebellum, and was highly correlated with neuron numbers in the deep cerebellar nuclei. The purpose of this experiment is to determine whether the L-NAP treatments of rats tested on eyeblink conditioning as juveniles also protects against neuronal loss in specific cerebellar populations of the essential circuit mediating acquisition and timing of eyeblink CRs—anterior and HVI lobules of cerebellar cortex and the IP nucleus. These neuroanatomical data will provide complementary structural data to ascertain the type and extent of cerebellar neuroprotection.

Experimental Design & Protocol. Experiment 2.2 will perform cell counts on cerebella of a subset of rats of each treatment group (n=8 per group). Total numbers of Purkinje cells of Lobule HVI and of anterior hemisphere lobules and total numbers of IP neurons will be counted on the side ipsilateral to the trained eye, using the optical fractionator (see Part IIi), by experimenters blind to group membership.

Analysis and Interpretation. Total cell numbers (coefficients of error typically <10%) for each neuronal population will be analyzed with planned comparisons testing three *a priori* hypotheses. For these planned comparisons, the error term will be the within-group mean square error of the 1-way ANOVA, presuming that the within-group variance for each population does not violate the homogeneity assumption. The first comparison will test the prediction that alcohol induces significant cell loss (in both populations), comparing the Vehicle/Alcohol group to the Vehicle/Sham Controls. The second comparison will test the prediction that L-NAP significantly prevents cell loss in each population by comparing the L-NAP/Alcohol to Vehicle/Alcohol group. The third comparison will assess whether L-NAP has any untoward effect by comparing the L-NAP/Sham group to the Vehicle/Sham group. In addition, we will compare Vehicle/Sham group with Normal Controls, to assess any differences between treated and untreated controls (none is expected). We also will determine whether there is a significant correlation between acquisition rate of ECC and cell numbers in the IP or Purkinje cell numbers. We previously found a strong correlation between CR acquisition rate and the number of neurons in the deep cerebellar nuclei (Green et al., 2002b). We expect the rate of CR acquisition, defined for each rat by the slope of the regression line of mean daily CR amplitudes over sessions 1 to 4 in Exp. 2.1, will be significantly correlated with the number of IP neurons (using rats from all 5 groups for which both ECC and cell counts are available). Similarly, correlational analyses will also be performed using the

Purkinje cell counts from anterior and HVI lobules, which likely are correlated both with IP counts and ECC acquisition.

Exp. 2.1a and 2.2a: Do structural derivatives of L-NAP that differ in their protective profiles in other models differentially protect against deficits in delay eyeblink classical conditioning and associated cerebellar damage induced by binge alcohol exposure on PD 4-9 in neonatal rats?

If positive results of Purkinje Cell neuroprotection are found with L-NAP or the derivatives (I6A-NAP; P7A-NAP) in Aim 1, then studies of either of the two derivatives will proceed. These derivatives are potentially quite informative, because the I6A-NAP does not show antagonism of ethanol's disruption of L1 or mouse teratogenesis (but does retain its neuroprotection against TTX-induced cell death), whereas the P7A-NAP retains most of its ethanol antagonism capacity but loses its neuroprotective effect on TTX cell death. If justified by outcomes of Aim 1 (or other studies within the Consortium), then these two compounds will be tested as described for L-NAP in Exp. 2.1 and 2.2. *Other candidate agents* may emerge as well over the course of this Consortium, and they can be tested, shown as Exps. 2.1b and 2.2b on the Timeline.

Exp. 2.3: Is the cerebellar neuroprotection identified in juvenile rats for a given candidate agent also evident in rats tested as adults? Does the protection extend both to CR acquisition of conditioning and to plasticity of CR timing when the ISI is shifted—a behavioral assay of cerebellar cortical function that is more sensitive than simple acquisition? Any candidate therapeutic agent found to have significant neuroprotective effects in Exps. 2.1 or 2.2 will be tested further by determining whether protective effects can be confirmed in adult subjects. In this study, not only will we evaluate acquisition of standard delay eyeblink conditioning, but we also will extend the analysis to assess regulation of CR timing by shifting the ISI to a new (longer) interval. This ISI shift manipulation with eyeblink conditioning appears especially sensitive to cerebellar cortical damage (Perrett *et al.*, 1993) making it ideal as a sensitive indicator of the extent of functional protection of cerebellar cortical circuits.

Rationale. Exp. 2.3 will be dependent on positive outcomes of a candidate agent in the juvenile studies of Exp. 2.1 or 2.2. If either study indicates functional or structural protection, the adult study will be pursued. The adult study will also add the ISI shift manipulation (after initial training on short-delay ECC), to assess the ability of the rats to regulate the timing of the CR after the ISI shift. CR timing is a specific feature of conditioned responding that appears to critically engage cerebellar cortex. Subjects trained at one ISI and then shifted to a second ISI typically show plasticity in CR timing after the ISI shifts. The time of the peak CR amplitude comes to approximate the time of the US onset at the new ISI. With the *short→long* ISI-shift procedure proposed in this study, the peak CR latency, which approximates the time of US-onset during short-delay acquisition (280 msec), typically shifts to approximate the time of US-onset at the longer ISI (880 msec). In studies of developmental hypothyroidism, another insult that targets the cerebellum, the ISI shift uncovered significant behavioral impairments in juvenile rats that were not evident during short-delay conditioning (Erwin et al., 2000). We reason that the ISI-shift manipulation will also be more sensitive than acquisition of short-delay conditioning for detecting alcohol-induced cerebellar damage and dysfunction, and therefore more capable of indicating the relative completeness of the extent of cerebellar cortical protection by the candidate agents. Exp. 2.3 will use those established ISI-shift procedures to assess the extent of protection on acquisition and timing of eyeblink CRs in adulthood.

Experimental Design & Protocol. Exp. 2.3 will have two grouping factors: Alcohol treatment (5.0 g/kg Alcohol; Sham Intubation) and Therapeutic Agent (Drug; Vehicle). A separate normal control group not given any neonatal treatments will also be included for comparison. There will be 12 rats of each sex per treatment condition. Although our studies of alcohol effects on ECC have not observed significant sex differences, n=12 should yield sufficient power to detect any Sex X Candidate Agent interactions of medium effect size (Cohen, 1988). The protocols for delivering alcohol and L-NAP, determining BACs, and assigning littermates to experimental groups will be exactly as described in Exp. 2.1 and Part IIc. The design and protocol for eyeblink conditioning are also as described in Exp. 2.1, except that after acquisition of short-delay training, all rats will be given subsequent long-delay training, imposing the *short→long* ISI shift manipulation. Acquisition of short-delay training will consist of six 100-trial sessions of training at the 280 msec ISI (over 3 days), followed by an additional six sessions of training at the 880 msec ISI, 2 sessions per day (total of 6 consecutive training days) At the end of training, all rats will be perfused and the cerebella collected, and counts of Purkinje cell counts and IP neurons will be performed on a subset of 8 rats of each treatment group.

Analysis and Interpretation. Measures of learning (CR percentage; amplitude, onset latency, peak latency) and performance (UR latency and amplitude) will be subjected to separate mixed ANOVAs for acquisition and post-shift phase. Data will be averaged within session (the trial-blocks factor will also be examined preliminarily but we expect to pool data across this factor). As in our previous studies, we expect UR measures to be unaffected by alcohol. Based on previous findings, CR acquisition during the *initial training phase* is expected to be impaired by alcohol, but that the alcohol group should show CRs on about 50% of the trials by the end of training. New information about the effect of alcohol will emerge from the *post-shift phase*. We expect both control groups to continue to perform CRs at a high level and adjust the CR peak latency to the new ISI, whereas the alcohol group is expected to continue to perform poorly, and show few if any properly-timed CRs at the long-delay interval. Performance of the alcohol group given the candidate agent is expected to confirm any neuroprotective effect on acquisition observed in the previous study in juveniles. More importantly, the post-shift perform will indicate whether the agent also protects against deficits in plasticity of CR timing, effects that are especially sensitive to damage to the cerebellar cortex. If the neuroprotective effect is sufficient to prevent acquisition deficits but the protection is still incomplete, then the group given the candidate agent will show impairments during the post-shift phase in the rate of change (and relative distribution over the ISI) of CR onset or peak latency--the primary dependent measures of CR timing. If no deficits in adjusting CR timing are observed in the group given the candidate agent, then we will conclude that the protection extends to the precise timing functions regulated by the cerebellum. Cell counts will be analyzed as in Exp. 2.2; correlational analyses will be performed separately for the acquisition and post-shift phases.

Potential Pitfalls for Aim #2: Based on data we have collected to date, we are confident that alcohol will induce relatively large deficits in CR acquisition. Existing data also assure that there will be an alcohol-induced loss of Purkinje cells and IP neurons (expected to be $\geq$ 40%), and loss in the two regions may be correlated due to their common anatomy and development. Although these studies should clearly indicated, one way or the other, whether or not L-NAP (or its derivatives) can protect against these effects, it is unknown whether these compounds will protect these cerebellar systems. If no protection is observed with the first dose and schedule of L-NAP treatment, we may use the caspase-3 activity screen of Aim 1 to try to identify more effective regimens. If none are found, we will then explore other candidate compounds, including other compounds that inhibit oxidative stress or that inhibit caspase-3. One other potential pitfall is that the neuroprotective agents may protect against one measure (e.g., ECC) but not another (e.g., cell loss in one of the cerebellar populations). In part, such an outcome may simply indicate that the functional status of the cerebellar circuit may not be predicted by the complement of cell numbers in the circuit. Nevertheless, protection observed in either measure (behavioral; neuroanatomical) will have general importance, even if the two outcomes cannot be linked within the set of studies proposed. For example, if protection of Purkinje cells but not IP neurons is observed, deficits in ECC may remain because the IP is critical for this learning. Nevertheless, significant protection against either neuronal population will be considered an outcome that encourages further study.

Time Requirements for Aim #2. The initial study (Exp. 2.1, n=120) of L-NAP effects on juvenile ECC acquisition will require about a year to complete, and the follow-up cerebellar cell counts (Exp. 2.2, n=40) will take about 3 months to complete (see Timeline at the end of Part II). Studies testing effects of derivatives of L-NAP or other candidate agents (labeled Exp. 2.1a,b and Exp. 2.2a,b in Timetable) will progress on approximately the same time scale for the juvenile outcomes. The adult study proposed to confirm neuroprotective effects (Exp. 2.3) will take about 14 months to complete the ECC and cell counts. It is proposed for year 03 of the Project, but that will depend on the outcomes of the juvenile studies.

SPECIFIC AIM 3: EXPERIMENTS TESTING THE HYPOTHESIS THAT THE CELL DEATH IN HIPPOCAMPAL CIRCUITS INDUCED BY BINGE ALCOHOL EXPOSURE ON PD 7 IN C57BL/6 MICE (CHARACTERIZED IN AIM 2 OF ZHOU'S MOUSE COMPONENT) WILL RESULT IN DEFICITS IN HIPPOCAMPAL-DEPENDENT LEARNING TASKS.

Exp. 3.1: Does binge alcohol on PD 7 in C57BL/6 mice induce selective deficits in reversal of discriminative eyeblink conditioning?

Rationale. The purpose of this experiment is to determine whether the cell death in hippocampus and subiculum induced in B6 mice by a single binge exposure to alcohol on PD 7 is sufficient to disrupt an ECC procedural variant that is known to require hippocampal interactions with the essential cerebellar-brain stem

circuit. Of the several different ECC higher-order conditioning procedures that are known to depend on this additional hippocampal engagement (Schmajuk & DiCarlo, 1991), we have chosen to use discrimination reversal, which is one that is disrupted by hippocampal damage (Berger & Orr, 1983; Schmajuk & DiCarlo, 1991). In discrimination training, acquisition sessions include trials with paired presentations of one CS (e.g., light or tone) with the US (CS+ trials), and other trials in which the alternative CS (tone or light) is presented but not followed by the US (CS- trials). After discrimination is acquired (CRs on the CS+ trials but not the CS- trials), the contingencies are then reversed (former CS+ becomes CS-, and vice versa; see **Fig. 13**, Preliminary Studies). We have chosen to use discrimination reversal because it offers several unique advantages that make it optimal for the goals of this Aim. It can efficiently assess the associative basis for acquisition of the initial discrimination learning (which does not require an intact hippocampus), because it includes a "built in" nonassociative control (CS-) for the effects of alcohol. It also does not require the use of long interstimulus intervals (as trace conditioning does), which can cause interpretive difficulties arising from performance factors inherent in sub-optimal ISIs. Finally, discrimination reversal is a within-subjects effect that does not require multiple training control groups, an important issue when considering the multiple treatment groups needed for the neuroprotection studies. The presence or absence of impairments in discrimination reversal learning may therefore help to characterize whether the PD 7 binge alcohol treatments in B6 mice induce functional impairments in learning and memory tasks that are specific for hippocampal-cerebellar interactions. The training is based on a tone-light discrimination procedure developed originally in weanling rats (Paczkowski et al., 1999) but with parametric adjustments that are more effective for mice (See Background & Significance). After four acquisition sessions, the CS contingencies are switched for eight reversal sessions. In this study, we will examine the effect of a single episode of binge alcohol exposure in B6 mice on PD 7 (5 g/kg), testing the prediction that the treatment will not impair acquisition of discriminative eyeblink conditioning but will significantly impair learning when the contingencies are reversed.

Experimental Design & Protocol. The B6 mice (see Part IIb) will be given PD 7 treatments (Ethanol, Saline Control, or Untreated Control) at IUPUI (see Part IIc), and blood alcohol concentrations will be sampled 2 hours after the 2nd injection (see Part IId). At ~60 days of age, the mice will be shipped overnight to the University of Delaware and housed in a quarantine facility for the duration of the experiment. About 5 days after arrival, the mice will undergo surgery to implant stimulating and recording electrodes for eyeblink conditioning. One week later, discrimination training will ensue (see Part IIg) The design of Exp. 3.1 will involve two grouping factors, PD 7 Treatment (Alcohol, Saline Control, Untreated Control) and Sex [male, female], with n=16 per group. CS Modality (Tone+/Light- vs. Light+/Tone-) will be a counterbalanced subgrouping factor (n=8) that we will examine, but we expect to pool data across this factor. Within-subject factors are stimulus (CS+ vs. CS-) and sessions (acquisition and reversal phases will be analyzed separately). The ECC training will use the same rodent eyeblink system (JSA Designs) used for the rat studies and in our preliminary studies in B6 mice (**Fig. 13**). The mice will receive 12 total training sessions, 2 sessions/day for 6 consecutive days. The first 4 sessions will be acquisition sessions; the last 8 will be reversal sessions.

Analysis and Interpretation. Similar to the previously described studies in rats, dependent measures of learning (CR frequency and amplitude) and performance (UR frequency and amplitude) will be analyzed, along with the other measures available (startle responses; CR onset and peak latency). The data for each mouse will be averaged within each sessions, then analyzed with separate mixed design ANOVAs for acquisition [3 (treatment) x 2 (sex) x 2 (stimulus type) x 4 (sessions)] and reversal [involving 8 sessions]. Based on published findings (Berger & Orr, 1983; Stanton & Goodlett, 1998; Paczkowski et al., 1999) and our preliminary studies with B6 mice (see **Fig. 13**), we expect that clear discriminative conditioning, i.e., robust CRs to CS+ but not CS-, will emerge during acquisition for all treatment groups, since the PD 7 alcohol treatment is not expected to damage the essential cerebellar circuits sufficiently to impair basic delay conditioning. During the reversal phase, we expect the Saline and Untreated Control mice to acquire CRs to former CS- and extinguish CRs to former CS+ such that clear divergence of responding reflecting reversed contingencies will be evident over the 8 reversal sessions. We also predict that, during reversal, the Alcohol group will acquire CRs to former CS- at a normal rate. However, they will show profound difficulty extinguishing CRs to the former CS+ (Berger & Orr, 1983). We have observed precisely this pattern of results in studies of genetically modified mice with forebrain dysfunction that were tested on this discrimination reversal task (Stanton et al., 2002). Such a reversal deficit would be confirmed statistically by significant interactions of treatment dose x stimulus, and/or treatment dose x stimulus x sessions in the ANOVA on the reversal session. Such an outcome would provide the first evidence that cell death in the hippocampal formation and other forebrain regions induced by a

single binge exposure on PD 7 in B6 mice is sufficient to disrupt hippocampal memory systems that interact with cerebellar substrates of eyeblink conditioning.

Exp. 3.2: Can selective deficits in ECC discrimination reversal induced by binge alcohol on PD 7 in B6 mice be prevented by pretreatment with L-NAP?

Rationale. In keeping with the collaborative goals of this consortium, we will assess L-NAP (or one of the other candidate neuroprotective peptides) for effectiveness in preventing or limiting structural and functional damage to hippocampal circuits that mediate discrimination reversal of ECC. We propose in this application to use L-NAP, but in practice that will depend on positive outcomes derived from Dr. Zhou's studies in Aim 2 of the Mouse component. If another peptide is found to be more effective in preventing alcohol-induced activation of caspase-3 in the hippocampus, subiculum, and retrosplenial cortex, then that compound will be used. If no peptide has been identified as being effective by the time this experiment is planned to begin, then we will start with anti-oxidant compounds (e.g., vitamin E), which have been found to be effective in several models of alcohol-induced cell death (see Background and Significance).

Experimental Design & Protocol. Exp. 3.2 will have three grouping factors: PD 7 Alcohol Treatment (5.0 g/kg Alcohol; Sham Intubation) and Therapeutic Agent (Drug; Vehicle). Separate groups of normal controls (both sexes) not given any neonatal treatments will also be included for comparison. There will be 12 mice of each sex per treatment condition (total n=120). Although our studies in rats of alcohol effects on ECC have not observed significant sex differences, n=12 should yield sufficient power to detect any sex differences in effects of the candidate agents. The protocols for delivering different alcohol doses, determining BACs, and assigning littermates to experimental groups will be exactly as described in Exp. 3.1 and Part IIc. L-NAP will be given in 2 injections, each 30 min before the alcohol/saline injection, as described in Part IIc. The general protocols for eyeblink conditioning are also as described in Exp. 1.1, and acquisition and reversal of discrimination training will follow procedures described for Exp. 3.1. At the end of training, all rats will be perfused and the brains collected, and cerebellar Purkinje cells and IP neurons, and neurons in hippocampal fields CA1, CA3 and the subiculum will be counted (see Exp. 3.3).

Analysis and Interpretation. ECC data analysis will follow that of Exp. 3.1, except that we will follow a 2-step process in subjecting the data to analyses of variance. In the first step, only the normal controls and the Vehicle/Sham (V/S)-treated controls will be analyzed, using separate mixed design ANOVAs for acquisition [2 (group) x 2 (sex) x 2 (stimulus type) x 4 (sessions)] and reversal [involving 8 sessions]. CS Modality (Tone+/Light- vs. Light+/Tone-) will be a counterbalanced subgrouping factor (n=6) that we will examine, but we expect to pool data across this factor. If there are significant main or interactive effects of group (i.e., if V/S group differs from Normals), then we will only use the V/S group in the subsequent ANOVAs of L-NAP effects. If no differences between Normal and V/S controls are seen, then their data will be combined into a single Control group. The L-NAP effects will be analyzed with separate mixed design ANOVAs for acquisition and reversal, with Alcohol Treatment (Alcohol, Control), L-NAP Treatment (L-NAP, Vehicle), and Sex as grouping factors, and Stimulus Type (CS+, CS-), Sessions (for acquisition; 8 for reversal) as within-subjects factors (pooling across CS modality). The Vehicle/Alcohol group should show profound difficulty extinguishing CRs to the former CS+. L-NAP protection would be indicated by extinction of CRs to the former CS+ that is comparable to the controls. Such protection of reversal learning deficit would be confirmed statistically by significant interactions of Alcohol X L-NAP x Stimulus Type in the ANOVA on the reversal session.

Exp. 3.3: Does L-NAP protect against hippocampal cell loss induced in B6 mice by PD 7 exposure? Does hippocampal structural neuroprotection correlate with protection of discrimination reversal performance?

Rationale. As discussed in the Background and Significance, the 5 g/kg binge exposure on PD 7 in B6 mice causes extensive apoptotic neuronal death in many forebrain regions, including the hippocampus, subiculum, and retrosplenial cortex. The purpose of this experiment is to determine whether the L-NAP treatments of mice tested on discrimination reversal ECC protects against neuronal loss in hippocampal and subiculum, populations that are part of the hippocampal circuit necessary for discrimination reversal. These neuroanatomical data will provide complementary structural data to ascertain the type and extent of hippocampal neuroprotection afforded by L-NAP.

Experimental Design & Protocol. Exp. 3.3 will perform cell counts on the hippocampi and cerebellum of mice from a subset of each treatment group (n=8 per group), by experimenters blind to group membership. Total numbers of Purkinje neurons in anterior and HVI lobules, neurons of the IP nucleus, pyramidal neurons in

CA1 and CA3, and neurons in the subiculum will be counted (all ipsilateral to the trained eye) using the optical fractionator (see Part IIi).

Analysis and Interpretation. Total cell numbers (coefficients of error typically <10%) for each neuronal population will be analyzed with planned comparisons testing several *a priori* hypotheses. For these planned comparisons, the error term will be the within-group mean square error of the 1-way ANOVA, presuming that within-group variance for each population does not violate the homogeneity assumption. First, no significant cell loss is expected for either cerebellar population, and the ANOVA on those populations should be non-significant. For the hippocampal formation, the first comparison will test the prediction that alcohol induces significant cell loss (in each population), comparing the Vehicle/Alcohol group to the Vehicle/Sham controls. The second comparison will test the prediction that L-NAP significantly prevents cell loss in each population by comparing the L-NAP/Alcohol to Vehicle/Alcohol group. The third comparison will assess whether L-NAP has any untoward effect by comparing the L-NAP/Sham group to the Vehicle/Sham group. In addition, although we do not expect significant effects of the treatment control conditions relative to normal controls, we also will test those two groups to assess any differences. We also will determine whether there is a significant correlation between reversal performance (rate of extinction of CRs to the previous CS+) with cell numbers in the hippocampal regions.

Potential Pitfalls for Specific Aim #3. Most of the experiments in this aim are direct extensions of procedures and logic from existing experiments that are clearly feasible and should yield an answer, one way or the other, concerning the major hypotheses of this Project. We know from the published studies of Olney and colleagues that the proposed alcohol exposure in B6 mice (the focus of Aim 2 of Zhou's mouse component of this Consortium) severely damages several regions of the circuit mediating higher-order conditioning phenomena, including the hippocampus, subiculum, and retrosplenial cortex. We have an effective protocol for studying discrimination reversal in these mice (see Preliminary Studies). We also know that this discrimination reversal procedure is sensitive to hippocampal damage in rabbits and humans and to developmental forebrain anomalies in mice (Stanton et al., 2002). Although it has not yet been empirically confirmed that hippocampal damage in mice also produces deficits in eyeblink discrimination reversal, the likelihood that the robust effects documented in other species will not generalize to mice seems rather remote. We also do not know whether alcohol-induced hippocampal damage in these mice will be sufficiently severe to produce the predicted reversal deficits. But this cannot be known without performing the proposed experiments. If the 5 g/kg dose of alcohol on PD 7 fails to induce deficits on discrimination reversal, we will then perform a follow-up experiment in which alcohol will be administered on 3 consecutive days (PD 7-9), a dosing regimen in rats which we have shown does result in deficits on hippocampal dependent learning task (Johnson & Goodlett, 2002). Thus, we may find that it is necessary to adjust experimental parameters (e.g., number of reversal training sessions, doses of alcohol or of L-NAP) to optimally demonstrate and characterize our predicted outcomes, but such parametric adjustments are readily accomplished. If none of these experimental modifications yields significant effects in ECC in Exp. 3.1, we with then test similarly- treated B6 mice on place learning in the Morris water task, which has been reported by Olney's group to reveal deficits in B6 mice after this PD 7 binge alcohol exposure (Dikranian et al., 2001; Olney et al., 2002a; Olney et al., 2002b). Taking together the existing empirical basis for pursuing Aim 3, it is difficult to imagine that useful new knowledge relevant to the overarching goals of this Consortium will not be forthcoming.

Time Requirements for Specific Aim #3. The first experiment characterizing alcohol-induced effects (Year 03 of the project, to take advantage of progress made in Zhou's mouse component of this Consortium), will involve about 96 mice, 4 replications of 24 mice each, with each replication requiring about 3 weeks to complete (after the mice are shipped). The high-throughput resource in the Stanton lab routinely completes testing on 32 rodents per week. By staggering the 4 replications by a few months (to accommodate dosing and data analysis schedules), these experiments will take about a year each to complete. The experiments testing L-NAP, then other candidate therapeutic agents, will occur over years 04-05 of the project. The cell count analysis of hippocampal neurons (Exp. 3.3) will be performed on mice from Exp. 3.2 (the L-NAP behavioral study). We have chosen to by-pass the labor-intensive cell counting of mice in Exp. 3.1, since our primary goal of Exp. 3.1 is to determine whether specific deficits in reversal learning are evident. If confirmed in Exp. 3.1, then the links between hippocampal cell loss and discrimination reversal learning can be established in the neuroprotection phase, which of course will have alcohol-treated groups either given vehicle or L-NAP. These cell count studies will use at least 10 mice from each the five treatment groups of Exp. 3.2 (5

male; 5 female, selected at random) 6 months to complete (about 2 hippocampal counts per week) once the brains are received from the Stanton lab.

PART II. DETAILS OF PROCEDURES AND METHODS (IN ORDER OF APPEARANCE IN PART I)

a. Long-Evans Rat Subjects and Breeders. Offspring of timed pregnant LE dams purchased from Harlan Laboratories (Indianapolis, IN) will be used. LE rats are a robust heterogeneous stock (Curley & Pelas, 1969; Lindsey, 1979), well suited for behavioral studies (Tonkiss et al., 1992). Pregnant dams will be purchased (rather than bred) for efficient, economic, and uniform animal procurement. Rats will be express transported to IUPUI with food/water on embryonic day (E) 10. Dams will be housed singly immediately upon arrival in a dedicated nursery (12 hr:12 hr light/dark cycle, lights on 0700 hr), with ad libitum chow and water. Cohorts of 6-10 pregnant dams for each delivery will be produced at Harlan by overnight mating; a sperm plug the next morning will designate E0 (Paxinos et al., 1994). Births will be monitored 3 times daily from E20 on; >80% occur between the evening of E21 and noon of E22. E22 is designated postnatal day (PD) 0, regardless of E day of birth. The age at the time of treatment is defined by E day (i.e., PD4 means E26). Pups will be culled to 8 (4 males, 4 females) the day after birth.

b. C57BL/6 Inbred Mice Subjects and Breeders. Offspring of timed pregnant B6 dams purchased from Harlan Laboratories (Indianapolis, IN) will be used. Dams will be purchased (rather than bred) for efficient, economic, and uniform animal procurement, and delivered by express transported to IUPUI with food/water on embryonic day (E) 10. Dams will be housed singly immediately upon arrival in a dedicated nursery (12 hr:12 hr light/dark cycle, lights on 0700 hr), with ad libitum chow and water. Cohorts of 6-8 pregnant dams for each delivery will be produced at Harlan by overnight mating; a sperm plug the next morning will designate E0. Births will be monitored 3 times daily from E16 on; most occur between the evening of E18 and noon of E19. E19 is designated postnatal day (PD) 0, regardless of E day of birth. The age at the time of treatment is defined by E day (i.e., PD 7 means E26). If necessary, pups will be culled to 8 (4 males, 4 females) the day after birth; litters with fewer than 6 viable pups will not be used.

c. Neonatal Alcohol and L-NAP Administration. Split litter designs will be followed in all studies, with no more that 1 male and 1 female assigned to each treatment condition. Rats given alcohol will be intubated twice a day with 2.5 g/kg of alcohol (total daily dose of 5.0 g/kg) administered as an 11.33% (v/v) solution in a milk formula (West et al., 1984) containing evaporated milk, corn oil, purified soy protein, minerals and salts, deoxycholic acid, calcium phosphate, tryptophan, methionine, and vitamins (ICN #999999). The solution is prepared daily by adding 6.77 ml of 95% ethanol USP to 50 ml of milk formula. The alcohol-treated pups will be given 4 intubations a day on the first day (3 on subsequent days), infusing 0.02778 ml/g bodyweight per gavage. The first 2 intubations of each day will contain alcohol, the others only milk. The additional milk feedings during intoxication are necessary to achieve normal somatic growth. Treatment control pups will be given sham intubations; milk infusion is omitted because the added calories significantly and persistently increase weights relative to suckle controls. All pups will be weighed daily (PD4-12), then every third day (until PD21). These intubations are routine; untoward effects are rare and the pups tolerate the procedure well. Note also that a normally reared control group that receives only the typical animal care (periodic cage changes after PD7) will be included in all studies. Mice will be given alcohol treatment only on PD 7. Mice within each treated litter will be randomly assigned to receive two subcutaneous injections (2 hours apart) of saline or alcohol (15% v/v in sterile saline, 2.5 g/kg per injection, delivered in volumes of 22.1 ul per g bodyweight.

L-NAP will be dissolved in 50 µl of dimethyl sulfoxide then diluted in sterile normal saline. The rats will be given subcutaneous injections of the designated dose (range from 1-50 ug per pup) or vehicle 30 minutes before each alcohol intubation. We will first screen doses of L-NAP between 1 to 50 ug per pup (about 0.1 to 5 ug per g bodyweight for PD 4 pups). If that dose range is ineffective in the caspase-3 studies of Aim 1, we will expand it over a larger range before pursuing other compounds. The mice will be injected subcutaneously with L-NAP or vehicle 30 minutes before each alcohol injection, using a dose to be determined from Aim 2 of Zhou's mouse component, but likely to be approximately 3ug per injection (total of 6 ug over the 2 injections).

d. Blood Alcohol Concentrations (BACs). A 20-µl sample of blood will be obtained from a tail-clip of each intubated pup on the first day of alcohol treatment (PD 4 in rats; PD 7 in mice), 2 hours after the second alcohol treatment. We have determined that this time point gives the best estimate of the peak BAC for the treatment. The blood will be immediately centrifuged at 4° C, and the plasma taken and frozen at –20° C until assayed for alcohol concentration. Plasma sample BACs will be analyzed using the Analox GL5 Analyzer.

e. Immunocytochemistry for active caspase-3. Eight hours after the first intubation on PD 4, the rats will be anesthetized with an overdose of sodium pentobarbital and perfused intracardially with 0.9% sodium chloride followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB). Cerebella will be post fixed at 4°C for 4 hours in the same fixative, then placed in 30% sucrose in PB overnight at 4°C. A minimum of 12 sagittal, vermal cerebellar sections (40 µm) will be collected in phosphate buffered saline (PBS) in Netwells (in 12-well culture trays) and

processed for active caspase-3 immunocytochemistry under constant motion on an orbital shaker table. The sections will first be washed 3X with PBS, incubated in 3% hydrogen peroxide (10 min), washed again (3X with PBS) and incubated in blocking solution (5% normal goat serum (NGS) containing 0.03% Triton X-100) for 1 hour at room temperature (RT). Sections will then be incubated overnight (at 4°C) in a polyclonal antibody raised against the cleaved (active) subunit of caspase-3 (#9661; Cell Signaling Technology, Beverly, MA) at 1:2000 in 1% NGS containing 0.3% Triton X-100. The next day, the sections will then be washed, incubated in 5% NGS blocking solution for 1 hour at RT, and incubated with secondary antibody (biotinylated goat anti rabbit IgG (H+L)) (BA-1000; Vector Laboratories, Burlingame, CA) at a concentration of 1:200 in PBS for 1 hour at RT. After additional washes (4X with PBS) and incubation in AB solution (Vector Laboratories) for 1 hour, the sections will be washed 3X with Tris-buffered saline (TBS) and then exposed to 3,3'-diaminobenzidine (Sigma Fast metal enhanced DAB, Sigma Chemical Co., St. Louis, MO) in the dark for 2 minutes, then washed 4X in TBS and mounted on double gelatin-subbed slides, dried overnight, dehydrated, and coverslipped using Permount.

f. Anesthesia and Surgery (rats and mice). All surgeries will be performed following aseptic technique under isofluorane inhalation anesthesia. Each animal will be surgically prepared for behavioral training with differential electromyogram (EMG) electrodes and bipolar periocular shock US electrodes. The EMG electrodes for recording activity of the external muscles of the eyelid, orbicularis oculi, are constructed of two strands of ultra-thin Teflon-coated stainless steel wire passed subdermally to penetrate the skin of the upper eyelid. Bipolar shock electrodes are positioned subdermally immediately caudal and dorsocaudal to, the eye. A ground electrode will be connected to a stainless steel skull screw (or back of the neck, for juvenile rats). The electrodes will be attached to a 10-pin Augut-style connector fixed to the skull with a cap of dental cement. The wound will be salved with antibiotic ointment and the animal will be given a single post-operative subcutaneous injection of buprenorphine, and the animals will be given either 2 days (juveniles) or one week (adults) to recover before the start of the training procedures.

g. Eyeblink Conditioning Procedures. All training will occur within darkened light- and sound-attenuating enclosures contained within electrically isolated chambers with background noise of 65-70 dB supplied from a circulating fan (Stanton & Freeman, 2000). Rats will be allowed to move unrestrained because the electrode leads are attached to the headstage via a 10-channel swivel/commutator.

Delay Conditioning (Rats), depicted in **Fig. 2** (see Background & Significance), will be performed using two different intervals between CS and US onset (the interstimulus interval, or ISI), either "**short delay**", with a 280-msec ISI, or "**long delay**" (used for the ISI shift in the adult studies), with an 880-msec-ISI. All acquisition (juveniles and adults) will use short-delay training; for the ISI shift studies of adults in Exp. 2.3, the rats will then be shifted to the long-delay training. For standard short-delay training, rats will be presented a 380 msec tone CS (2.8 kHz, 90 dB SPL) that overlaps and coterminates with a 100 msec eye-shock US. For long-delay training, a 980 msec tone CS that overlaps and coterminates with a 100 msec eye-shock US will be used. The short-delay trial epochs, 800 msec in duration, will consist of a 280-msec pre-CS "baseline period", a 280-msec "CS period" (divided into an 80-msec "startle period" after CS onset and a 200-msec "CR period" before the US), and a 240-msec "US period" (beginning with the 100-msec shock). The long-delay trial epochs, 1400 msec in duration, will consist of a 280-msec pre-CS "baseline period", an 880-msec "CS period" (divided into an 80-msec startle period, a 600-msec "non-adaptive CR period", and a 200-msec "adaptive CR period" just before the US), and a 340-msec "US period"). In all procedures using rats, eye-shock level will be set at 1.5 mA, the minimum intensity required to produce consistent, vigorous eyeblinks. Conditioning sessions will occur twice a day, 5 hr apart. Sessions will be composed of 100 trials separated by 20-40 sec intertrial intervals (pseudorandomly determined). The 100 trials will be divided into 10 blocks composed of 9 paired trials and 1 CS-alone presentation. No explicitly unpaired training control groups are proposed, since we have repeatedly found that alcohol-treated and control rats do not differ with unpaired training.

Discrimination Reversal (Mice). For the discrimination reversal procedure, the mice will be habituated to the conditioning chamber for 2 consecutive days before the condition procedures are started. The discrimination training involves two different CSs, one paired with the shock US (CS+) and presented without the shock (CS-). A tone (2.8 kHz, 70 dB) and a light (15 watt chamber illumination) will be used as the CSs (Paczkowski et al., 1999). Each CS will be 380 msec in duration, but for a given subject, only one (CS+) will overlap and coterminate with the 100 msec shock US (stimulus contingencies counterbalanced across subjects of a given group). Eye-shock level for the mice will be set at 0.75 mA, the minimum intensity required to produce consistent, vigorous eyeblinks. Sessions will be composed of 100 trials separated by 18-42 sec variable intertrial intervals, with each session having 50 CS+ trials (including 5 trials of CS+ alone) and 50 CS- trials, with the order pseudorandomly determined within blocks of 20 trials (10 of each type). **Fig. 13** (Preliminary Studies) shows typical results from this procedure.

Eyelid responses will be measured as EMG activity recorded from the orbicularis oculi muscle of the left eye. EMG activity will be differentially recorded from the Teflon-coated wires implanted during surgery. The activity will be amplified, filtered routed to an integrator that converts the filtered signal to an integrated dc signal in arbitrary units. *Conditioned responses* (CRs) are defined as integrated EMG activity (in arbitrary units) that exceeds the

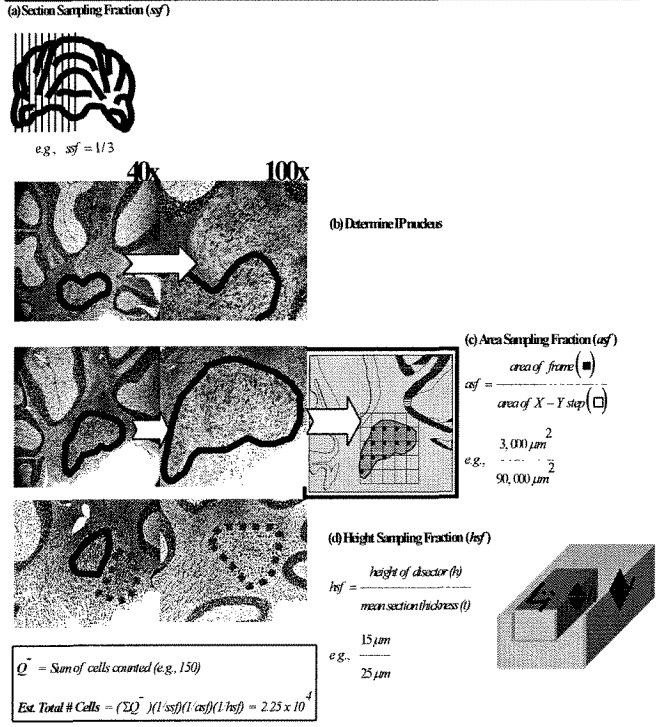
resting baseline EMG level by more than 0.4 arbitrary units (see **Fig. 2**). CR scoring will not include the startle period (see above) because nonassociative "alpha" or startle responses to the CS presentation are occasionally evident during this period. For all studies, CR timing characteristics will be assessed by analyzing responses on CS-alone trials, i.e., the 200 msec *before and after* when the US would occur (if it was presented), because these trials contain responses that are free from US/UR contamination. Separate analyses of acquisition of CRs (CRs during the adaptive CR period) will be also performed on paired and CS-alone trials.

h. Tissue Preparation and Sectioning for Cell Counts. All aspects of cell counting will be done with experimenters blind to treatment and training conditions. Under terminal anesthesia, rats will be perfused intracardially with aldehydes, and the cerebellum, brain stem and forebrain weighed and stored in fixative at 4°C. For cerebellar cell counts, the vermis and one hemisphere will be isolated into a tissue block by a standardized cut at one vermal-hemisphere juncture, and embedded in glycol methacrylate (Technovit®, Leica Corp.) by dehydrating and infiltrating with increasing concentrations of embedding solution with constant shaking, then polymerized and hardened in small molds. Each cerebellum (vermis + hemisphere) will be sectioned exhaustively in the sagittal plane at 30 μm using glass knives and an automated rotary microtome. A predetermined ratio of sections with a designated periodicity will be saved (1 out of 3), with a random start in the first interval (a "systematic random" sample).

For hippocampal cell counts, the hippocampal formation will be isolated into a tissue block by blunt dissection away from the underlying thalamus and a coronal cut just anterior to the septal pole of the hippocampus, and one hemisphere will be embedded in glycol methacrylate with constant shaking, then polymerized and hardened in small molds. The hippocampal formation will be sectioned exhaustively in the horizontal plane at 30 μm using glass knives and an automated rotary microtome. A predetermined ratio of sections with a designated periodicity will be saved (1 out of 6), with a random start in the first interval (a "systematic random" sample).

i. Optical Fractionator Cell Counting. Cerebellum (Rats of Aim 2). Stereological counting using the optical fractionator will estimate the total number of neurons (identified by histological criteria) in the anterior hemispheric lobules (HI-HV) and lobule simplex (HVI), and the deep interpositus nucleus (Gundersen, 1986; Gundersen et al., 1988a; Gundersen et al., 1988b; West et al., 1991), using sagittal sections. **Fig. 14 (see Appx for full color)** shows sample boundaries for IP, and illustrates the fractionator approach. Systematic random samples of a predetermined fraction of the structure will be obtained at each of three levels of the structure: 1) the section sampling fraction *(ssf)*, the proportion of sections taken from an exhaustive set, 2) the area sampling fraction *(asf)*, the proportional area of each section sampled, determined by counting frame size relative to X-Y step size that determines the sampling locations, and 3) the thickness sampling fraction *(h/t)*, or height of the counting box ("disector") relative to the thickness of the section, measured optically. Total neuron number is obtained by multiplying the summed count over all disectors (ΣQ^-) by the inverse of the 3 sampling fractions {$N = \Sigma Q^- * 1/hsf * 1/asf * 1/ssf$}.

Fig. 14: Optical Fractionator Counts of IP Nucleus
(a) The cerebellar hemisphere is cut in the sagittal plane, saving a known fraction, termed section sampling fraction *(ssf)* = 1/3.
(b) The anatomical boundaries of IP (bold outline) are determined, isolating IP from the medial (top, green-stippled) and dentate (bottom, red-stippled).
(c) A predetermined fractional area is sampled from each section, i.e., the counting frame area divided by area of the X-Y stepper, or area sampling fraction (*asf*)
(d) Cell counts using unbiased rules in known volume of the tissue (*h* height of box / *t* thickness)--height sampling fraction *(hsf)*.



Neurons in each region of interest will be counted using the Olympus CastGrid computer-driven microscope fitted with a 100X oil objective (n.a.=1.4) and a Heidenhain optical vernier for z-axis measurements (±0.5 μm). Cells will be counted when they come into focus within a unbiased counting frame of known X-Y dimensions as the optical plane of focus (<1 μm) is moved through a known depth. A guard zone of at least 4 μm will be set from the top of the section surface to the top plane of the optical disector. When efficient sampling parameters are set (minimum samples needed to limit sampling error of the estimate), about 200-400 total locations are counted, yielding 100-200 cells counted per region (Gundersen, 1986; Gundersen & Jensen, 1987), with a coefficient of error of about 5%, calculated by the formula describe by West et al., 1991.

Hippocampus (Mice of Aim 3). Stereological counting

using the optical fractionator will estimate the total number of neurons in the hippocampus (Gundersen, 1986; Gundersen et al., 1988a; Gundersen et al., 1988b; West et al., 1991) using horizontal sections. Total numbers of cells will be determined for pyramidal cells in CA1, CA3, and subiculum, using the optical fractionator sampling design described previously in detail (West et al., 1991). **Fig. 15 (see full version, Appx. #2)** illustrates the fractionator approach in the hippocampus and shows sample boundaries for CA1. Systematic random samples of a predetermined fraction of the structure will be obtained at each of three fractions of the structure (see previous section for more details): 1) the section sampling fraction *(ssf)*, 2) the area sampling fraction *(asf)*, and 3) the height sampling fraction *(hsf)*. Total neuron number is obtained by multiplying the summed count over all disectors (ΣQ^-)

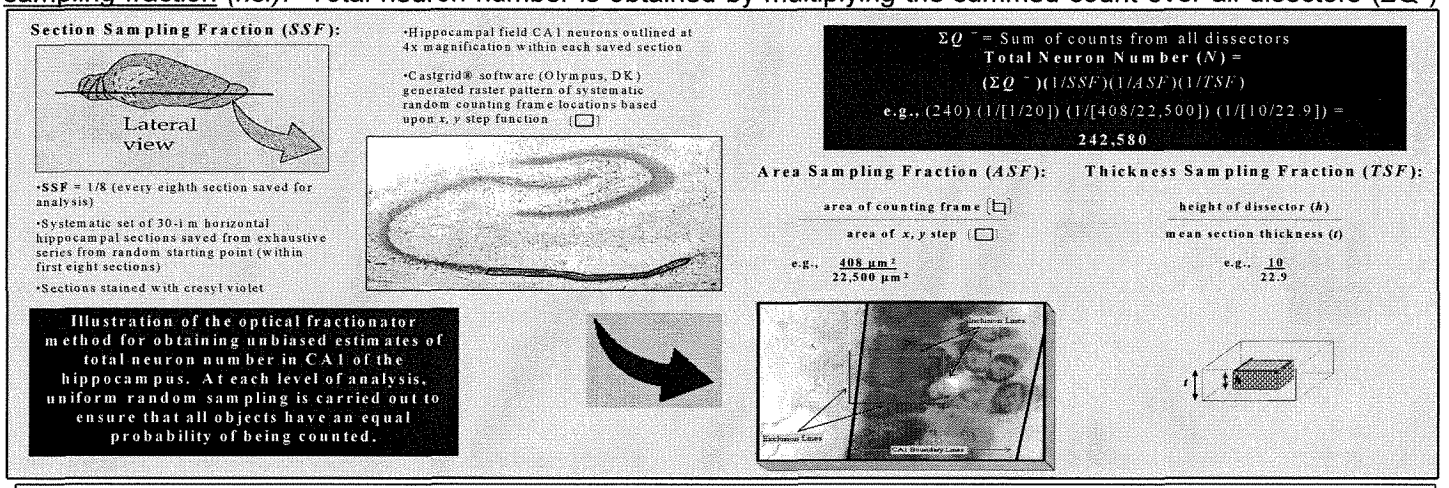


Fig. 15. Hippocampal cell counts with the optical fractionator See Appx. for full size]

by the inverse of the 3 sampling fractions {$N = \Sigma Q^- * 1/hsf * 1/asf * 1/ssf$}. Neurons in each region of interest will be counted using histological criteria. We will need to identify the optical parameter for adult mice. When efficient sampling parameters are set, about 200-400 total locations are counted, yielding 100-200 cells counted per region, with a coefficient of error of about 5%, calculated by the formula describe by West et al., 1991. The subdivisions of the hippocampal formation will be identified based on Blackstad's definitions (Blackstad, 1956). Hippocampal region CA1 will be distinguished from CA3 based on looser packing density and larger cell size of CA3 pyramidal neurons than those of CA1; the neurons of the narrow transition zone (CA2) whose cells are similar to those of CA3 and thus included in the CA3 counts. Region CA1 will be distinguished from cells of the subicular layer by identification of an abrupt end in the continuity of the pyramidal cells that comprise the tail end of this cell layer. Glial cells will be distinguished from the principal neurons within their respective cell layers and excluded from the counts. Basket cells are difficult to discriminate from pyramidal cells, so they are inevitably included in the estimates of CA3 & CA1.

Timeline. The Timeline for completing all experiments.

	Exp #	Objectives & (# of Rats)	Year 1	Year 2	Year 3	Year 4	Year 5
Aim 1 (Protection of activate caspase-3 in Purkinje cells)	#1.1a	Screen L-NAP (n~36)	◄►				
	#1.b	Screen derivatives of L-NAP (n's ~ 36 per compound)		◄►			
	#1.1c	Screen Other Candidate Agents			◄►	◄►	
Aim 2 (Protection against delay ECC deficits & cerebellar cell loss in rats)	#2.1	Delay conditioning with L-NAP (juv., n=120)	◄►				
	#2.2	Counts of Purkinje cells & IP neurons (juv, n=40)		◄►			
	#2.1a	Delay conditioning with L-NAP derivatives (juv, n=60)		◄►			
	#2.2a	Counts of Purkinje cells & IP neurons (juv, n=40)			◄►		
	#2.3	Delay conditioning in adults (n=120)			◄►	◄►	
	#2.1b	Delay conditioning with other candidates (juv, n=120)				◄►	
	#2.2b	Cerebellar cell counts for other candidates (juv, n=40)					◄►
Aim 3 (Effects on hippocampal-dependent learning in B6 mice)	#3.1	Discrimination Reversal in B6 mice (Ethanol Effects) n=96			◄···►		
	#3.2	Discrimination Reversal in B6 mice testing L-NAP, derivatives, or other agents (n=120)				◄···►	
	#3.3	Hippocampal Cell counts on B6 mice of Exp. 3.2 (n=40)					◄►

e. HUMAN SUBJECTS N/A

f. VERTEBRATE ANIMALS

1. Approximately 630 Long-Evans rats and 300 C57BL/6 (B6) inbred mice, half males and half females in each species, will be used as subjects in the experiments in this proposal. The proposed studies are based on the use of neonatal outbred rat and neonatal inbred mouse models of developmental exposure to alcohol, studying the effects on eyeblink classical conditioning combined with neuroanatomical measures of alcohol-induced cerebellar and hippocampal damage and behavioral dysfunction. The goals of the proposed studies are to screen and identify candidate molecular agents that may show efficacy in antagonizing or preventing the neuroteratogenic effects of binge alcohol exposure during the neonatal period of rodent brain development that is comparable to that of the human third trimester. These tests of neuroprotective effects of anti-oxidant treatments capitalize on the well-studied effects on cerebellar systems in the neonatal rat, and on the enhanced vulnerability of the B6 mouse to limited episodes of binge alcohol exposure during this period. Alcohol will be administered either via intragastric intubation (5 g/kg/day in milk formula on postnatal days 4-9 in rats) or via subcutaneous injection (5 g/kg divided into two injections, 2 hours apart, on PD 7 in B6 mice). Control treatments of littermates will involve sham intubations or saline injections. Alcohol exposure of neonatal rats is used because brain development in general (and cerebellar and hippocampal development in particular) during this period corresponds to developmental events that occur in humans over the last 15 weeks of pregnancy.

2. Prenatal exposure to alcohol damages the brain and leads to cognitive and behavioral dysfunction, but the specific consequences on neural circuits leading to learning disabilities are not known, nor are treatments to prevent developmental brain damage available. Animal models must be used to control the exposure conditions, evaluate the efficacy treatment with potential therapeutic agents, and allow quantitative evaluation of behavior and anatomy. Rats and mice have major advantages of being well suited for developmental studies, with relatively short periods to reach adulthood, and much is known about the anatomy and development of the rat and mouse brain and about their behavior. There is a large neurobehavioral literature on the effects of alcohol on rats (and a rapidly growing literature in mice), and a growing literature on eyeblink conditioning in both species. Long-Evans rats will be used because they are a robust, heterogeneous stock of laboratory rat that have several advantages in terms of visual and behavioral capabilities over albino stocks. B6 mice will be used because they have been shown to be extremely vulnerable to binge alcohol-induced neuronal cell death in limbic and cortical regions following a single exposure on postnatal day 7. The group numbers proposed (8-12) per treatment and training condition, are known or anticipated to be sufficient to detect important main and interactive effects of the alcohol treatments.

3. The animal care facilities at IUPUI are AAALAC-approved, and the attending veterinarian is Dr. Laurel Schantz. At the University of Delaware, the attending veterinarian is Dr. George Keech.

4. Surgery to prepare animals for eyeblink conditioning involves placement of the EMG recording electrodes in the orbicularis oculi muscle, a ground electrode, and a periocular bipolar stimulating electrode for delivering the shock US, and, in the IUB studies, unit recording electrodes placed in the cerebellum and hippocampus, all secured to an implanted head stage. All surgical procedures will be performed using aseptic conditions. All rats will be anesthetized with isofluorane. The electromyogram (EMG) electrodes for recording activity of the external muscles of the eyelid are constructed of two strands of ultra-thin Teflon-coated stainless steel wire passed subdermally to penetrate the skin of the upper eyelid. Bipolar stimulating electrodes will be positioned subdermally immediately caudal and dorsocaudal to the eye. The ground electrode will be connected to a stainless steel skull screw. All the electrodes will be attached to a 10-pin Augut-style connector and fixed into a cap of dental cement. The wound will then be salved with antibiotic ointment, the subjects given a single injection of buprenorphine (0.05 mg/kg) for post-operative analgesia, and then monitored for recovery from anesthesia to full mobility before returning them to their home cage with ad lib access to food and water for the duration of the experiment. The adults will be given 1 week to recover before the start of the training procedures; the

juveniles will be tested the day after surgery (since age is the key variable), a condition they can tolerate well. The behavioral eyeblink procedures involve delivery of the minimal shock to the bipolar periocular electrode necessary to elicit a vigorous eyeblink. For rats, this is a 1.5 mA, 60 Hz, 100 msec, constant current square wave; for mice, the shock will be 0.75 mA. These are standard procedures that utilize the minimum shock needed to support effective eyeblink classical conditioning in the two species.

5. The majority of experimental animals will be perfused following a lethal overdose of sodium pentobarbital (120 mg/kg, IP). Animals not perfused for anatomical studies will be euthanized by CO2 gas followed by bilateral pneumothorax to insure death. This method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

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h. CONSORTIUM/CONTRACTUAL ARRANGEMENTS Aim 3 of this component will be conducted by Dr. Mark Stanton (Deparment of Psychology, University of Delaware) over years 03-05, under a subcontract agreement with the University of Delaware. Dr Stanton has developed and successfully performed eyeblink classical conditioning studies in C57BL/6 mice and in genetically modified mice including the discrimination reversal training studies proposed in this component. For that reason, he is one of the most qualified investigators in the world to conduct and interpret the mouse model studies of Aim 3. This effort constitutes about 20% of the effort of the entire project, and needs to be included as part of this project because of the links to the other two Aims and the goals of protection against FASD.

i. CONSULTANT A letter from Dr. Mark Stanton confirming his role as Co-Investigator is included on the following page