THERAPEUTIC EFFECTS OF ANIRACETAM ON COGNITIVE

DEFICITS INDUCED BY ETHANOL TERATOGENICITY:

A NOVEL TREATMENT APPROACH THROUGH

SYNAPTIC AMPA RECEPTOR MODULATION

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Nayana Wijayawardhane, daughter of Mr. and Mrs. Wijayawardhane, was born on December 31, 1972. She graduated from The Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka in 2000, with her Bachelor's of Veterinary Science. Shortly after graduation, she was recruited to the Department of Veterinary Clinical Sciences of the same college where she earned her degree. She worked there as a lecturer for about four years until she joined the doctoral degree program in pharmacology, at Auburn University in January 2004. Nayana was born with other two siblings, a sister and a brother. She was married to Vajira Jayasinghe in December, 2004. During her stay in Auburn University she obtained several awards and prizes at international, regional and University levels. She was awarded as the 2004-2005 outstanding graduate student in Auburn University and the outstanding graduate student in the Harrison School of Pharmacy, 2006.

DISSERTATION ABSTRACT

THERAPEUTIC EFFECTS OF ANIRACETAM ON COGNITIVE DEFICITS INDUCED BY ETHANOL TERATOGENICITY: A NOVEL TREATMENT APPROACH THROUGH SYNAPTIC AMPA RECEPTOR MODULATION

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Ethanol exposure during fetal development can adversely affect the outcome of the offspring. Numerous mechanisms are likely to contribute to these damaging effects of ethanol on the fetus and particularly the developing central nervous system. The hippocampus which is critically involved in learning and memory formation is most vulnerable to ethanol exposure *in utero,* resulting in persistent memory and learning deficits commonly observed in fetal alcohol syndrome (FAS). FAS is assumed to be mediated partially via alterations in glutamatergic synaptic transmission. The α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) subtype of glutamate receptors plays a crucial role in learning and memory. However, the damaging effects of ethanol on AMPAR-mediated synaptic transmission in the hippocampus are not well studied.

Therefore, this study investigated the hypothesis that *in utero* exposure to alcohol can impair the AMPAR function, altering the normal neurobehavioral function and targeting the AMPARs would provide a new therapeutic approach in the treatment of FAS. The identification of this new mechanism and its contribution to ethanol-induced fetal damage led to the development of a rational approach using aniracetam that targets AMPARs, for the treatment of alcohol-related cognitive deficits associated with FAS.

Developmental reflexes, plus-maze test and active avoidance tests were carried out to assess the behavioral teratogenicity in Sprague Dawley rat offspring exposed to moderate ethanol (4 g/kg/24h; 38% v/v), throughout pregnancy. The whole-cell-patch clamp technique and bilayer reconstitution of synaptosomal AMPARs were used to study the AMPAR-mediated currents in the hippocampus. Growth retardation, impairments in learning and memory, and enhancement of anxiety were noticed after ethanol exposure. Significant reduction of AMPAR-mediated currents suggested impairment of post synaptic AMPARs as well as glutamate release from presynaptic nerve terminals. Ten day aniracetam treatment (50 mg/kg) during preadolescence effectively ameliorated the neurobehavioral deficits of ethanol exposure *in utero*. The results of this study clearly demonstrate for the first time that the AMPAR-mediated synaptic transmission is impaired by moderate prenatal ethanol exposure, which results at least partially in behavioral teratogenicity in FAS. Our findings emphasize the importance of targeting AMPARs using aniracetam in developing an effective intervention approach to address this major public health problem.

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Style manual or journal used

The Journal of Neuroscience

Computer software used

Microsoft Word

Microsoft Excel

Microsoft Power Point

Microcal Origin 6.0

pClamp 9.0

Mini analysis program

Paint

Infranview

Adobe Photoshop

Statistical Analysis Software (SAS)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1 INTRODUCTION

Ethanol exposure during development produces cognitive deficits, including impaired learning, memory and attention problems as hallmarks of fetal alcohol effects on the central nervous system (CNS) (Warren and Foudin, 2001). Damage to the hippocampus acts as an important mediator of some of the behavioral and cognitive deficits associated with FAS. These deficits result in long-lasting neurobehavioral disabilities such as problems in school performance, dependent living, substance abuse and mental health disorders (Larkby and Day, 1997).

Recent literature reviews confirm that no single putative mechanism can account for all the components and variations of these neurophysiological and anatomical characteristics found in children exposed to ethanol *in utero* (Tan et al., 1990; Guerri, 1998). However, identifying the mechanisms contributing to ethanol-induced damage on the developing brain is particularly complex. For certain groups of brain cells alcohol can induce cell death, whereas for other cell groups it interferes with cellular functions. Ethanol may even deplete cells in a given cell population, depending on the developmental stage of the cells (West et al., 1994; Guerri, 1998), sometimes causing alterations in the cell division and migration to appropriate locations. Neurons may also die because one stage of cell development (e.g., before neurons migrate to their final location) interferes with subsequent developmental stages (e.g., migration or

differentiation). There is mounting evidence indicating that ion channels/receptors in the brain are also affected by ethanol exposure. The effects of ethanol on fetal ion channel function in the brain are likely to result in abnormal CNS functions in FAS, since these channels play a key role in the developing and mature nervous systems. Therefore, understanding how different ion channels of the hippocampus are affected by prenatal ethanol exposure would improve the diagnosis of FAS and provide insight into the nature of neurobehavioral deficits. The development of more effective treatments for fetal alcohol effects might then be possible.

In the mammalian brain, ionotropic glutamate receptors mediate a significant proportion of excitatory synaptic transmission (Monaghan et al., 1989; Boulter et al., 1990) Glutamate interacts with three classes of ionotropic receptors (Dingledine et al., 1999): NMDA receptors (NMDARs), AMPA receptors (AMPARs) and kainate receptors (KARs). NMDARs are involved in the encoding and retrieval process of memory and AMPARs are involved in memory consolidation/retention. Studies suggest that the effects of ethanol on the developing CNS are to some extent results from alterations in neurotransmission at glutamatergic synapses which are critical for the maturation of the neuronal circuits. Consumption of moderate quantities of ethanol by rat dams during pregnancy reduces NMDAR number (Abdollah and Brien, 1995), and function (Morrisett et al., 1989). However, the extent to which other ion channels are affected is largely unknown.

The functional significance of the AMPARs is known to provide a key element in synaptic plasticity. The synaptic plasticity/ long term potentiation (LTP) is impaired in the subjects exposed to ethanol *in utero* (Swartzwelder et al., 1988; Tan et al., 1990).

These observations prompted us to ask whether AMPARs are also affected by prenatal ethanol exposure. Therefore, characterizing the mechanisms of action of ethanol on AMPARs in developing neuronal circuits is important to further understanding of the pathophysiology of FAS. Also understanding the exact mechanism responsible for cognitive impairments associated with prenatal ethanol exposure, is important to define effective treatment strategies.

Efforts have been carried out using psychotherapeutic CNS stimulants (Riley et al., 2001; Lynch, 2004), including methylphenidate (Ritalin), d-amphetamine (Dextrine), premoline (Cylert) and in some cases caffeine and choline, in treating the children affected by fetal alcohol effects. However, currently there are no effective treatments for the neurobehavioral problems associated with prenatal ethanol exposure (Riley et al., 2003). Aniracetam, a pyrrolidonic cognition-enhancing drug has been found to be effective in the treatment of cognitive deficits in a rat model exposed to mild doses of ethanol *in utero* (Vaglenova and Vesselinov Petkov, 2001) and in the treatment of certain other CNS dysfunctions: (i) by enhancing the learning and memory possibly by facilitating the flow of information between cerebral hemispheres, (ii) by enhancing the resistance towards chemical and physical injuries. They are also being widely used in pediatrics and geriatrics in the treatment of cerebral ischemia and encephalopathic disturbances.

Animal models, particularly those using rodents have been used as powerful tools in determining the mechanisms and outcomes of early ethanol exposure, because the physiological responses to alcohol in development are similar to those in humans (Hannigan and Abel, 1996), in addition to the existing similarities in physiology,

biochemistry and genetic factors. The neurobehavioral outcome of prenatal ethanol exposure in animals is also remarkably similar with the clinical and behavioral sequelae in humans. Therefore, findings from such studies can often provide key information about the effects in humans. Nevertheless, the results should be interpreted with some caution, because species differences in vulnerability to ethanol can exist. In order to investigate this therapeutic agent in treating the cognitive deficits associated with ethanol exposure, we used a sensitive rat model of moderate ethanol exposure.

One of the most widely used procedures among alcohol research is the use of chronic ethanol exposure paradigms in animal models. In order to investigate the neurobehavioral deficits that result from chronic moderate ethanol exposure, administration of ethanol to pregnant animals has been accomplished in diverse manners by different investigators. Since, rats do not normally voluntarily consume enough alcohol to maintain chronically high blood alcohol concentrations (BAC) during pregnancy, intragastric intubation (gavage) procedures have been developed for alcohol administration to pregnant rats. This technique makes this ethanol exposed rat model especially useful and relevant in clinical studies. One of the objectives of this study is to assess the development and neurobehavioral functions at a later postnatal time point in relation to the prenatal ethanol exposure. This assessment helps in comparison of the model before and after the treatment with the therapeutic agent.

Furthermore, studying the behavior aspect alone does not allow a detailed understanding of the ethanol and therapeutic agent's actions on the individual cells of the brain. Such detailed experimental analysis to investigate the molecular mechanisms of actions of ethanol and aniracetam on a single neuronal level is possible using live brain

cells of the treatment groups. Determining the synaptic transmission of hippocampal neuronal cells allows researchers to understand the mechanisms underlying the developments of these effects. The study of molecular level changes using novel experimental techniques allows the determination of the changes that occur at the single receptor level. This allows us a possible extrapolation of the findings from the molecular actions observed in receptors obtained from single neuronal cells to the more complex mechanisms that simultaneously determine the behavior of the animal.

Correlating the alterations of the animal behavior with changes in single neurons and receptor action in these neurons may give us a more detailed understanding of the complex process occurring in the presence of fetal ethanol. Therefore, this study focused on investigating the changes in the animal behavior using an array of developmental and behavioral experiments followed by AMPAR-mediated synaptic transmission using whole cell configuration in hippocampal slices. Further, the single channel properties of synaptic AMPARs were also investigated using isolated synaptosomes reconstituted in lipid bilayers.

In summary, this study was designed to elucidate the developmental, learning and memory process of individual animal to quantify the deficits, induced by prenatal ethanol exposure and also to study the clinical relevance of the new therapeutic regimen to bring about the desired cognitive enhancement. Therefore, using our model we propose to investigate the consequences of exposure to moderate doses of ethanol *in utero* on the function of AMPARs which is involved in the various aspects of learning and memory. Further, we propose to utilize this model to screen the effects of aniracetam in ameliorating the behavioral deficits by modulating the AMPAR-mediated synaptic

transmission. The aims proposed in this study will provide important information concerning the action of ethanol and the underlying causes of neurobehavioral problems associated with fetal ethanol exposure. Most importantly, successful use of aniracetam in this model could lead to therapeutic advancement of the neuropathologies in children exposed to ethanol *in utero* as well as for children exposed to other toxic agents *in utero*.

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2 REVIEW OF LITERATURE

Outline

2.1 Overview of fetal alcohol spectrum disorder (FASD)

Since the early 1970s the scientific literature on the effects of prenatal alcohol exposure on the fetus has been rapidly expanding. However, public and scientific attraction to the long-term consequences of prenatal alcohol exposure began after 1973 when Jones and Smith (1973) and Jones et al., (1973) made a detailed descriptions of eleven children born to alcohol-abusing women. Prenatal exposure to alcohol has been demonstrated to produce a range of subtle alcohol-related neurodevelopmental disorders (ARND, fetal alcohol effects, and alcohol-related birth defects) to a combination of characteristic abnormalities described as fetal alcohol syndrome (FAS) (Larkby and Day, 1997). The most widely known consequence of prenatal alcohol exposure is FAS. Diagnosis with FAS requires the following criteria: (i) prenatal and/or postnatal growth retardation, (ii) a distinct facial appearance (three facial characteristics required for diagnosis- smooth philtrum, thin vermolin, and short palpebral fissures and other characters such as epicanthal folds, strabismus, ptosis, low nasal bridge, ear abnormities (Fig. 2.1), and (iii) some evidence of a central nervous system (CNS) dysfunction. The revised Institute of Medicine (IOM) guidelines include the confirmed history of maternal alcohol exposure, presence of two of the facial characteristics, and one other characteristic such as growth retardation, evidence of deficit brain growth or a pattern of behavioral or cognitive abnormalities. The majority of children with substantial prenatal alcohol exposure (about 3 times as many children as those with FAS), however, show only some of the above features (Sampson et al., 1997), and they are referred to as having ARND. For convenience, the term FASD shall be used from henceforth to refer to the full spectrum of morphological and cognitive-behavioral outcomes following exposure to

alcohol prenatally and the terms “ethanol” and “alcohol” in similar meaning.

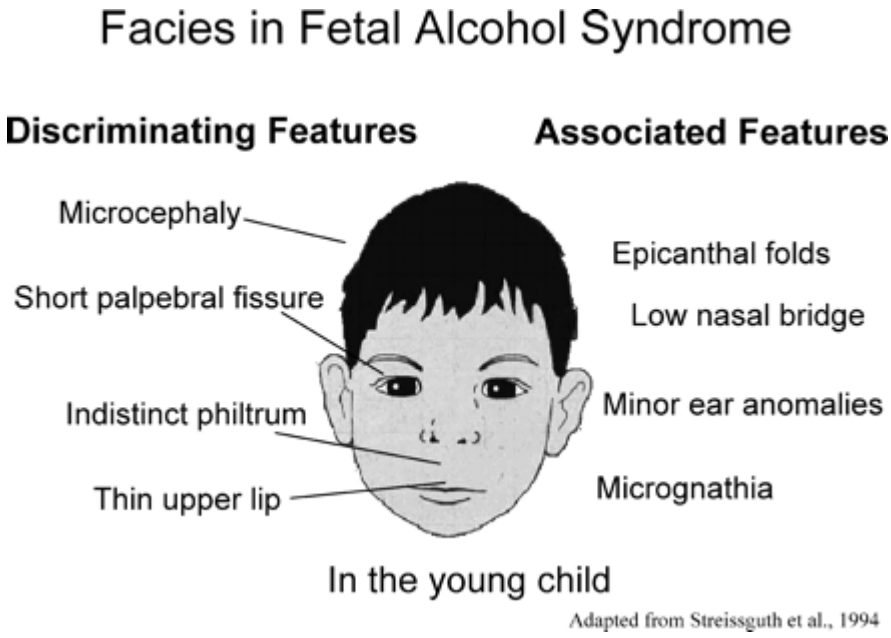

Figure 2.1. The facial phenotype of FAS in a young child. A smooth philtrum, thin vermillion or upper lip, and short palpebral fissures are typically used in the diagnosis of FAS, although the other features listed are common. Adapted from Streissguth AP. A long-term perspective of FAS. Alcohol Health Res World 18:74–81, 1994.

The term fetal alcohol spectrum disorders (FASD) has been recently introduced to describe the range of effects (physical, mental or behavioral, with possible lifelong implications) that can occur in an individual whose mother consumed alcohol during pregnancy. The range of outcomes seen in FASD is varied due to several reasons (Table 2.1). One of the major factors that influence these outcomes is the quantity of alcohol that comes in contact with the developing fetus, which would be largely dependent on the

dose and the pattern of alcohol consumption. Genetic factors also play a major role since metabolism of and/or sensitivity to alcohol is determined by the genetic pool. Metabolism of the alcohol is mainly by the liver enzymes (95%), which oxidize the alcohol (Fig 2.2). Asian individuals (e.g., Japanese, Korean, and Chinese) typically have genes that code only for an inactive form of the enzyme which is not efficient in metabolizing alcohol; thus, resulting in very high levels of acetaldehyde even with a small intake of alcohol. Nutritional factors also could directly or indirectly influence the blood alcohol level. Timing of exposure mainly determines which developing structures are affected and the severity of the effect. For example prenatal alcohol exposure during the first trimester interferes with the migration, and organization of brain cells (Cook et al., 1990; Livy et al., 2003). During the third trimester, alcohol exposure directly leads to the damage of cerebellum, hippocampus and prefrontal cortex (Coles et al., 1991; Sutherland et al., 1997; Livy et al., 2003). Age of the mother has also been identified as another risk factor for FAS.

The most noticeable and devastating outcomes of prenatally alcohol exposed offspring are cognitive-behavioral deficits. The teratogenic effects of alcohol cause abnormalities in brain development, thus producing cognitive social and motor dysfunction. As shown in figure 2.3, these dysfunctions lead to a range of negative life outcomes, including academic, social, and emotional problems. Cognitive functioning of children with FASD includes mainly deficits in intellectual functioning attention and information processing, executive function, language, visual-perception, number processing, and memory.

Table 2.1. Risk factors associated with FASDs (Adopted from Riley and Mcgee., 2002)

Dose of alcohol	e.g., Mild, moderate, high
Pattern of exposure	e.g., Binge, chronic
Developmental timing of exposure	e.g., First, second, third trimester
Genetic variation	e.g., Hetero/homozygous for alcohol dehydrogenase
Maternal characteristics	e.g., Malnutrition, age, cultural-racial facts
Socioeconomic status	e.g., Education, economy
Synergistic reactions with other drugs	e.g., Hypnotics
Interaction with nutritional variables	e.g., Vitamin B1

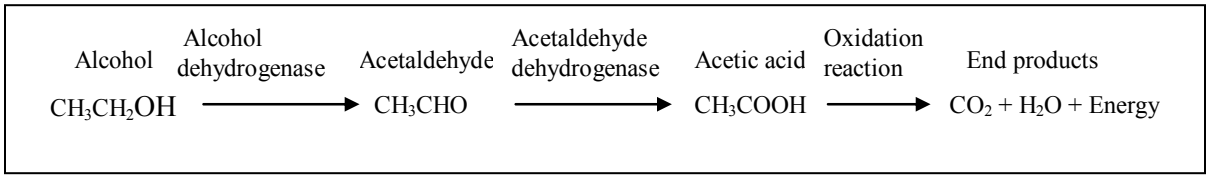

Figure 2.2. Metabolism of alcohol. The principal metabolic pathway of alcohol involves formation of the toxic metabolite acetaldehyde, which must be further degraded to acetic acid.

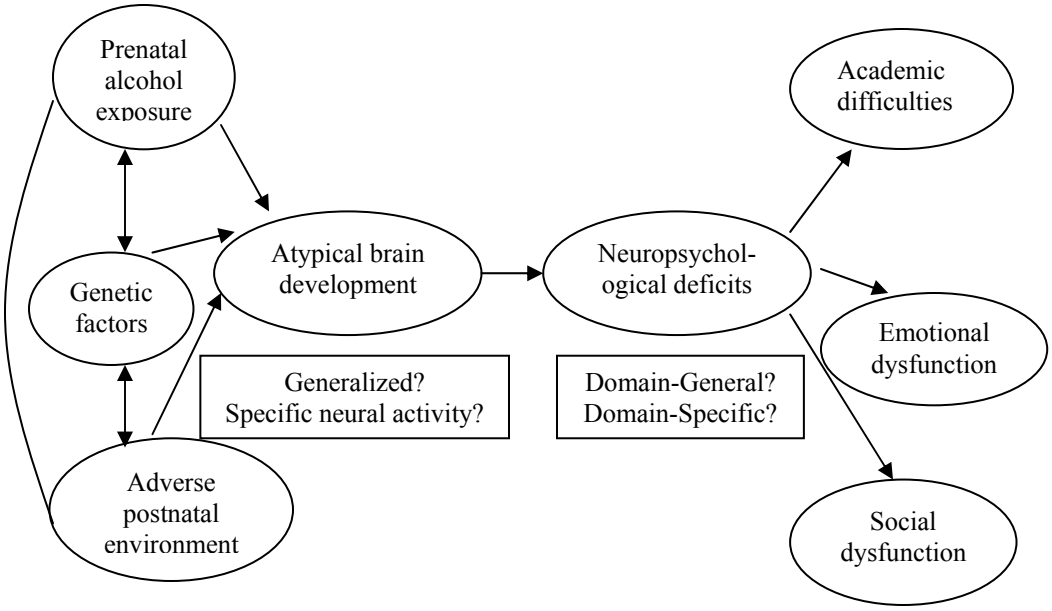

Figure 2.3. A neuropsychological model of cognitive and behavioral outcomes of prenatal alcohol exposure. Link between prenatal ethanol exposure and cognitive-behavior functioning depends on environmental and genetic factors, in addition to the exposure (e.g. quantity and frequency) and maternal variables (e.g. age, body weight). Adapted from Kodituwakku PW. Neuroscience and Biobehavioral Reviews. 2006.

2.2 Alterations in cognitive functions associated with FASD in children

2.2.1 Intellectual ability

FASD has been reported to be associated with diminished intellectual functioning in children (Streissguth et al., 1990; Mattson et al., 1997; Mattson and Riley, 1998). Average IQs of affected children range from mildly retarded to borderline range (Mattson and Riley, 1998), and there is a correlation between significant deficits and heavy exposure during prenatal development (Mattson et al., 1997). Furthermore, a dose-dependent decrement of intellectual ability has been suggested: exposure to one ounce of

absolute alcohol a day decreased nearly 5 full-scale IQ points (Streissguth et al., 1990). Intellectual deficits have been reported to cause slower processing speed in infants (Jacobson, 1998), in young children (O'Connor et al., 1986; Coles et al., 1991; Mattson et al., 1997; Larroque and Kaminski, 1998), and in school age children (Kodituwakku et al., 1995; Mattson et al., 1997; Adnams et al., 2001).

2.2.2 Attention and speed of information processing

Deficits in attentional skills have been considered pronounced effects in prenatal alcohol exposure (Streissguth et al., 1986; Nanson and Hiscock, 1990). Children with FASD like those with attention deficit disorder (ADD), report difficulty in focusing, organization, and maintenance of attention over time and difficulty in response inhibition (Nanson and Hiscock, 1990). Infants and children, exposed to alcohol prenatally have been observed to have slower processing speed and slower reaction time using different paradigms (Jacobson, 1998; Roebuck et al., 2002; Simmons et al., 2002; Burden et al., 2005).

2.2.3 Executive functioning

Prenatal alcohol exposure is associated with deficient executive functioning such as conscious goal-oriented behavior, planning, set shifting, inhibition of goal-irrelevant responses, and holding goals in working memory (Kodituwakku et al., 2001). Children with FASD have also been found to be deficient in non-verbal and verbal fluency (Schoenfeld et al., 2001), particularly in letter fluency (generation of words beginning with certain letters under specific conditions) (Kodituwakku et al., 1995; Kodituwakku, 2006).

2.2.4 Language

Prenatal alcohol exposure is associated with deficits in naming (Mattson and Riley, 1998), word comprehension (Conry, 1990), grammatical and semantic abilities (Becker et al., 1990), and pragmatics (Abkarian, 1992).

2.2.5 Visual perception and visual construction

Researchers have documented that children with FASD are impaired at visual perceptual tasks (Kodituwakku, 2006), and had difficulty in drawing designs that required planning and visual-motor integration (Uecker and Nadel, 1996).

2.2.6 Learning and memory

There is increasing evidence that learning and memory are specially vulnerable to the effect of prenatal alcohol exposure. Conditioning and habituation were diminished when infants were exposed to alcohol prenatally (Streissguth et al., 1986). Animal research has also provided evidence that the hippocampus, a region important for learning and memory is specially vulnerable to the toxic effects of alcohol during brain development (Berman and Hannigan, 2000). Using spatial navigation (to study the association between hippocampal damage and the learning and memory deficits), in children with FASD, (Hamilton et al., 2003) has demonstrated impaired spatial learning (finding a hidden plat form) in Morris water maze.

Most studies of leaning and memory of children with FASD show a deficiency in verbal learning (recall of word lists) as well (Mattson et al., 1996; Willford et al., 2004). Interestingly, inspite of deficient initial learning, Kaemingk et al., (2003) have reported that FASD children have been successful in retaining the newly learned information

whereas Mattson and Riley (1999) reports impaired explicit memory and unimpaired implicit memory in these children.

2.2.7 Number processing

There is increasing evidence suggesting that children with FASD perform poor in complex number processing tasks such as calculation and cognitive estimation (Streissguth et al., 1994; Kopera-Frye et al., 1996; Burden et al., 2005).

2.3 FASD associated behavioral dysfunction in children

Altered cognitive functions associated with prenatal alcohol exposure contribute to a range of behavioral outcomes, including academic difficulties, social skills deficits and emotional problems. Maternal binge drinking and drinking during early pregnancy has been shown to associate with poor classroom behaviors such as distractibility, lack of persistence, restlessness and difficulty in information processing and reasoning (Olson et al., 1998).

Children with FASD experience greater difficulty in relative complex adaptive behaviors and social interactions such as facial and vocal affective expressions, reading body language and understanding the pragmatic aspects of language (Kodituwakku, 2006). Therefore, it is reasonable to assume that children with FASD experience greater difficulty with relatively complex adaptive tasks during adolescence when social demands are higher. In addition to these deficits in adaptive behaviors in FASD children, numerous reports suggest symptoms of mood disorder (O'Connor et al., 2002) believed to result from an interaction of multiple variables including CNS damage due to prenatal ethanol exposure, familial genetic factors and adverse postnatal experiences

(Autti-Ramo, 2000; Streissguth and O'Malley, 2000).

2.4 Changes in brain structure of children affected with FASD

The most devastating consequences of prenatal alcohol exposure are related to changes in the brain thus leading to the behavioral sequelae. Therefore, these changes have the greatest impact on the lives affected by prenatal alcohol exposure. Changes in brain structure include microcephaly, errors in migration, agenesis of the corpus callosum in addition to anomalies in the anterior comissure, cerebellar basal ganglia, and the brainstem (Mattson, 1996). Prominent changes have been noted in certain parts of brain cortices especially in perisylvian cortices in the parietal and temporal lobes (Archibald et al., 2001), and there are apparent relative increases in gray matter and reduction in white matter in these areas (Sowell et al., 2001). Shape abnormalities of the brain were also noted; specifically, a narrowing of the brain, reduced brain growth in the ventral portions of the frontal lobe mainly in left hemisphere. These findings suggest that the impaired cognitive and behavioral function difficulties are related to frontal lobe function (i.e., response inhibition, behavioral control, and executive functioning) (Olson et al., 1998; Mattson and Riley, 1999). Cortical surface gray matter asymmetry found in control brains is reduced in prenatal alcohol exposure indicating the adverse effects of alcohol persist long after the initial prenatal insult on the developing brain.

Reductions in the size of cerebellum, especially the anterior vermis has been reported in FAS (Sowell et al., 1996). In addition, most significant changes have been reported in the corpus callosum, the fiber tract that connects the two hemispheres. Changes included agenesis or thinning of corpus callosum (Mattson et al., 1992), which explains the deficits

in verbal learning tasks (Kodituwakku, 2006). Also, a reduction in the size of basal ganglia, a group of subcortical neuclei, has been observed in the children with FASD (Mattson et al., 1992).

The hippocampus is one of the brain regions most vulnerable to ethanol exposure *in utero* (Guerri, 1998), and it has been associated with the behavioral deficits. Decreased size of the hippocampus has been observed in some children exposed to alcohol *in utero* (Autti-Ramo, 2000), as well as a reduction in pyramidal cell number (Barnes and Walker, 1981), depression of glutamate release and decrease in glutamate binding (Farr et al., 1988), and alteration of neurotrophic activities (Heaton et al., 1995) in the hippocampus have been observed. The hippocampus is critically involved in memory formation, and such anatomical and neurophysiological changes may underlie the persistent memory and learning deficits commonly noted in FASD children (Streissguth et al., 1990; Uecker and Nadel, 1996, 1998). Dysfunction in the dorsal hippocampus is also suspected since spatial learning, which is dependent upon this structure (Johnson et al., 1997; Hannesson et al., 2001) is affected following prenatal exposure to ethanol (Blanchard et al., 1987; Kim et al., 1997).

2.5 Models of prenatal ethanol exposure

Rodents (rat, mice and guinea pig) have been used as a popular group in alcohol research. In order to use animal model systems to identify and evaluate alcohol-related changes during development and to extrapolate the results to humans, it is important to consider equivalent periods of brain development in the two species. Although all mammals pass through the same stages of brain development, the timing of those stages,

relative to birth, varies among species (Dobbing and Sands, 1979). During the first ten days of gestation in rats (equivalent to the first trimester in humans), neurulation occurs. The initial stage of rat brain development occurs between gestational days (GD) 11-14 (equivalent to the initial part of the second trimester of humans), after the closure of the neural tube on GD 10 (Bayer, 1980). During this period, the neuroepithelium proliferates rapidly and post mitotic neurons appear in the caudal and ventral parts of the developing CNS. Equivalent to the latter part of second trimester in humans, an intermediate stage of brain development takes place in rats between GD 15-18 where the neuroepithelium remains prominent and active and secondary germinal matrices expand. During this period, differentiating neurons are detectable and the brain parenchyma enlarges until all major brain systems are identifiable. Between GD 19-21 and during the first 10-14 postnatal days (PND), the final stage of the brain development occurs and this is equivalent to the third trimester in humans. During this stage, neuroepithelium starts to dissolve and brain parenchyma starts to grow rapidly. In the rat even after birth, neurogenesis occurs, and neurons complete their differentiation and myelination (Bayer, 1994). Therefore, in order to model effects of alcohol exposure during different developmental stages of human brain, rat models are being exposed to ethanol during different perinatal time periods.

Patterns of ethanol consumption have also been demonstrated to play a key role in the resulting teratogenicity. Concentrated bouts (binge-like exposure) are more detrimental than equivalent amounts distributed in lower concentrations over long periods (West, et al., 1989). Maternal alcohol abuse that extends throughout the second and third trimester produces more severe effects on behavioral and cognitive development than drinking

which ends in the second trimester. Therefore, animal models used in evaluating the ethanol insult have been subjected to different paradigms of ethanol concentrations such as single ethanol concentration in the liquid diet, gradual increase of concentrations of ethanol throughout pregnancy. These paradigms mainly focused on mimicking ethanol exposure during the first and second trimester of pregnancy in humans.

A variety of techniques have been used to expose the neonatal rats to ethanol. One of the most popular paradigms is the administration of ethanol containing liquid diet (table 2) to pregnant animals at different time points of their pregnancy. Administration of ethanol-containing liquid diets that contain various concentrations of ethanol ranging from ~3-7% [v/v, blood ethanol concentration (BEC) ranging between 32 and 146 mg/dl, 6-30 mM] has been reported by different investigators.

Studies focused on the late fetal development and/or the neonatal period to mimic exposure during the equivalent of the human third trimester of gestation used paradigms such as intubation of pregnant rats (Diaz-Granados et al., 1997) or guinea pigs (Abdollah and Brien, 1995) during the latter part of pregnancy and early postnatal period which coincides with the brain growth spurt of the hippocampal pyramidal neurons. Artificial "pup-in-the-cup" paradigm was used to rear and feed the pups intragastrically during the first 2 weeks of postnatal period (Bayer, 1980). This artificial rearing technique has been used to minimize the undernutrition as a confounding variable which would have effects on behavior and growth of the animal. Exposure of neonates to ethanol vapor chamber (Gruol et al., 1998; Bellinger et al., 2002) has also been used to mimic the exposure to ethanol during the brain growth spurt period. Although a great majority of published studies of fetal alcohol effects have relied on these procedures, direct intraperitoneal

injections (Pal and Alkana, 1997), and subcutaneous injection of ethanol to the neonatal rats (Ikonomidou et al., 2000) have also been used.

2.6 Behavior alterations in animal models of FASD

Even prior to the definitions of FASD, placentally transferred alcohol was known to accumulate in the fetal hippocampus of rodents and primates (Ho et al., 1972). It has been reported that damage to the vulnerable hippocampus may be responsible for many of the behavioral sequelae (Suzuki et al., 1993). In regard to the hippocampal damage, deficits in spatial navigation (place learning) have been of major concern in prenatal ethanol exposure. In T-maze test, rats exposed to alcohol via maternal liquid diets show a dose-dependent increase in error (Lochry and Riley, 1980), deficits in spontaneous alteration (Zimmerberg et al., 1989; Tan et al., 1990), deficits in spatial reference and working memory (Zimmerberg et al., 1991) and in B6D2F2 mice with deficits in learning (Wainwright, 1990).

In the basic Morris maze task, where rodents are required to swim to a hidden escape platform submerged in a large tank of opaque water, hippocampal damage shows substantial impairments in ability to locate the platform. Perinatal ethanol exposure has shown deficits in spatial learning in the Morris water maze (Blanchard et al., 1987). The radial arm version of the maze test also has shown deficits associated with spatial learning after prenatal ethanol exposure in rodents (Reyes et al., 1989; Pick et al., 1993; Staubli et al., 1994b). In Stone et al., (1996), where deficits in spatial learning was detected, no impairments in passive avoidance were reported suggesting that spatial performance may be more sensitive to behavioral measure of alcohol teratogenicity in the

hippocampus (Greene et al., 1992).

The spatial deficits revealed in T-mazes, Morris mazes, and radial arm mazes illustrate the variable age-dependency of fetal alcohol effects. Some experimental studies show a "catching up" of alcohol-exposed animals to alcohol-naïve animals in some tasks (Meyer et al., 1990). Eventhough effects of fetal alcohol exposure in children and rodents can be long-lasting (Riley, 1990; Maier and West, 2001), this "catching up" observation may explain the reported transient effects of fetal alcohol exposure (Meyer and Riley, 1986). Such reports support the possibility that some fetal alcohol effects are modified with age, and treatment.

Spatial and temporal serial patterns of learning and memory (Riley et al., 1993; La Fiette et al., 1994), sometimes called working memory, have been shown to be impaired as a result of prenatal alcohol exposure. These learning task impairments may also indicate attention problems, although such exact attention deficits have not being reported from rats (Hayne et al., 1992). In addition to spatial learning and memory, Clausing et al., (1995), reported behavioral signs of increased stress/anxiety in the rats exposed to prenatal ethanol. Gender differences as well as time of exposure also show some changes in the extent of the damage caused by alcohol *in utero*. Blanchard et al.,(1987) found that male rats exposed to alcohol prenatally (35% EDC, throughout gestation), were more impaired than females in spatial memory tested in Morris water maze. In contrast, Minetti et al., (1996), found that using a single prenatal alcohol exposure on GD 8 produced greater deficits exist in females than in males in Morris water maze. However, alcohol exposure during PND 4-10 have resulted in impairments in the Morris maze acquisition equally in both genders (Goodlett et al., 1987). But, females were reported to show

greater deficits when high, repeated, binge-like, peak BECs were generated during this same postnatal period (Tran and Kelly, 2003). The timing and duration of exposure may also affect gender differences in the extent of ethanol teratogenicity. Goodlett and Peterson (1995) reported spatial learning deficits in males exposed to ethanol over 2 PNDs (i.e., PND 4-6 or PND 7-9), whereas for females required a full exposure period (i.e., PND 4-9). In males, even a single neonatal day of alcohol exposure on either PND 5 or PND 10 has been reported to be sufficient to impair Morris maze performance in male rats (Pauli et al., 1995).

2.7 Neuroanatomical findings of brain in animal models of FASD

2.7.1 Cell loss

Ethanol exposure during hippocampal development has been shown to delay the generation of neurons or proliferation, rather than causing cell death or changes in density due to changes in area or volume. Studies show that reduced neuronal populations in the hippocampus following prenatal ethanol exposure are regionally selective. Reduction in the number of dorsal hippocampal CA1 region neurons (20%) on PND 60 rats (Barnes and Walker, 1981; Livy et al., 2003), and a 10% reduction in all CA fields on PND 36 (Staubli et al., 1994b) has been reported in ethanol exposure *in utero*. Perez, (1991) reported that with the increase of age (PND 65), reductions ranged from 31% in CA3 to 46% in CA1. Similar to the effects of high BEC binge exposure on behavior, 35% EDC liquid diet influenced significant reductions in the CA1 area ,the pyramidal neuronal counts and the granule cells on PND 21 (Wigal and Amsel, 1990). In Diaz-Grandos et al., (1993), the combined prenatal and neonatal ethanol exposure as well as prenatal or

neonatal exposure alone has been reported to reduce the density of mature granule cell density. In contrast, Miller (1995) reported prenatal ethanol exposure did not affect the numbers of granule cells in dentate gyrus while postnatal exposure to ethanol affected granule-cell numbers in a biphasic pattern: At "moderate" BECs, dentate gyrus-cell numbers were significantly higher; at "moderately high" BECs, there was no effect. And only at the higher BECs, were cell numbers significantly reduced. Lobaugh et al., (1991) reported that changes in cell counts in offspring prenatally exposed to ethanol can vary as the animals age. This study concluded that these offspring did not have significant changes in counts of hippocampal pyramidal cells at PND 21 and 180, following non-spatial behavioral testing.

2.7.2 Neuronal branching and spines

Teratogenic effects of ethanol on hippocampal spine density have been shown to be persistent according to multiple studies. West et al., (1981) first reported that prenatal ethanol (35% EDC) caused abnormal branching of mossy fibers in the ventral hippocampus where they invaded the CA3 infrahippocampal region. Davies and Smith, (1981) reported dendritic arbors which were "simpler" in configuration had a 20% "stunning" in total basilar dendritic length. Numerous studies report a significant decrease in the number of dendritic spines in the CA1 hippocampal region (Ferrer et al., 1988; Tan et al., 1990; Staubli et al., 1994b) after exposure to ethanol in different dosage regimens. However, in contrary to Abel et al., (1983) and Perez et al., (1991) there were no significant differences in dendritic spine densities on PND 90, suggesting a recovery from the teratogenic effects of ethanol on the hippocampus during postnatal maturation

between PND15-90.

Prenatal ethanol exposure induced changes in the hippocampus even in the ultrastructural level. Hippocampal pyramidal cells of adult rats after exposure to prenatal ethanol have shown dense package with less elaborate dendritic arbors in a manner similar to younger animals, suggesting developmental delay. There were also fewer dendritic spines, with no changes in spine length or number of microtubules. Morphological changes have been found in mitochondria and in the distribution of endoplasmic reticulum in CA1 (Smith and Davies, 1990). Rough endoplasmic reticulum in the hippocampus has shown to be dilated upon ethanol exposure prenatally (Suzuki et al., 1993). Changes in numbers of dendritic spines, synapses, or complexity of dendritic fields have suggested the alterations of synaptic plasticity following prenatal ethanol exposure. This is supported by findings in electron microscopy studies where a decrease in synapse turnover was noticed (Hoff, 1988) and several other findings which reported a reduction in collateral sprouting of hippocampal fibers in response to unilateral enterohinal lesions and a decrease in neurite outgrowth in prenatal ethanol exposed rats (Dewey and West, 1984, 1985; West et al., 1984).

2.8 Electrophysiological alterations of hippocampus in FASD rodents

Evidence for the effects of prenatal ethanol exposure on electrophysiology suggests that abnormal neural electrical activity is consistent with the behavioral and neuroanatomical studies. Electrophysiological studies have been carried out at several levels ranging from EEG measurements to *in vitro* hippocampal slice studies. *In vitro* rat brain slice preparation has been used extensively for electrophysiological studies. Hablitz

(1986) reported changes in CA1 hippocampal region stimulation-evoked extracellular field potentials at the age of PND 40-60. Paired-pulse response inhibition at shorter intervals (< 50 msec) was absent after prenatal ethanol exposure but paired-pulse potentiation at longer inter-pulse intervals (> 100 msec) was greatly enhanced. Eventhough the effects of prenatal ethanol on input/output curves were less significant compared to controls, findings suggest ethanol exposed neurons are less responsive for evoked responses (Hablitz, 1986; Tan et al., 1990), when they were exposed to 35% EDC liquid diet from GD 3-21.

Long term potentiation (LTP), a cellular model of synaptic plasticity has been shown to be lower in hippocampal CA1 (Swartzwelder et al., 1988; Tan et al., 1990) of prenatally ethanol exposed rats. Savage et al., (1998), demonstrated that the rate of decay in LTP was accelerated in hippocampal slices from prenatal ethanol exposed rats. The study done by Krahl et al., (1999) reported 6 g/kg/day ethanol administration from GD 8-21 significantly reduced maximal evoked population spike amplitudes of PND 25-32 rats. The same study showed that lower alcohol doses on PND 70 produced no differences in input/output profiles or paired-pulse responses, suggesting that those effects of prenatal alcohol exposure are both dose-related and age-dependent.

The route and pattern of prenatal alcohol exposure also have led to the variable electrophysiological outcomes reported in literature. The liquid diet procedures produced abnormal paired-pulse responses and LTP in CA1 (Hablitz, 1986; Swartzwelder et al., 1988; Tan et al., 1990), whereas the intragastric gavage technique (Krahl et al., 1999), showed intact evoked responses in CA1. Similarly, field EPSPs in the dentate gyrus of rats exposed to 5% ethanol containing liquid diet which led to moderate BEC (~83 mg/dl)

has shown impaired LTP, but not the input/output curves (Sutherland et al., 1997). Another study by Bellinger et al., (1999) suggested that higher BEC (350 mg/dl) achieved after postnatal vapor exposure showed similar reductions in input/output characteristics without changing LTP or PPF. Liquid diet procedures would be more representative of steady ethanol consumption in humans, while gavage procedures may be more representative of binge-drinking patterns resulting in higher BECs. Therefore, pattern of ethanol consumption which would result in varying BECs exposing the fetus to different levels and peaks might have led to the different neurobehavioral, anatomical and electrophysiological effects (Berman and Hannigan, 2000).

EEG studies carried out in animals exposed to ethanol *in utero* have shown latencies of the P1 and N1 of auditory event-related potentials (ERPS) (Kaneko et al., 1993), altered theta activity and slow rhythmic EEG activity indicative of hippocampal damage (Cortese et al., 1997).

In summary, *in vivo* and *in vitro* findings demonstrate abnormal hippocampal electrophysiological activity resulting from prenatal ethanol exposure are consistent with neuroanatomical (i.e., loss of principal neurons in the hippocampus, mossy fiber projections) and behavioral alterations (i.e., deficits in spatial learning task). Considered together, evidence suggests that at least some of the cognitive deficits seen in FASD may result from hippocampal damage.

2.9 Theories of FASD

There are two major and widely accepted theories of FASD: 1) Synaptic dysfunction associated with glutamatergic neurotransmission and 2) Alterations in GABAregic

transmission. These dual mechanisms- blockage of NMDA glutamate receptors and hyperactivation of $GABA_A$ receptors (Ikonomidou et al., 1999) result in reduced brain mass and lifelong neurobehavioral disturbances associated with FASD. However, much emphasis is placed on the glutamatergic neurotransmission theory, which is the most common and widely studied.

2.10 Glutamatergic Synaptic Transmission

Glutamate is a major neurotransmitter that mediates synaptic excitation at a vast majority of synapses in the CNS. Glutamate is involved in many important brain functions, such as differentiation, neuronal cell survival and death, proliferation and the development of neuronal and glial cells, and plastic changes in efficacy of synaptic transmission underlying memory and learning and formation of neural networks during development (Mayer and Westbrook, 1987; Dingledine et al., 1988; Monaghan et al., 1989).

Glutamate receptors (GluRs) are categorized into two distinct classes, ionotropic and metabotropic receptors (Nakanishi, 1992; Seeburg, 1993; Hollmann and Heinemann, 1994). The ionotropic receptors (iGluRs) which contain cation-specific ion channels, are further subdivided into three groups on the basis of agonist specificities; α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptor channels. Metabotropic receptors (mGluRs) are coupled to GTP-binding proteins (G-proteins) and modulate the production of intracellular messengers. Fast glutamatergic neurotransmission is mediated via iGluRs.

2.10.1 Ionotropic glutamate receptors

Glutamate binding to iGluRs opens the receptor channel permitting the flux of Na^+ and K^+ ions down their electrochemical gradients. As a consequence of this ion flow, plasma membranes depolarize and generate excitatory postsynaptic potentials (EPSP) causing membrane potential to shift from -70 mV towards more positive values. Depolarization generates an electrical current that propagates down the dendrites and axons of the neuron.

Structures of ionotropic glutamate receptors

Ionotropic glutamate receptor subunits possess an extracellular amino terminal domain, followed by a first transmembrane domain and then a pore forming membrane-residing domain that does not cross the membrane but forms a reentrant loop entering from and exiting to the cytoplasm (Fig. 2.4). The second and third transmembrane domains are linked by a large extracellular loop and the third transmembrane domain is followed by an intracellular carboxy-terminus (Dingledine et al., 1999; Mayer and Armstrong, 2004). The agonist binding domain is located in a pocket formed between the extracellular N-terminal region (S1 region) and the extracellular loop between transmembrane domains 3 and 4 (S2 region).

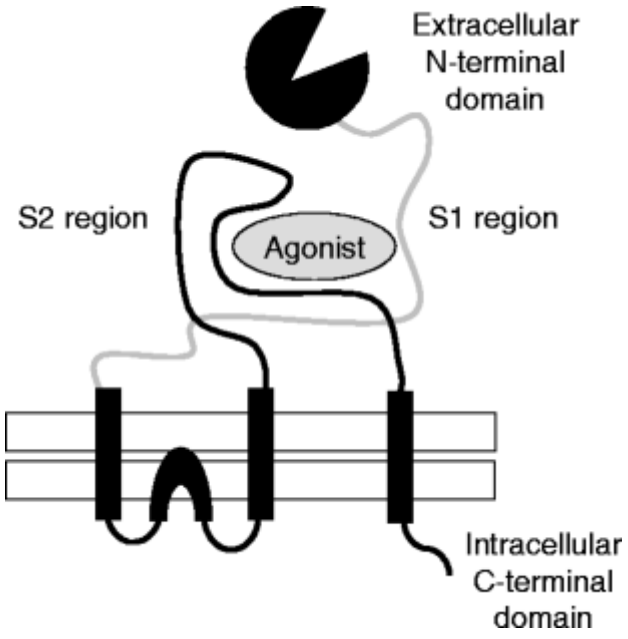

Figure 2.4 Schematic representation of an iGluR family member. The extracellular amino terminal domain is followed by a first transmembrane domain and then a pore forming membrane-residing domain that forms a re-entrant loop. The second and third transmembrane domains are linked by a large extracellular loop and the third transmembrane domain is followed by an intracellular carboxy-terminus. The agonist binding domain is located in a pocket formed between the extracellular N-terminal region and the extracellular loop between transmembrane domains 3 and 4

2.10.1.1 AMPA receptors

AMPARs are composed of a four-subunit family (GluR1-4) that are products of separate genes and are believed to assemble as functional tetramers (Rosenmund et al., 1998). They are heteromeric in composition. Each AMPAR subunit (GluR1-4) has ~900 amino acids and has a molecular weight of ~105 kDa (Rogers et al., 1991). Eventhough there is approximately 70% sequence homology between genes encoding each subunit, at two distinct sites genes may undergo alternative splicing. This results in subunits that have either long or short C termini, and flip or flop variants in the extracellular domain (Black and Grabowski, 2003). Whereas flip variants are predominantly prenatal, flop variants become expressed postnatally and reach levels of those of flip in the adult. The flip and flop splice variants have effects on the rate and extent of desensitization of heteromeric AMPARs and also influence their sensitivity to allosteric modulators.

Desensitization rate of flip variants is about four times slower than flop (Mosbacher et al., 1994; Koike et al., 2000) (discussed in detail below). Short or long intracellular regions in GluR2 and GluR4, resulting from C-terminal splice variants, play a role in intracellular protein-protein interactions and receptor clustering (Dingledine et al., 1999) because the PDZ binding motif is only present in the short form (Dev et al., 1999).

Cation pore channel formed by the reentrant loop (Kuner et al., 2003) of the AMPAR subunits contributes to the gating of Na^{+} and Ca^{+2}, perhaps due to the larger pore size (Tikhonov et al., 2002). Na^{+} and Ca^{+2} gate through the AMPARs, in response to ligand binding, with conductance and kinetic properties of the receptor depending on the subunit composition (Mat Jais et al., 1984; Hollmann et al., 1991; Jonas, 1993). Influx of ions causes a fast excitatory postsynaptic response. The GluR2 subunit plays a key role in determining the permeability of Ca^{+2}. Thus, GluR2 containing AMPARs have a low permeability to Ca^{+2} and low single channel conductance (Burnashev et al., 1992; Heaton et al., 1995; Burnashev, 1996).

Post-translational modifications that occur in the AMPARs are glycosylation and phosphorylation. Glycosylation of different AMPAR subunits possesses different functional effects. Only surface and synaptically expressed AMPARs have been proposed to have the mature glycosylated form (Weaver et al., 1993; Standley et al., 1998). Phosphorylation of AMPARs by calcium and kainase profile regulates the properties of the channel, its intermolecular interactions, and trafficking of the protein (Swope et al., 1999).

AMPARs start to appear at mRNA levels at embryonic stages of rat brain development with GluR2 subunit being ubiquitous (Monyer et al., 1991). Expression

levels of GluR1-4, increase gradually, and peak in the third postnatal week (Morrisett et al., 1989; Insel et al., 1990; Durand et al., 1996; Arai et al., 1997). AMPAR incorporation into the plasma membrane occurs prior to synaptogenesis when GluR1-containing AMPARs cluster at potential postsynaptic sites (Morrisett et al., 1989).

Several studies indicate that AMPARs are widespread and varied in distribution (Hollmann and Heinemann, 1994). GluR1, GluR2, GluR3 are widely spread in the hippocampus, outer layer of cortex, olfactory regions, lateral septum, basal ganglia and amygdala (Keinanen et al., 1990; Beneyto and Meador-Woodruff, 2004), whereas the GluR4 is less well pronounced in rat CNS, except in the thalamic nuclei and the cerebellum (Morrisett et al., 1989; Petralia and Wenthold, 1992; Spreafico et al., 1994).

AMPARs are distributed in the synaptic membrane, postsynaptic density (PSD) (Rogers et al., 1991; Archibald and Henley, 1997) extrasynaptically and within the cytoplasm of individual neurons (Baude et al., 1994, 1995). AMPAR insertion and removal at the postsynaptic membrane and maintenance of functional clusters play an important role in synaptic plasticity (Lu et al., 2001). Two basic processes that are involved in delivery to the correct postsynaptic location are direct exocytosis to the site of action, or insertion into the membrane at a separate location followed by subsequent diffusion to the post synaptic density. AMPAR internalization and reinsertion into the postsynaptic membrane occurs in 10-20 minutes time. GluR2 appears to be the dominant subunit when deciding the fate of internalized receptors (Lee et al., 2004).

Synaptic expression of functional AMPARs is highly regulated during development and by neuronal activity. Silent synapses which contain NMDARs but no AMPARs (Liao et al., 1999) recruit AMPARs to the synapses soon after the NMDARs get activated

(Fitzjohn et al., 2001; Liao et al., 2001; Lu et al., 2001; Pickard et al., 2001), and this "unsilencing" of AMPARs plays a key role in NMDAR dependent synaptic plasticity and neuronal development (Durand et al., 1996). Long-term potentiation (LTP), a cellular mechanism for learning and memory (Bliss and Lomo, 1973) has been suggested to be a result of strengthening of synaptic transmission due to high frequency stimulation of glutamate receptors of which modulation of AMPARs has a key role.

The potential to pharmacologically influence the AMPARs is being clinically exploited. Three binding sites of AMPARS which are pharmacologically studied are (1) glutamate binding site (agonist AMPA and various clinically effective antagonists bind to this site i.e., topiramate, YM90 K), (2) an uncompetitive binding site at which several agents including piracetam and aniracetam intervene by positively modulating the AMPARs (which is important in physiological processes such as memory and cognition, and (3) ion channel binding site where various insect toxins mediate the action (Bleich et al., 2003).

Exposure to ethanol has been shown to produce profound functional and structural alterations in CA1 region of rat hippocampus, and these alterations may contribute to the behavioral deficits associated with FASD. Blockage of NMDARs during development was shown to produce apoptotic neurodegeneration in several regions of the CNS, including the hippocampus (Ikonomidou et al., 1999, 2000). The neuroteratogenic effects of alcohol are thus assumed to be mediated, in part, via this NMDAR-dependent mechanism. Little is known on the effects of ethanol on AMPA and/or kainate receptors which also have important roles during CNS development and function (Ozawa et al., 1998). Therefore, it is reasonable to suggest that ethanol may actually damage CA1

pyramidal neurons via inhibition of postsynaptic AMPARs and a decrease in glutamate release, due to the expression of AMPAR subunits even during the embryonic stage. Therefore, AMPARs may represent a potential target for the development of a specific treatment strategy to counteract the loss of synaptic AMPAR function in prenatal ethanol exposure.

2.10.1.2 NMDA receptors

The NMDAR family is composed of seven subunits, NR1, NR2A-D and NR3A and B, which are all products of separate genes. The expression of these subunits is developmentally regulated. NR2B subunit expression is higher in the fetus and at early postnatal ages, whereas expression of NR2A and NR2C does not become prominent until PND 2 (Zukin and Bennett, 1995). Functional NMDARs appear to be composed of NR1 and at least one NR2 subunit, or NR1 and both NR2 and NR3 subunits. Therefore, expression of the NR1 and NR2 subunit together is essential to form functional channels, since the glutamate binding domain is formed at the junction of NR1 and NR2 subunits. The NMDAR is unique among ligand-gated ion channels since it requires two obligatory co-agonists, glycine and glutamate which bind to sites at NR1 (Kuryatov et al., 1994; Wafford et al., 1995; Hirai et al., 1996; Kew et al., 2000) and NR2 subunits (Heaton et al., 1995; Laube et al., 1997). NMDAR activation has been shown to require occupation of two independent glycine sites and two independent glutamate sites (Benveniste and Mayer, 1991; Clements et al., 1992). The N-terminus and C-terminus of the NMDAR subunit contains the sites for splice variation, which are important in the regulation of intracellular interactions with PDZ. Application of L-glutamate produces a considerably

shorter offset decay time in the NR2A subunit (Monyer et al., 1994). Thus, physiology of cells containing NMDARs is mainly determined by the NR2A and NR2B subunits. At resting membrane potentials, NMDARs are inactive due to a voltage dependent Mg^{2+} block. Depolarization of the postsynaptic membrane activates the NMDAR by removing this channel block permitting the flow of both monovalent and divalent ions.

Normal functioning of NMDARs is critical for certain aspects of neurodevelopment, including cell growth, proliferation, differentiation, migration and plasticity. Alterations in NMDAR function and subunit expression can seriously impact on normal neurodevelopment. Extensive studies have been conducted on the effects of ethanol exposure *in utero*, on NMDAR neuro anatomy in many brain areas (Ikonomidou et al., 2000) expression of NR subunits (Spuhler-Phillips et al., 1997; Nixon et al., 2004) and NMDAR function (Morrisett et al., 1989; Weaver et al., 1993). Therefore this study did not focus on the investigation of expression and function of NMDARs.

2.10.1.3 Kainate receptors

Kainate receptors are composed of two related subunit families, GluR5-7 and KA1-2. Evidence suggests that in the absence of GluR5-7 subunits, KA-2 cannot achieve cell surface expression but it is retained in the endoplasmic reticulum (Gallyas et al., 2003). In the hippocampus, GluR6 appears to be the critical subunit in both pre- and post-synaptic kainite receptors at mossy fibre synapses in the CA3 region (Contractor et al., 2003). Kainate receptors typically exhibit fast desensitization upon agonist binding. However, physiological importance of kainate receptors still remains largely unknown.

2.10.2 Determining synaptic transmission

At most central synapses, both AMPA and NMDARs are activated during synaptic transmission. AMPARs mediate the fast neurotransmission thus enabling the use of their rapid kinetics in the evaluation of the properties of synaptic AMPARs. The time course of EPSCs mediated by AMPARs depends on two factors: glutamate concentration at the synapse and the properties of the postsynaptic receptors. The amount of glutamate released from the presynaptic terminal and the rate at which it is removed by diffusion and/or uptake determines the transmitter concentration in the synaptic cleft. The affinity of the receptors for glutamate and their deactivation and desensitization kinetics control the time course of synaptic currents produced by the available transmitter in the synaptic cleft. The duration of the transmitter in the synaptic cleft is very brief in most synapses, and the deactivation rather than desensitization plays a major role in determining the decay rate of EPSCs. However, there is evidence that both deactivation and desensitization play a major role in determining the time course of EPSCs in the presence of pharmacological compounds such as aniracetam. In addition to deactivation and desensitization, changes in number of functional AMPARs, channel kinetics such as conductance, probability of channel opening, and open and close time of AMPARs may also trigger synaptic activity.

Techniques such as LTP, LTD used in recording synaptic transmission mainly target the electrical activity of neural soma. A major limitation of these methods is that the site of interest (synapse) and the site of recording (soma) are two different locations. When recording EPSCs from a highly branched dendritic tree of a CA1 pyramidal cell, this problem becomes more important. Therefore, infrared video microscopy has been used to

record from dendrites (Stuart et al., 1993). However, these methods are rather relative and do not represent realistic synaptic transmission at basal level and/or at single channel level. It is of great interest to determine the synaptic parameters at cellular level and subcellular level which would alter the behavior of the animal. Therefore, in this study we have used a unique approach to address the issue of alterations in animal behavior, using a single neuronal response (miniature and spontaneous currents) to single channel properties of hippocampal synaptic receptors. To further support our *in vitro* and *in vivo* observations, we investigated the expression levels of proteins responsible for the observed synaptic and behavior alterations.

2.11 Prenatal ethanol exposure and glutamatergic transmitter system

Studies suggest that the effects of ethanol on developing CNS are, to some extent, the result of alterations in neurotransmission at glutamatergic synapses which are critical for maturation of neuronal circuits (Costa et al., 2000; Ikonomidou et al., 2001; Zhang and Poo, 2001; Hua and Smith, 2004; Olney, 2004). Three major types of channels (NMDA, AMPA and KA) important in the glutamatergic transmitter system have been shown to be affected in prenatal ethanol exposure to variable extents depending on the location, ethanol paradigm, etc (Table 2.2).

2.11.1 Prenatal ethanol exposure and NMDA receptors

Studies to date principally report the effects of prenatal ethanol exposure on the NMDA subtype of glutamate receptors. Blockage of NMDA receptors by ethanol in neonates has resulted in apoptotic neurodegeneration in many brain areas including the hippocampus, cortex, hypothalamus, thalamus and caudate nucleus (Ikonomidou et al.,

2000). NMDA sensitive component of ^{3}H-glutamate binding sites has been shown to be reduced in the dentate gyrus and the dorsal hippocampus following prenatal ethanol exposure (Savage et al., 1991). Decreases in glutamate and NMDA receptor number (B_{max}), without altering the affinity (K_d) has been shown in the hippocampus of guinea pig (Abdollah and Brien, 1995). A significant reduction in expression levels of NR2A in, and NR2B in forebrain and hippocampus has been reported after ethanol exposure *in utero* (Nixon et al., 2004). Functional alterations of NMDA receptors include the reduced sensitivity of the hippocampus to NMDA and increased inhibitory effects of Mg^{2+} (Morrisett et al., 1989). The NMDA mediated (Weaver et al., 1993; Spuhler-Phillips et al., 1997) as well as resting intracellular Ca^{2+} levels (Gruol et al., 1998) have been shown to be reduce upon ethanol insult *in utero*.

Table 2.2. Effects of prenatal and/or early postnatal ethanol exposure on ligand-gated ion channels

NMDA					
Liquid diet (3.5 or 6.7%) GD 1-21	39-70	45 PND hippocampus	^{3}H-Glutamate binding	↓	Farr (1998)
Liquid diet (3.5 %) GD 1-21	39	Hippocampal slices-adult	NMDA currents Mg^{2+} currents	↓ ↑	Morriset (1989)
Liquid diet (3.5 %) GD 16-21	39	45 PND hippocampus	NMDA-sensitive ^{3}H-Glu binding	↓	Savage (1991)
Liquid diet (GD 1-3, 0%, GD 4-6, 3.85 %, GD7-9, 5.8% -and 7% until birth)	32-52	Neonatal dissociated brain -neurons	NMDA-dependent Ca^{2+} -elevations	↓	Weaver (1993)
Liquid diet (GD 1-2, 0%, GD 3-4, - 3.85 %, GD5-6, 5.8% - and 7% until birth)	120-144	Neonatal dissociated brain -neurons	NMDA-dependent Ca^{2+} -elevations	↓	Lee (1994)
Guinea pig, oral intubation -(4g/kg/day) GD 2-62	270	Near-term fetal hippocampus	Glu and NMDA binding	↓	Abdollah (1995)
Intubation (5g/kg/day) GD 12-18 followed by artificial rearing (6.4 ml/kg 95% EtOH) PND 4-9	143 429	20-23 PND hippocampus - and cortex	^{3}H-MK-801 binding	↓	Diaz-Grandoz (1997)
Liquid diet (GD 1-2, 0%, GD 3-4, -3.85 %, GD5-6, 5.8% - and 7% until birth)	130-146	Neonatal dissociated brain -neurons	NMDA-dependent Ca^{2+}	↓	Spuhler-Phillips(1997)
Artificial rearing (6g/kg) at PND 6	310	Live adult PND 40	Serial spatial discriminate -ion task	Deficits	Thomas (1997)
Liquid diet (GD 3-4, 3.85 %, -GD5-6, 5.8% - and 7% until birth)	119-138	1-21 PND membranes from forebrain and hippocampal neurons	Subunits (WB): NR1, - NR2A and NR2B	No effect ↓ some - regions	Hughes (1998)
Chronic exposure, vapor-Chamber (PND 4-7)	318	Neonatal granule cerebellar -neurons	NMDA-mediated, Ca^{2+}	↓	Groul (1998)
Non-NMDA					
Liquid diet (3.35%) GD1-21	39	Hippocampus of adult neurons	AMPA and VKA binding and currents	no effects	Martin (1992)
Liquid diet (3.35 or 6.7%) -GD1-21	39-70	45 PND hippocampus	^{3}H-VKA binding	↓	Farr (1988)
$GABA_A$					
Gastric intubation (9.6 g/kg/day -GD1-3, 0%, GD 4-5, 2%, -GD 15-18, liquid diet (5%) -GD 6-7, 3% and GD 7-21, 5%	Not determined 83 mg/dl	Cortical neurons-adult Membrane vesicles-frontal -cortex, cerebellum, hippo-adult	GABAergic response GABA-stimulated ^{36}Cl flux $GABA_A$ R stimulation	↑ No effect	Janiri (1994) Allan (1998)
Artificial rearing (4.5g/kg/day), -in 2 of 12 daily feeds between -PND 4-9	Not determined	Medial septum /diagonal band neurons (PND 4-10, 11-16, 25-35) Purkinje cells from juveniles PND 12-16) and young adult -(PND 25-35)	GABA currents ↓ GABA R maximum current--densities	↓	Hsiao (1998) Hsiao (1998)
Guinea pig-Oral intubation-(4g/kg/day) GD2-67	235	Cortical cell membrane--preparation (PND 11,21, 61)	^{3}H-Flunitrazepam--binding to -GABA Rs	↑ GABAR - number, ↓ affinity for	Bailey (1999) flunitrazepam

2.11.2 Prenatal ethanol exposure and non-NMDA glutamate receptors

Little is known about the effects of prenatal ethanol exposure on AMPA and/or kainate receptors. A decrease in specific ^{3}H-vinylidene kainate binding in hippocampal CA1 stratum lucidum has been reported (Farr et al., 1988). However, studies of AMPA-induced or kainate-induced depolarizations in hippocampal CA1 pyramidal neurons found no differences in prenatal ethanol exposed conditions. No significant differences in the density of ^{3}H-AMPA binding sites in the hippocampus have been reported (Martin et al., 1992). Therefore, reports suggest that effects of prenatal or early postnatal ethanol exposure do not have a significant effect on non-NMDA receptors in the same manner as NMDA receptors. However, Bellinger et al., (2002) reported that expression of GluR1 subunit of AMPA receptors are decreased upon ethanol vapor exposure to neonatal rats.

There is evidence that the number of molecules needed for the proper function and expression of AMPA receptors are being altered by prenatal ethanol exposure. Neural cell adhesion molecule (NCAM) which is important in the proper targeting of AMPA receptors to the active zone of the synapse has been shown to be altered. Failure to down regulate PSA-NCAM during postsynaptic elaboration has been shown to promote the migration of the synapses to ectopic locations thereby resulting in gross structural brain deficits, and plasticity impairments observed in FASD (Edelman and Choung, 1982; Minana et al., 2000). Alternatively, other cell adhesion molecules such as L1 which is important in cell surface expression of NCAM, neurite outgrowth (Bearer et al., 1999), and cell adhesion or aggregation (Charness et al., 1994) has been shown to be impaired by prenatal ethanol exposure. Protein kinase C (PKC) activity, expression of presynaptic GAP-43 which is important in the proper function of AMPA receptors and synaptic

plasticity have also been shown to be impaired in prenatal ethanol exposure (Perrone-Bizzozero et al., 1998; Tanner et al., 2004).

2.12 Epidemiology and management of FASD

FASD has been reported across the world (Tan et al., 1990). In the United States, this is a public health problem and an important cause of morbidity and mortality. Prevalence estimates for FAS is 1 to 1.5 cases per 1000 live births (Sampson et al., 1997; Bagheri et al., 1998). However, less complete and widely variable manifestations (FAEs) that do not meet the full criteria for FAS are six to eight times more prevalent (Sampson et al., 1997; Bagheri et al., 1998). Thus, even assuming 1.0 case of FASD per 1000 live births, the number of new cases in the United States would be 39,000 per year and accordingly the total number of affected people (children and adults) would exceed 2.6 million. Annual mortality rate from FASD is over 6% or about 2100 to 2300 adults.

2.12.1 Costs of FASD

Annual cost estimates range from US$74.6 million (Tan et al., 1990) to US$9.7 billion per year (Bagheri et al., 1998). A lifetime cost of care (neonatal care, management of developmental delays, and birth defects, years of special education, decades of developmental disabilities services, costs to the criminal justice system, alcohol and drug abuse treatment, mental health services, health care costs, and a life time of supported living costs) per case is estimated to be about US$1.4 million (Bagheri et al., 1998).

2.12.2 Treatment of FASD

At present there are no specific pharmacological treatments available for children or pregnant women to treat the effects associated with FASD (Riley et al., 2003). The first attempts to influence some behavior reactions resulting from prenatal ethanol exposure were with psychotherapeutic CNS stimulants. Clinical efficacy of methylphenidate (Ritalin), d-amphetamine (Dextrine), premoline (Cylert), and in rare cases caffeine has been used to treat the attention disorders and hyperactivity in children associated with FASD. Early dietary supplementation of choline, the precursor of acetylcholine as an essential nutritional supplementation has been shown to enhance memory capacity of young adults and appears to prevent age related memory and attention decline. However, no specific pharmacological treatment has been reported to improve more complicated behavior impairments such as learning and memory in FASD victims.

2.13 Potential role of nootropic pyrrolidone class of AMPA receptor potentiators in the treatment of brain disorders

Nootropics are substances which boost human cognitive abilities. The word nootropic was coined by Giurgea in 1972, using the Greek words *noos* or mind and *troops*, a bend. The class of AMPAR potentiators described to date includes the nootropic pyrrolidones (piracetam, oxiracetam and aniracetam), related piperadine compounds (1-BCP, BDP12, or CX516, CX546), the benzothiodiazenedioxides (diazoxide, cyclothiazide, S189861, IDRA-21) and the birrylsulphonamides (LY392098, LY395153, LY414187, LY503430). Almost thirty years have passed since the “Nootropic Revolution” began with the discovery of the piracetam-like nootropics, in the late 1960’s. In the late 1970’s the newer

compounds such as Oxiracetam, Pramiracetam and Aniracetam were discovered.

2.13.1 Pyrrolidones

The most investigated compounds in this category are piracetam, oxiracetam, pramiracetam, etiracetam, niferacetam, aniracetam and rolziracetam (Fig. 2.5). This group of drugs shares a common pyrrolidone ring. Small changes in structure often lead to compounds with different chemical properties (i.e., poor hydrolytic stability of aniracetam and rolizaracetam compared to the highly stable piracetam). The mechanisms of action of this group of drugs are quite broad. They work by: increasing the brain's supply of neurochemicals (neurotransmitters, enzymes, and hormones), increasing the brain's oxygen supply, or by stimulating nerve growth, enhancing learning and memory. General properties of these compounds are: enhancement of learning and memory, facilitation of flow of information between the cerebral hemispheres, enhancement of the resistance towards chemical and physical injuries, lack of usual physiological and general cardiovascular pharmacological activity of psychopharmaca and being harmless in clinical practice and having no side effects (Coper and Herrmann, 1988). They are usually prescribed to treat brain disturbances and intellectual disorders caused by alcohol, tranquilizers, neuroleptics, depressants, barbiturates and agents impairing the brain circulation. These cognitive enhancers are also widely used in pediatrics and geriatrics for the treatment of cerebral ischemia and encephalopathic disturbances (Platt et al., 1993). They also have been used in the prematurely born infant to treat intrauterine hypoxia and other illnesses (Foltyn et al., 1983; Coper and Herrmann, 1988; Gamzu, 1989; Voronina, 1989; Canonico, 1991; Levinson, 1991; Senin et al., 1991; Domna,

1998).

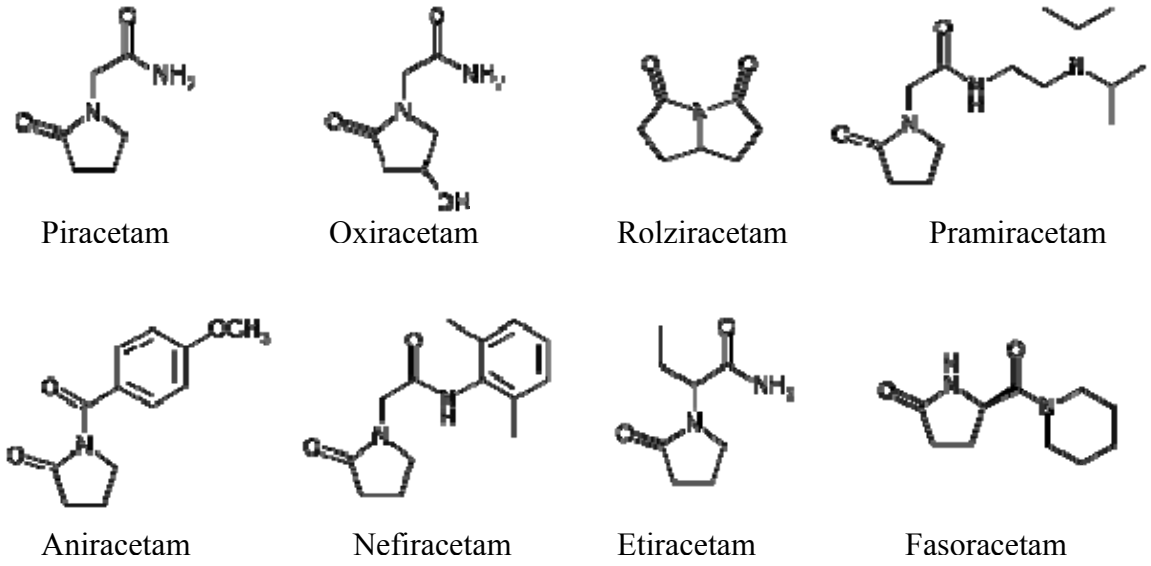

Figure 2.5. Most investigated pyrrolidineacetamide compounds

2.13.2 Nootropic pyrrolidone: Aniracetam ($C_{12}H_{13}NO_3$)

Aniracetam [1-(p-anisoyl)-2-pyrrolidinone, Draganon, Memodrin, Sarpul/Sarple, Ampamet, Reset] was first reported as a nootropic in 1979. In 1993, it was first introduced into clinical practice. Like other "racetams", aniracetam contains the pyrrolidinone ring, but does not contain an acetamide group.

Pharmacokinetics

This drug is absorbed very rapidly from GI after p.o. administration but its bioavailability is low due to extensive first-pass metabolism. In rats, peak plasma levels of aniracetam are reached 20-30 minutes after oral administration and the half life is

1.7-2.1 hours (Ogiso et al., 1998). In humans, two hours after administration, the metabolites reach the highest blood concentration and plasma levels of the metabolites reach baseline within six hours (Endo et al., 1997). Total body clearance is as high as 10 l/min (Guenzi and Zanetti, 1990).

In rodents, effective doses are usually in the range of 10-100 mg/kg and the commonly recommended clinical dose is 1500 mg daily (Manufacture's insert for Draganon). This compound is quite unstable *in vivo* and is transformed to the more stable amide and acid. In humans the main metabolite of aniracetam is *N-anisoyl*-GABA (70%) and the remaining (30%) appear as 4-methoxybenzoic acid and 2-pyrrolidinone. In rodents the main metabolite appears to be p-anisic acid. All of these metabolites, along with aniracetam itself, have been implicated in the activity profile of the drug (Suzuki et al., 1993; Shirane and Nakamura, 2001; Ghelardini et al., 2002; Miyamoto et al., 2003). The multiple metabolites and their different activity profiles, may be one of the reasons this drug has such diverse benefits (Tolson, 2006)

The nootropic properties of aniracetam have been studied using animal models. In tests categorized as "tests of learning", aniracetam minimized the negative effects of scopolamine and hypoxia in rats and scopolamine in monkeys. In maze tests, aniracetam prevented the effects of scopolamine and basal forebrain lesions in rats. In passive avoidance tests and active avoidance tests aniracetam has shown anti-amnesic effects (Gouliaev and Senning, 1994). Other research using animal models have shown the effect of aniracetam to block the amnestic effect of 6-hydroxydopamine, ischemia, methamphetamine treatment, apomorphine, low-intensity electromagnetic fields, motion sickness, fetal alcohol syndrome, aging, and alprazolam (Stancheva et al., 1993; Suzuki

et al., 1993; Himori and Mishima, 1994; Zivkovic et al., 1995; Bartolini et al., 1996; Iasnetsov et al., 1996; Vaglenova and Vesselinov Petkov, 2001). Aniracetam has been shown to be effective in the two-lever choice task, the radial arm maze (which tests working memory and spatial memory), the Y-maze, and object recognition (which tests episodic memory) (Lebrun et al., 2000; Rao et al., 2001; Shirane and Nakamura, 2001). Moreover, effects of aniracetam on healthy adult or young animals have shown positive or equivocal results (Gouliaev and Senning, 1994; Zajaczkowski and Danysz, 1997). Thompson et al., (1995) reported that aniracetam improved conventional learning in monkeys only when the complexity of the task was increased. In addition to the effect of aniracetam on improving learning and memory, it has been reported to be beneficial in the animal models of depression and anxiety (i.e., forced swim test, the reduction of submissive behavior model, the social interaction test, the elevated plus-maze, and conditioned fear stress) (Suzuki et al., 1993; Knapp et al., 2002). Aniracetam has also been shown to improve the experimentally-induced deficits in vigilance, age-related deficits in temporal regulation of behavior (Suzuki et al., 1993), motivation in animals (Suzuki et al., 1993), and REM sleep (Kimura et al., 2000).

Using human studies, aniracetam has been effective in the treatment of mild to moderate dementia of vascular origin (Yu and Cai, 2003), Manufacturer's insert for Draganon and Ampamet), Alzheimer's disease, and in patients with brainstem infarction (Zajaczkowski and Danysz, 1997; Kihara et al., 2001). In patients with Alzheimer's disease, Parkinson's and cerebral infarction, aniracetam has been shown to reduce anxiety, depression, and sleep disorders while improving the vigilance. It is also reported to be a very effective treatment for the post-stroke depression and sleep disorders (Suzuki

et al., 1993). To date there are no studies concerning the use of aniracetam in healthy, unimpaired humans. However, Gouliaev and Senning (1994) reported that aniracetam reduced learning deficits induced by hypoxia in healthy humans. Since piracetam, an analogue of aniracetam has been shown to improve learning and memory in healthy humans, it is reasonable to speculate that aniracetam is superior to piracetam (Tolson, 2006)

Safety

Like other nootropics, aniracetam is very safe. LD50 is 4.5 g/kg orally in rats and 5.0 g/kg orally in mice (Gouliaev and Senning, 1994) and for a man of 80 kg, this would equate to over 500 times the standard dose of 1.5 g. No toxic or teratogenic effects have been reported (Gouliaev and Senning, 1994; Shorvon, 2001). One potential problem reported with aniracetam is excitotoxicity in an animal model of multiple sclerosis; this has been presumed to be due to the effects of aniracetam on AMPA transmission (Groom et al., 2003). However, other literature contradicts this notion regarding the aniracetam induced excitotoxicity. Thompson et al., (1995) suggested that aniracetam does not disrupt the physiological oscillation of glutamatergic transmission, since it is an allosteric modulator with a relatively low intrinsic activity at AMPA-sensitive glutamate receptors. Multiple studies indicate that aniracetam protects against excitotoxicity (Pizzi et al., 1995; Shirane and Nakamura, 2001; Shorvon, 2001; Yu and Cai, 2003). It also has other neuroprotective effects such as reducing free radical formation and improving glucose metabolism (Himori et al., 1995; Ouchi et al., 1999). Induction of release of inhibitory neurotransmitters during neuronal injury has also been reported (Yu and Cai, 2003).

Mode of action

Aniracetam interacts with γ-aminobutyric acid (GABA) neurotransmission (Nabeshima et al., 1990a, 1990b) glutamatergic neurotransmission, cholinergic transmission, dopamine transmission, and steroid and protein/lipid metabolism. Aniracetam has a weak effect on GABA receptors. Aniracetam exerts its effect on acetylcholine by increasing the ACh content in hippocampus and cortex in the presence and absence of scopolamine-induced depletion of ACh (Spignoli and Pepeu, 1987; Toide, 1989). Aniracetam has been reported to decrease the dopamine (DA) level in the striatum and the hypothalamus (Petkov et al., 1984) by inhibiting monoamine oxidase A (MAO_A) (Stancheva and Alova, 1988). Inhibition of steroid biosynthesis has been shown to suppress the memory improving effect of aniracetam, thus indicating an interaction with steroids (Mondadori and Petschke, 1987; Mondadori et al., 1989; Mondadori et al., 1990). The antagonizing effect of aniracetam on amnesia induced cycloheximide, a protein synthesis inhibitor, has been suggested to prove the interaction of aniracetam with protein/lipid metabolism (Nabeshima et al., 1991). However, the main mode of action of aniracetam has been shown to be due to the interaction with glutamatergic transmission by positively modulating metabotropic glutamate receptors and AMPA receptors.

Aniracetam has been shown to enhance the efficacy but not the potency of AMPA-induced calcium influx in cerebellar granule cells (Triggle, 1990), while not changing the receptor binding affinity for AMPA nor the ion conductance (Ito et al., 1990). Aniracetam has been shown to affect only fast synaptic currents mediated by AMPA, but not NMDA or kainate (Ito et al., 1990; Kaneko et al., 1991; Tang et al., 1991; Ozawa et

al., 1998). Aniracetam has also been reported to have selectivity for flop splice variants vs. flip splice variants (Johansen et al., 1995; Partin et al., 1996). Aniracetam appears to exert its effects on AMPA receptors by slowing the rate of deactivation of the receptor (channel closing as consequence of transmitter dissociation) and slowing the desensitization (Partin et al., 1996; Arai and Lynch, 1998) (Fig 2.6).

Deactivation and desensitization are two mechanisms by which glutamate receptors terminate the flow of ions through the channel. These processes involve transduction of an allosteric signal from the agonist binding pocket to the pore. Previous work has demonstrated that the flip/flop domain (Sommer et al., 1990) which forms the part of the extracellular M3-M4 loop (Hollmann et al., 1991; Bennett and Dingledine, 1995) of AMPARs, is necessary for signal transduction between the agonist-binding pocket and the pore (Partin et al., 1996), and regulates the kinetics and the onset of and recovery from desensitization. This region has been shown to play a critical role in AMPAR synaptic function (Partin et al., 1996) and determine the AMPAR sensitivity to aniracetam and cyclothiazide (Sommer et al., 1990; Johansen et al., 1995). Aniracetam has been shown to modulate desensitization as a consequence of slowing channel closing (Partin et al., 1996) by selectively potentiating the responses of flop isoforms (Tomita et al., 2006) which desensitizes and deactivates faster than the flip version, on which cyclothiazide selectively acts (Partin et al., 1996).

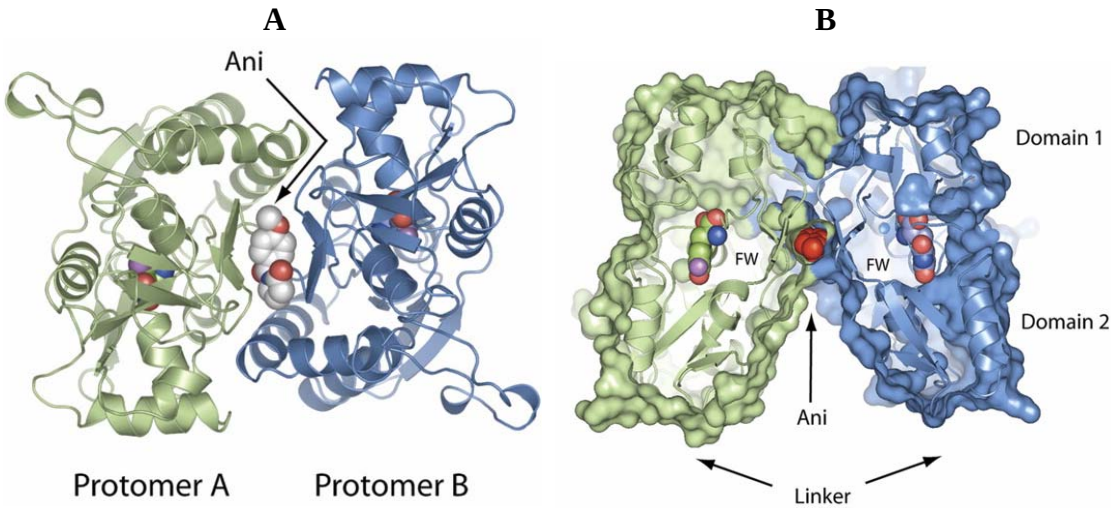

Figure 2.6. Aniracetam binds in the dimer interphase in a crevice at the clamshell hinge. A: View down the twofold axis, showing the binding of aniracetam, in one of its two equivalent orientations. Protomer A is in green, protomer B is in blue, and aniracetam is drawn in Corey, Pauling, and Koltun (CPK) representation, as is the partial agonist fluorowillardiine. B: View of the Ani/FW complex perpendicular to the molecular twofold axis. Here, aniracetam is red and in CPK representation; the approximate positions of the "linker" that connects the ligand-binding cores to the transmembrane domains is shown.

In addition to the effect of aniracetam on the deactivation and desensitization of AMPARs, it has been shown to increase synaptic potentiation in a fashion similar to long-term potentiation (LTP; a model mechanism for learning and memory which require PKC; (Colley et al., 1990; Abeliovich et al., 1993; Matsuyama et al., 1997) and in some instances enhances LTP itself (Satoh et al., 1986; Ito et al., 1990; Vyklicky et al., 1991; Xiao et al., 1991; Staubli et al., 1994a; Boxall and Garthwaite, 1995; Kolta et al., 1998; Shen et al., 1999; Black et al., 2000; Nomura and Nishizaki, 2000). Reports suggest aniracetam activates PKC (Lucchi et al., 1993; Lu and Wehner, 1997) and γ-PKC enhancing the contextual learning in mice (Smith and Wehner, 2002). They further reported an increase in membrane/cytosolic γ-PKC in the hippocampus 30 min after the

drug administration (when the drug reaches the maximum plasma concentration). This study further supported the view of postsynaptic localization of γ-PKC (Suzuki et al., 1993) suggesting a possible mechanism of postsynaptic action of aniracetam (Vyklicky et al., 1991; Xiao et al., 1991). This information suggests a possible mechanism of alteration of AMPA receptor kinetics by an increase in γ-PKC activity (Smith and Wehner, 2002). Also it explains a possible increase in the phosphorylation of GluR2 AMPAR by PKC (McDonald., 2000)Therefore, an alteration of γ-PKC activity by aniracetam is also likely to improve hippocampal learning and memory (Smith and Wehner, 2002).

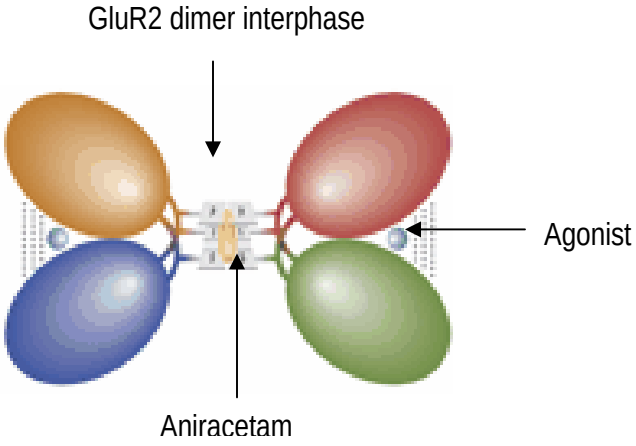

Figure 2.7. A simple model to describe the mechanism of action of positive allosteric modulators such as aniracetam on AMPA receptors. The diagram depicts a side view of the GluR2 ligand-binding core dimer in which domains 1 and 2 of protomer A are orange and blue, and domains 1 and 2 of protomer B are red and green, respectively. Agonists are represented by small blue spheres that bind between domains 1 and 2 and stabilize the closed-cleft conformation. Aniracetam (yellow oval), binds on the backside of the ligand-binding core through interactions with a proline ceiling and a serine floor, at the interdomain hinge in the dimer interface, and stabilizes the closed-cleft conformation of the ligand-binding core (dashed lines).

2.13.3 Therapeutic potential of positive allosteric modulators of AMPA receptors

Considerable evidence has indicated that dysfunction of glutamatergic signaling in the CNS may contribute to cognitive deficits associated with a variety of neurological and psychiatric disorders (Yamada et al., 2000). Several therapeutic approaches have been designed to enhance the activity of glutamatergic neurotransmission. One such treatment strategy involves augmentation of AMPAR function using positive allosteric modulators. Support for the therapeutic potential of the AMPAR modulators was generated from preclinical and clinical studies demonstrating that modulators, including aniracetam, improve performance on a variety of cognitive tasks that require multiple types of mnemonic processes (Staubli et al., 1994a; Hampson et al., 1998; Quirk and Nisenbaum, 2002). Collectively these studies support the possibility that positive modulation of AMPARs may be a novel therapeutic approach for cognitive deficits in a variety of disorders, particularly those that are associated with reduced glutamatergic signaling.

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3 POSTNATAL ANIRACETAM TREATMENT REVERSES LEARNING AND MEMORY DEFICITS CAUSED BY PRENATAL ETHANOL EXPOSURE

Abstract

Specific pharmacological treatments are currently not available to address problems resulting from fetal ethanol exposure described as ftal acohol sndrome (FAS) or ftal acohol sectrum dsorder (FASD). However, aniracetam treatment given at the adolescence of the offspring has previously been demonstrated to alleviate the cognitive impairments caused by mild ethanol exposure in pregnancy. The current study evaluated whether preadolescence aniracetam treatment can also ameliorate cognitive impairments caused by moderate ethanol exposure *in utero* using a well-characterized and sensitive animal model of FASD. Ethanol, administered orally at a moderate dose (4 g/kg/24h; 38 % v/v) during the entire course of pregnancy caused severe cognitive deficits in offspring. Both progeny genders were affected by a spectrum of behavioral abnormalities such as a delay in the development of righting reflex, poor novelty- seeking behavior and high anxiety levels in female rats. Adult rats exposed to ethanol *in utero* demonstrated impaired cognitive functions with a decrease in avoidance/ escape, measured by two-way active avoidance task. In comparison, offspring given oral ten day (PND 18-27) aniracetam treatment (50 mg/kg) were not cognitively impaired performing at the same level as control rats in the active-avoidance task indicating a significant increase in the number of avoidances and the number of "good

learners". Significant anxiolytic effects on PND 40 also preceded acquisition improvements in the avoidance task. These findings indicate the evidence for the therapeutic potential of aniracetam in reversing of the cognitive deficits associated with FASD.

Introduction

The teratogenic nature of ethanol has been well documented in humans and experimental animals (Streissguth et al., 1980; Dreosti, 1993; Becker, 1996). Cognitive and behavioural impairments such as deficits in attention, learning and memory (Mattson and Riley, 1998), are much more commonly observed in children exposed prenatally to ethanol and hence are more significant and costly to both the individual and the community. However, the extent and severity of a child's condition depend on several factors such as the amount of alcohol intake by the pregnant mother, the frequency and the period of pregnancy. The mechanisms, involved in ethanol-related neurobehavioral abnormalities are unclear. Although it is likely that many factors are involved, information obtained to this date has not led to establishment of specific pharmacological treatments that could ameliorate or reverse defects resulting from fetal ethanol exposure (Jones et al., 1973) or FASD (Clarren and Smith, 1978).

Chronic maternal ethanol abuse during pregnancy is associated with important teratogenic effects on the offspring and this is one of the leading causes of mental retardation and congenital malformations in humans (Ernhart et al., 1987; Tan et al., 1990). Experimental models of FASD showed cognitive deficits at all ages, from pre-

weaning life through late adulthood (Barr et al., 2005). Deficits have been observed in a wide variety of learning and memory tasks, including spatial reference memory (Kim et al., 1997; Matthews and Simson, 1998; Zimmerberg and Weston, 2002; Gabriel et al., 2005), working memory (Neese et al., 2004) and associative memory (Clausing et al., 1995; Wainwright, 1998; Vaglenova and Vesselinov Petkov, 2001; Weeber et al., 2001).

Currently there are no effective pharmacological agents available for improving complex behavioral abnormalities resulting from chronic prenatal alcohol exposure. Psychotherapeutic CNS stimulants were the first agents used in an attempt to influence some of the behavioral deficits (Ulug and Riley, 1983; Riley et al., 2001). Recent experimental data suggest that early dietary intervention by choline supplementation may reduce the severity of FASD (Thomas et al., 2000). A fragment of the glial-derived activity dependent neuroprotective protein, NAP has been shown to act against alcohol-induced embryo toxicity and growth retardation in mice (Spong et al., 2001). Aniracetam, among other compounds, has been categorized as a piracetam analogue (Gouliaev and Senning, 1994) and has been investigated in our lab (Vaglenova and Petkov, 1988; Petkov et al., 1991) and the minimum effective doses to reverse prenatal alcohol-induced deficits have been well characterized. The major advantage of this agent is its ready availability and oral dosage forms, particularly compared to NAP.

Aniracetam, a 2-pyrrolidinone derivate [1-(4-methhoxybenzoyl)-2-pyrrolidinone] has minimal side effects (Foltyn et al., 1983; Gouliaev and Senning, 1994). It has been used in clinical practice and usually prescribed to treat brain disturbances and intellectual disorders caused by alcohol, tranquilizers, neuroleptics, depressants, barbiturates and agents impairing circulation in the brain.

Starting on the gestation day (GD) 3, pregnant rats were exposed to ethanol throughout their pregnancy and this corresponds approximately with first and second trimester of human pregnancy (Olney, 2002, 2004). To establish whether postnatal aniracetam treatment during preadolescence can protect against spatial learning and memory deficits caused by chronic ethanol exposure, offspring were tested for cognitive impairments using an array of developmental and behavioral experiments. In the present study, aniracetam was administered before adolescence and cognitive parameters were investigated one month later in adult offspring. We also compared the effect of gender of the offspring in the outcome of the moderate prenatal exposure and aniracetam treatment. Thus, the objective of the present work was to develop a therapeutic protocol for attenuating the cognitive deficiencies caused by prenatal ethanol exposure at a moderate dose.

Research Design and Methods

Experimental animals and chronic treatment regimens

On gestation day (GD 3), pregnant Sprague Dawley rats (n = 24) (Zivic-Miller Laboratories, Pittsburgh, PA) were randomly assigned to one of the two treatment groups; ethanol, iso-caloric sucrose. Each pregnant animal in the ethanol group received a moderate dose of ethanol (4 g ethanol/kg maternal body weight/day as an aqueous ethanol solution (38% v/v, prepared with tap water) and had *ad libitum* access to food. This regimen mimics a moderate drinking pattern and once a day dosing reduces the stress effects involve in repeated intragastric intubation (Keshavarzian et al., 2001) while

minimizing the withdrawal episodes (Maier and West, 2001). In the isocaloric-sucrose group, each pregnant rat received isocaloric sucrose and food. Each treatment was given daily from GD 3 to 20 by gavage in once a day dosing. On GD 14, a blood sample was taken from tail vein at 1 h after the ethanol dose, when the apparent maximal blood ethanol concentration is achieved. On postnatal day (PND) 1, litters were culled to an equal number of males and females whenever possible, with an equal number of 10 pups per mother. The progeny was weaned on PND 25 and in order to control litter effects, no more than 1 mate per gender was placed into a particular group (Wainwright, 1998). The colony room was maintained at 22-24º C and kept under 12-hr light/dark cycle.

On PND 18, randomly selected offspring from each litter was treated with aniracetam (commercially available as Ampamet) at a single dose of 2 ml/kg at a 50 mg/kg, dissolved in distilled water, by gavage for 10 days (PND 18-27) (Spear 2000). The maximum benefit of the dose of 50 mg/kg, the duration and the route of administration (the agent is orally active) were chosen in accordance to prior investigations in our laboratory over the past 20 years and supported by numerous other authors (Giurgea, 1980; Cooper and Herrman, 1988; Petkov et al., 1991; Gouliaev and Senning, 1994; Vaglenova and Vesselinov Petkov, 2001). Ampamet tablets were broken and powdered in a glass mortar and the resulting powder stirred in dichloromethane for 24 hours. The suspension was then filtered and the filtrate evaporated to dryness under reduced pressure. The resulting white powder was recrystallized from a mixture of n-heptane and benzene to yield white crystals. The purity and identity of the material was confirmed by GC-MS analysis.

Twenty animals per group have been required to detect a 20% difference between treatment groups (Vaglenova et al., 2004). A total number of 240 pups were grouped in 4 treatment groups: prenatally sucrose exposed (control) (C), prenatally ethanol exposed (E), prenatally sucrose exposed, treated with aniracetam postnatally (C-A) and prenatally ethanol exposed group treated with aniracetam postnatally (E-A). Each group (n = 20/sex) of pups, each one with an identical number, was used for developmental milestones measures, locomotor activity, plus-maze and active avoidance.

Behavioral and developmental analysis

Developmental analysis

After birth, animals from ethanol and sucrose groups were observed for mortality rates, body weights, incisor eruption and eye opening. Righting reflex and negative geotaxis were performed from the third day of age and continued until all tested animals in the various treatment groups meet the criteria. Righting reflex was tested by placing the animal on its back on a smooth surface and recording the time required to right him to a position where all four paws touched the surface. Negative geotaxis was measured by measuring the time required to reorient to a head-up position after placing the animal in a head-down position on a 30^0 inclined plane. All control animals achieved the criterion of 45 seconds of the task after 6 days of the trial (Bonthius and West, 1988).

Measurement of exploratory activity and novelty-seeking behavior

On PND 18 (Table 3.1), the pups used in developmental milestone measures were assessed for exploration, in an open field and "hole" board activity Plexiglas chamber (46 cm x 46 cm) with 5 holes (3 cm diameter) located on the floor. The apparatus uses a computer controlled photo beam activity system (Omithech Electronics Inc., Columbus, OH) to measure the horizontal and vertical movements, the total distance, the rest time, the time spent in corners, and numbers of head dips (Palanza et al., 2001; Vaglenova et al., 2004) in the course of a 10 min trial, performed between 09:00 and 11:00 AM in a soundproof room.

Plus Maze activity

Subjects from all four groups were tested for 5 minutes at the age of 40 days for anxiety using an elevated plus-maze. This was performed in 50 cm elevated black Plexiglas apparatus consisting of 2 opposite open arms (50 cm x 10 cm) and 2 opposite arms enclosed with 30 cm high walls, connected in the middle with 10 cm x 10 cm square center area forming a "plus" shape. The trials started after a rat was placed in the center, facing an open arm. The trials were videotaped and the time spent (seconds) in the various arms, and number of entries into different arms were recorded (Escorihuela et al., 1995; Vaglenova et al., 2004).

Two-Way Active Avoidance (Shuttle-Box)

On PND 60, the learning and memory of individual offspring from each group were measured by active avoidance task in a shuttle box apparatus using repeated training procedures (Vaglenova and Petkov, 1988; Vaglenova and Vesselinov Petkov, 2001; Vaglenova et al., 2004). The computer controlled apparatus (Columbus Instruments) consists of two equally divided compartments (47.5 x 27 x 22.5 cm) connected by an opening at the center. The chamber rested on a grid of metal rods (1.5 mm diameter, spaced 1.05 cm apart which delivered the electric shock. A combination of a tone (80 dB) and light (5 W), presented together but alternatively in each compartment, was used as the conditioned stimulus (CS) for 5 seconds during which the rat could avoid the following unconditioned stimulus (US). US was a foot shock of 10 seconds, elicited by an electric current (0.5-0.7 mA, ac), delivered to the grid floor of the apparatus. During the session the subjects learn to escape the US moving into the other compartment. The intertrial interval was 15 seconds, and each rat was trained for 5 consecutive days with 30 trials in each session. To estimate the long term memory (retention) in these individuals, a trial was carried out 7 days after the last day of training (Heise, 1984; Yonkov et al., 1989; Vaglenova et al., 2004).

Data analysis

Prenatal and postnatal treatment and genders were analyzed as factors for exploratory activity, anxiety test and active avoidance test (2 x 2 x 2), using ANOVA. Training days were presented as a within factor (when it was appropriate). Specific

contrast was investigated by Bonferroni post hoc analysis to identify the source of variance. Furthermore, the rats in each treatment group were stratified into "helpless", "poor", and "good" learners which allowed a qualitative measure of the performance (Vaglenova et al., 2004). Briefly, "good" learners were defined as the animals that perform at or above the control group mean and showed an increase in the number of avoidances during the last two days of the test compared to the "poor" learners who exhibited a decrease in the number. "Helpless" animals were those who completely failed to perform the task, displaying one or less avoidances during each trial (Vaglenova and Vesselinov Petkov, 2001; Vaglenova et al., 2004). Chi-square analysis was used to test the effect of treatment in groups and gender on the stratification of learners and developmental reflexes. Results are presented as mean ± S.E.M. The differences were considered to be significant at $P < 0.05$.

Results

Maternal blood ethanol concentration

The maternal blood ethanol concentration at 1 h following the ethanol dose on GD 14 was 185.0 ±0.3 mg/dl (n = 6).

Pregnancy outcome

There were no maternal deaths or spontaneous abortion in any of the treatment groups. The ethanol dose and gavage procedure did not adversely affect the overall

dietary consumption in the ethanol treated mothers. No significant differences were observed in their body weights during gestation or lactation when compared to the isocaloric-sucrose treated control group. There were no birth defects, still born pups, cases of maternal cannibalism, or significant postnatal mortality rate in any of the experimental groups. However, birth weights of the offspring were significantly reduced in the prenatally ethanol exposed group compared to the isocaloric-sucrose exposed group and they failed to recover until PND 5 (data not shown).

Developmental tasks

Chi-square analysis indicated significant deficits in righting reflex of prenatally ethanol exposed group when compared to isocaloric sucrose exposed (control) group (X^2 = 66.0; $P < 0.001$) (Fig. 3.1). All animals in the control groups reached criteria (> 90% of the animals performing the righting reflex in less than 2 seconds) within 5 days, compared to 8 days for the group exposed to ethanol *in utero*. Latencies to righting were also statistically higher in the prenatal ethanol exposed progeny compared to controls ($P < 0.05$) (data not shown). There was no effect of gender on offspring task performance ($P > 0.05$) in any of the two treatment groups. All control animals achieved the criterion of 45 seconds on the negative geotaxis task after 5 days of trials. Pups in prenatally ethanol exposed group reached the criterion of 45 seconds after 6 days of the trials and this was not significantly different compared to the control group.

Behavioral tasks

Several behavioral parameters were investigated at different postnatal developmental time points. Those included the spontaneous locomotor activity and novelty-seeking behavior in pre-weanling animals on PND 18 and 25, plus-maze test to investigate the levels of anxiety in adolescence (PND 40), and active avoidance task to study the cognitive functions in adulthood (PND 60-72). These experiments are summarized below (Table 3.1).

Exploratory activity and environmental adaptation

There were no significant abnormalities in ambulance activity in both 18- and 25-day- old prenatally ethanol exposed animals (data not shown). As shown in figure 3.2, hole-board exploratory activity, as defined as the number of head-dips into the holes located on the floor, was significantly reduced in both genders of 18-day-old ethanol exposed offspring compared to controls (treatment $F(1,79) = 5.05$; $P < 0.05$). All animal activity in this task was mostly noticeable during the first 5 minutes of the trial followed by significant habituation to the environment during the last 5 minutes (treatment/periods $F_{(1, 79)} = 5.01$; $P < 0.02$). These results confirmed that the ethanol exposure *in utero* significantly decreased the novelty-seeking activity, poor adaptation and neophobia.

Effects of aniracetam on anxiety in elevated plus maze

The plus maze is based on the rodent's natural aversion of being exposed to an elevated space, and serves as a generalized test of measuring anxiety levels. These

levels were assessed by measuring the number of entries and length of time spent in the open arm of the maze, or the "aversive area." Forty-day-old rats were used in this experiment since the adolescent rat is especially vulnerable to anxiety and stress, possibly due to the immaturity of brain mechanisms involved in the regulation of anxiety (Doremus et al., 2004; Vaglenova et al., 2004). Table 3.2 shows the effects of prenatal ethanol exposure and postnatal aniracetam treatment on the plus maze test in 40-day old rats. Results indicate that only female offspring from dams exposed to ethanol showed significant reduction in time spent in unprotected arms suggesting a possible anxiogenic effect of ethanol at 4 g/kg dose. Statistical comparison analysis, using prenatal exposure and postnatal treatment design, demonstrated a primary effect of postnatal aniracetam treatment for the number of entries into the open arms for both genders (males: $F_{(1,84)} = 31.0$, $P < 0.0001$; females: $F_{(1,84)}=62.6$, $P < 0.0001$). These results indicate a strong anxiolytic effect of aniracetam. There was no effect of treatment or interactions for entries with prenatal drug exposure (all $P > 0.05$). On the contrary, primary effects of postnatal aniracetam treatment were observed for the time spent in the open arms for both genders (male: $F_{(1,84)} = 15.5$, $P < 0.0003$; female: $F_{(1,84)}= 31.6$, $P < 0.0001$). This was also indicative of the anxiolytic effect of aniracetam; however, there was a strong interaction for the time spent in the open arms in prenatal ethanol exposure and postnatal aniracetam treatment in both genders, with the simple main effects detecting a further improvement as a result of aniracetam treatment in the ethanol-exposed offspring as compared to controls (interaction prenatal drug exposure x postnatal aniracetam treatment, males: $F_{(1,84)} = 5.6$, $P < 0.03$; female: $F_{(1,84)} = 4.1$, $P < 0.05$).

Effects of prenatal ethanol exposure on learning and memory

There was a significant effect of prenatal ethanol exposure on learning and memory of offspring without having a significant gender effect. Each sex of offspring (PND 60) exposed to ethanol *in utero* had decreased numbers of avoidances during the training sessions as well as on the test day for memory retention (Fig. 3.3) (male: $F_{(1, 250)} = 13.6$, $P = 0.003$; female: $F_{(1, 263)} = 14.0$, $P = 0.0002$). These changes are a result of an increase of animals classified as poor or helpless learners in both genders (Fig. 3.4). It is interesting to note that there was a specific gender effect, seen in the higher percentage of helpless male learners in the ethanol exposed offspring as compared to females, while less than 10% of both genders in the control group were considered "helpless." Surprisingly, 25% of ethanol-exposed males and 20% of ethanol-exposed females were not affected by prenatal ethanol exposure, demonstrating a high number of avoidances and qualifying as "good learners." This result corresponds to human outcomes showing that alcoholic mothers give birth to otherwise healthy children. In contrast, more than 50% of control animals of either gender were considered "good learners."

Effects of aniracetam on learning and memory of offspring exposed to ethanol in uetro

As shown in figures 3.3 and 3.4, cognitive function in both genders exposed to ethanol significantly benefited from chronic aniracetam administration. In contrast, primary effects of postnatal aniracetam treatment were not observed for acquisition and retention for both genders (male: $F_{(1, 216)}=2.7$, $P = 0.1$; female: $F_{(1, 240)}= 0.2$; $P = 0.6$); however, there was a strong interaction between the number of avoidances with prenatal

ethanol exposure and aniracetam treatment in both genders, with the simple main effect indicating an improvement as a result of aniracetam treatment in the ethanol-exposed offspring as compared to controls (interaction prenatal drug exposure x postnatal aniracetam treatment, males: $F_{(1, 473)}$=6.5, P = 0.01; female: $F_{(1, 502)}$ = 43.3, P < 0.0001).

Further analysis based on the stratification of rats as learners gave other interesting information. Measures of learning (e.g., numbers of avoidances and number of "good learners") indicated that the female offspring recovered more completely than their male counterpart when treated with aniracetam postnatally (Fig. 3.4). However, they were also outnumbering those parameters of the control group. The number of "helpless" learners in the aniracetam-treated groups was significantly lower than those exposed to ethanol alone. Surprisingly, their number was higher in aniracetam treated males compared to controls, although, there were no differences between 'mean' avoidances, further supporting the finding that aniracetam treatment improved these parameters in ethanol-exposed offspring.

We also studied the effect of gender on the therapeutic efficacy of aniracetam using different parameters associated with the performance of the active avoidance task. Analysis of the frequency of escape failures (EFs) revealed acquisition problems in all male groups (X^2 males/females = 19.0; P < 0.02) (Fig. 3.5). EFs were used as a measure of the offspring's inability to overcome the stress and anxiety associated with the unconditioned stimulus which was elicited as electrical shock (Ferguson et al., 2000). Table 3.3 shows that the majority of EFs were in the group of "helpless" animals, although there was a significant effect of gender on this activity. However, no significant difference was observed between control and control-aniracetam treated groups (P =

0.28). There was a dramatic increase in the number of male EFs in ethanol exposed group and this corresponded well with the increase in the number of "helpless" learners in this group (Table 3.3, Fig. 3.4).

The significant decrease in escape latency (EL) and EFs in males exposed to ethanol and aniracetam, coincided well with an increase in the learning and memory performance. This further suggests that decreased cognitive performance in ethanol exposed animals may have resulted in part from increased stress and anxiety in the poor and helpless learners of both genders. In contrast, a significantly higher number of female helpless animals in the prenatal ethanol exposed group corresponded with the negligible number of animals with >15 EFs ($X^2 = 3.89$; $P = 0.28$). Aniracetam was able to improve disrupted fixed-interval performance in the ethanol exposed male and female animals as evident by the elimination of escape failures during the last learning and retention phases of the experiment (treatment $F_{(3, 316)} = 11.0$; $P < 0.0001$), thereby significantly increasing the number of good learners (Fig. 3. 4).

Discussion

Prenatal ethanol effects in the rat model

Physical development

Therapeutic effects of aniracetam were evaluated on a well-characterized and sensitive animal model of FASD. Body weights and early developmental reflexes were taken into account as a fairly accurate index of prenatal or early postnatal development. Moderate prenatal ethanol exposure had various effects on pregnancy outcome in

the rats. Chronic prenatal exposure to ethanol decreased the birth weights relative to the isocaloric sucrose exposed group, however, ethanol exposed offspring could compensate this by PND 5. Physical retardation and neuronal incapacity for motor control were expressed as significant delay in developing only of righting reflex that suggests impairment in the required motor coordination. Hannigan et al., (1993) reported that lower birth weight was a common consequence of prenatal ethanol exposure while other authors (Trofimov et al., 1996) did not find changes in the body mass and elementary inborn reflexes during the first three weeks of postnatal life, using ethanol at 5 g/kg/day, dose. Endocrine alterations can impact fetal and postnatal growth and development in prenatally ethanol exposed offspring. There is evidence indicating that prenatal alcohol or nicotine exposure can reduce postnatal plasma concentrations of insulin-like growth factor-1 (IGF-1), a growth promoting peptide, with levels correlated with somatic and brain growth deficits in these offspring (Breese et al., 1993, 2000), providing biochemical evidence for specific long-term alterations related to somatic growth development.

Exploratory activities

Before aniracetam treatment was initiated, locomotor activity and novelty seeking behavior were assessed. Our results demonstrated that *in utero* exposure to ethanol did not cause significant abnormalities in horizontal and vertical activity of 18- and 25-day-old ethanol exposed rats when compared to isocaloric-sucrose exposed controls. However, one study reported increased activity during the pre-weaning period (Doremus et al., 2004). In fact, 18-day-old, ethanol exposed offspring of both genders showed a significant decrease in exploratory activity, novelty-seeking behavior in the hole-board

when compared to their sucrose exposed controls. Novelty seeking decrements are indicators for neophobia, anxiety and stress disposition (Arevalo et al., 2001; Palanza et al., 2001; Vaglenova et al., 2004). Activity of all animals was mostly during the first 5 minutes of the trial, with significant habituation to the environment during the last 5 minutes suggesting the adaptation to the environment. The present study shows that there was no difference in the development of the habituation process among control and prenatally ethanol exposed groups.

Therapeutic effects of aniracetam

Anxiolytic effects

The present study also examined the anxiety level after aniracetam treatment at a later time point, forty day of their age, using the plus maze test. Since, fear, stress and anxiety are considered to be harbingers of cognitive problems (Ho et al., 2002; Vaglenova et al., 2004), the assessment of the level of anxiety by measuring the number of entries and the length of the time spent in the open arms ("aversive area") of plus maze was used . Forty-day-old animals were used in the experiment to minimize the effect of adolescence induced anxiety and stress (Doremus et al., 2004; Vaglenova et al., 2004). This investigation also found that ethanol exposure elevated "anxiety state" only in female offspring. A recent study (Carneiro et al., 2005) reported anxiolytic effect of prenatal ethanol exposure in an earlier stage of development (21 day old rats) while increased anxiety is shown in adolescent human and rats (Day, 1997; Osborn et al., 1998). The discrepancy between that study and our investigation on the effects of chronic

prenatal ethanol exposure related anxiety may be the result of the age difference in the two studies resulting in changes in brain mechanisms involved in the regulation of anxiety are immature (Doremus et al., 2004) and acquiescent to the intensive hormonal alterations at this specific age.

Aniracetam treatment during preadolescent period resulted in strong anxiolytic effects in their adolescence (10 days after the last dose of the treatment). The present results also showed that in both genders of the sucrose-aniracetam treated group, the number of entries to the open arm significantly increased when compared to the sucrose alone exposed group. This indicates that aniracetam treatment during preadolescence also results in reducing the natural levels of anxiety during their adolescence. Although such effect is demonstrated for the first time in a model of fetal ethanol exposure, other authors (Suzuki et al., 1993) have reported anxiolytic effects of aniracetam in many other models such as the condition fear stress test, forced swim test and submissive behavioral model (Knapp et al., 2002). These results indicate that aniracetam possesses a wide range of anxiolytic properties, which may be mediated by an interaction between cholinergic, dopaminergic and serotonergic system (Suzuki et al., 1993) and thus, is able to reduce fetal ethanol induced anxiogenic effects in female offspring.

Cognitive enhancing effect of preadolescent aniracetam treatment

The major finding of this study is the ability of preadolescent (PND 18-27) aniracetam treatment to improve/reverse the learning and memory deficits resulting from chronic moderate prenatal ethanol exposure. In a previous investigation (Vaglenova and

Vesselinov Petkov, 2001) piracetam (Pyramem), aniracetam, and meclophenoxate (Centrophenoxine) have been shown to be therapeutically efficacious on a different model of perinatal ethanol exposure (4 times lower than the current study). In the same study aniracetam was given to adolescent rats (PND 30-40) and learning and memory were also examined in adult rats. Taken together, these results provide the wide therapeutic window of aniracetam in the treatment of ethanol exposure. Our present results demonstrate that aniracetam is effective on both genders; however the recovery is more complete in females and even their learning indicators (number of avoidances and number of "good learners") are outnumbering those in the control group. Male offspring exposed to ethanol *in utero* and aniracetam postnatally also showed restored learning and memory when compared with prenatal ethanol exposed group. However, it was not expected to see an increase number of "helpless" and "poor learners" in the sucrose exposed male animals once treated with aniracetam. This would be possibly due to the fact that aniracetam, like other nootropics, does not enhance learning in healthy individuals (Gamzu et al., 1989; Vaglenova and Vesselinov Petkov, 2001). Eventhough it is possible to speculate that anxiolytic effect of aniracetam would have led the prenatally ethanol exposed female offspring to learn to avoid the foot shock, such explanation is not appropriate for the sucrose-aniracetam treated male offspring. An increased number of helpless male animals in this group consisted of a higher number of subjects with escape failures although mean escape indicators did not significantly differ from those of control group. Therefore, it is possible that animals classified as poor or helpless learners may have deficits in either general learning and memory from those classified as good learners, or may indicate significant differences in anxiety, fear or emotional state

associated with the learning task (Escorihuela et al., 1995; Clausing et al., 2000); either of which would be observed and defined as a deficit in cognitive function. It could also be possible that aniracetam is exhibiting an overdose effect only for male Sprague Dawley rats or this may be a result of elevated blood corticosteroid and testosterone levels in male animals. The cause for this existing gender difference in response to aniracetam treatment remains to be determined.

Conclusion

The current study was carried out in a rat model of FASD. The data of the present study demonstrated the spectrum of developmental and behavioral modifications in juvenile, adolescent and aged animals. The potential risk of prenatal ethanol exposure was clearly defined in both genders by measuring the changes in developmental reflexes, exploratory activity, novelty seeking behavior, "anxiety state", and the heterogeneity of individual and group responses in learning and memory. The effects of the drug provide promising possibilities in the treatment of cognitive impairments associated with fetal alcohol exposure. Additionally, given the abundant evidence that aniracetam modulates AMPA receptor function, it is reasonable to speculate whether that the aniracetam induced increase in AMPA receptor mediated synaptic transmission may determine the behavior modifications in the groups of interest. Despite the likelihood of the AMPA receptor involvement in the mechanism of action of aniracetam in these groups, it is yet to determine whether the AMPA receptor synaptic transmission is impaired in prenatal ethanol exposure. Currently we are investigating the alterations of these AMPA receptor

mediated synaptic transmission in the hippocampus of ethanol neurobehavioral teratogenicity.

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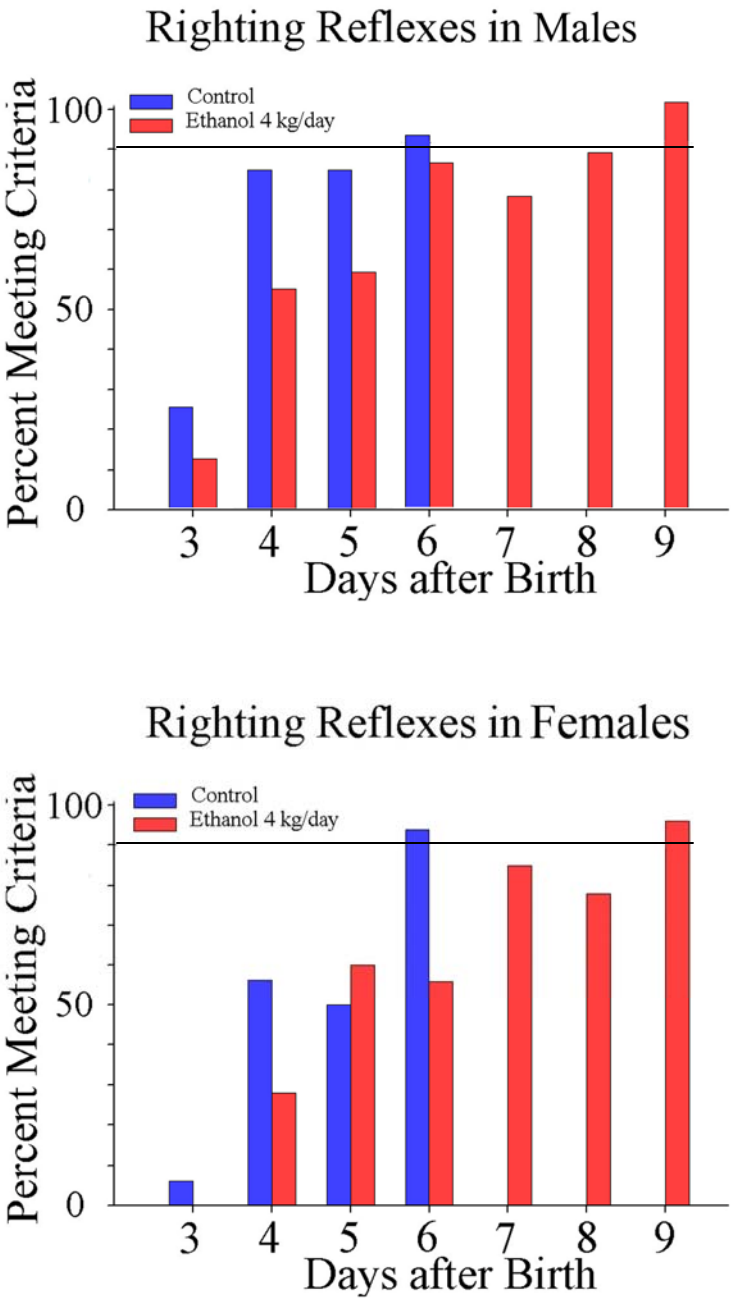


Figure 3.1. Prenatal ethanol exposed animals exhibited delayed righting reflex. Percentage of males (right) and females (left) exposed to ethanol/isocaloric sucrose *in utero*, eliciting a fully righting reflex plotted as a function of days after birth. > 90% in the control group could perform the righting reflex under 2 seconds, in 5 days after birth compared to the 8 days taken by the prenatal ethanol exposed group. Differences between groups were investigated by Chi-square, taking into account sex and treatment. $X^2 = 66.0$ with 24 degree of freedom; ($P < 0.001$).

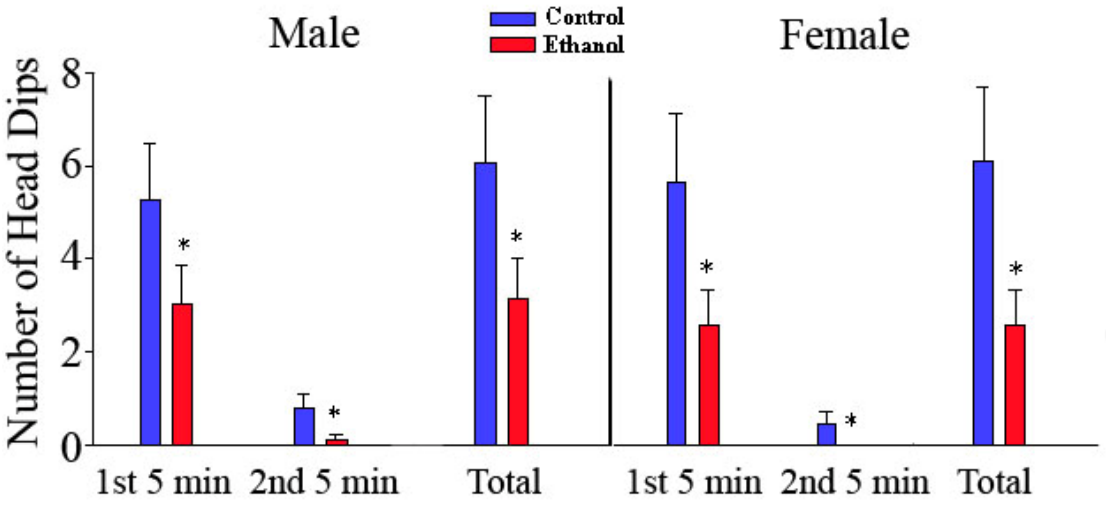
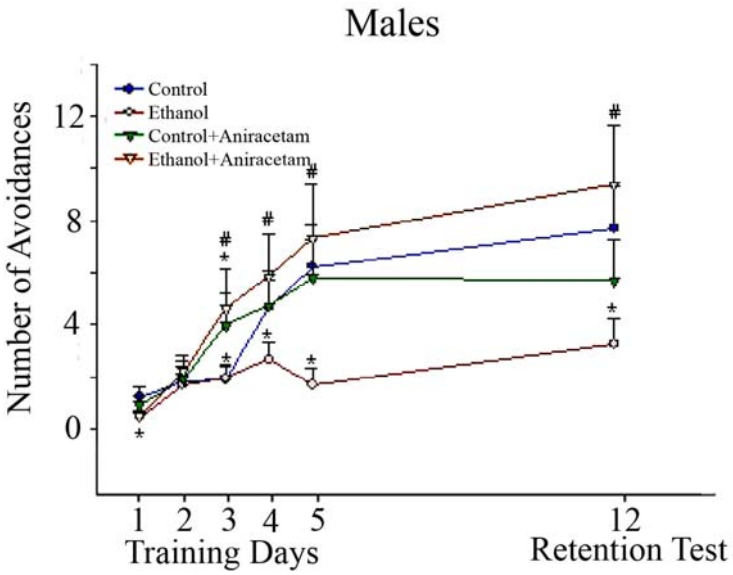


Figure 3.2. Prenatal ethanol exposure decreased the number of head dips (mean ± S.E.M.) indicating poor novelty seeking behavior. Offspring (prenatally ethanol/isocaloric sucrose exposed) were tested for novelty seeking behavior using the hole board apparatus on PND 18, during the 10 minutes session. The number of head dips into the holes located on the floor of the apparatus was significantly reduced in both genders of the prenatally ethanol exposed animals compared to the control. Most of the exploratory activities of the animals in ethanol/sucrose groups were evident during the first 5 minutes, indicating a significant habituation to the environment during the last 5 minutes of the session. Data were statistically analyzed by ANOVA, using treatment and sex as factors. (treatment $F(1, 79) = 7.49$; $P = 0.007$). *$P < 0.05$ ethanol vs. sucrose.


Males
Control
Ethanol
Control+Aniracetam
Ethanol+Aniracetam
Number of Avoidances
0
4
8
12
1 2 3 4 5
Training Days
12
Retention Test

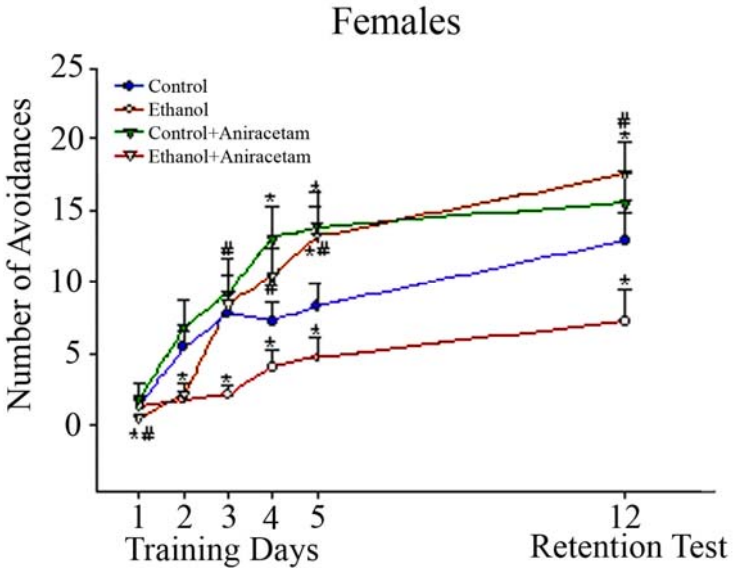

Females
Control
Ethanol
Control+Aniracetam
Ethanol+Aniracetam
Number of Avoidances
0
5
10
15
20
25
1 2 3 4 5
Training Days
12
Retention Test

Figure 3.3. Prenatal ethanol exposure decreased and postnatal aniracetam treatment increased the number of avoidances (mean ± S.E.M.) indicating alterations in cognitive functions. Offspring (ethanol/sucrose/aniracetam treated) from the four treatment groups were tested for learning and memory using the active avoidance task carried out in the computer controlled shuttle box apparatus from PND 60. A combination of tone (80 dB) and light (5 W) were used as conditioned stimuli and during this period the animals learn to avoid the subsequent foot shock given by an electric shock (0.5-0.7 mA, ac) (unconditioned stimuli). Animals were trained for five consecutive days and tested for retention of memory after one week of resting period. The number of avoidances during the training and test date were significantly decreased in the ethanol exposed group. Postnatal aniracetam treatment given to the ethanol exposed offspring could significantly increase the number of avoidances. Data were statistically analyzed by ANOVA, using treatment and sex as factors. (treatment/sex $_{(18, 444)} = 2.91$; $P = 0.00006$).

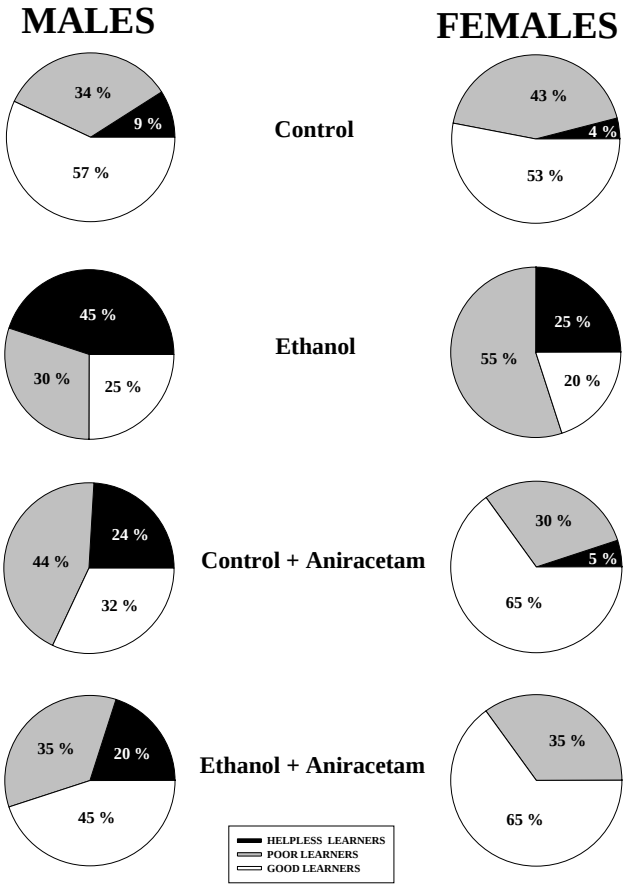


Figure 3.4. Stratification of helpless, poor and good learners among subjects exposed to ethanol (4g/kg; 38% v/v /day) or sucrose *in utero* and aniracetam postnatally. Prenatal ethanol exposure increased the number of helpless learners while aniracetam (50mg/kg) treatment could significantly increase the number of good learners assessed by the active avoidance task. Recovery was complete when aniracetam treatment was given to the female offspring exposed to ethanol prenatally (n=20). Data were statistically analyzed by ANOVA, using treatment and sex as factors. Significance by $\chi 2$ males = 45.89; $P < 0.001$; females = 79.84; $P < 0.001$.

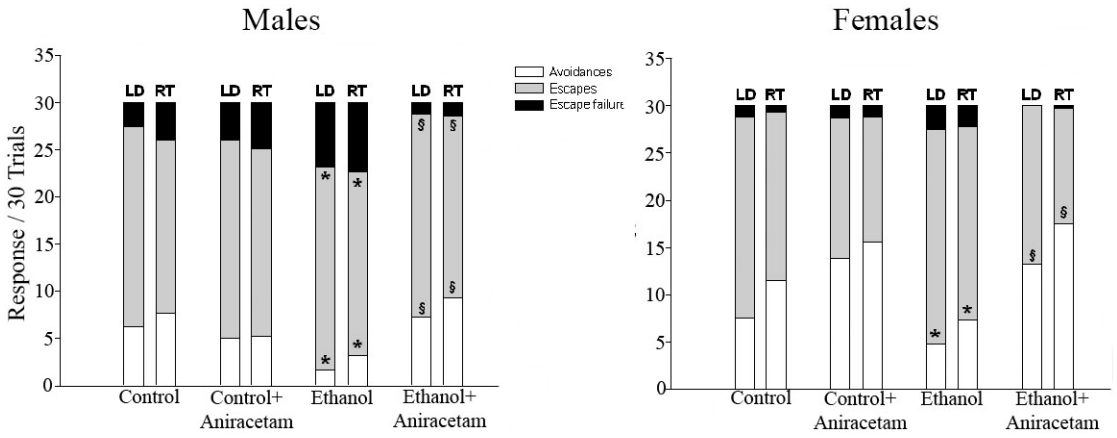


Figure 3.5. Prenatal ethanol exposure resulted in increased escape failures (EFs) in males indicating acquisition impairments. EFs were used as a measure of the animal's inability to overcome the stress and anxiety associated with unconditioned stimuli initiated by the active avoidance task. LD = learning days, RT = retention test day. Data were statistically analyzed by ANOVA, using treatment and sex as factors. Significance by $\chi 2$ males/females = 19.0; $P < 0.02$).

Table 3.1. Outline of the experiment paradigm

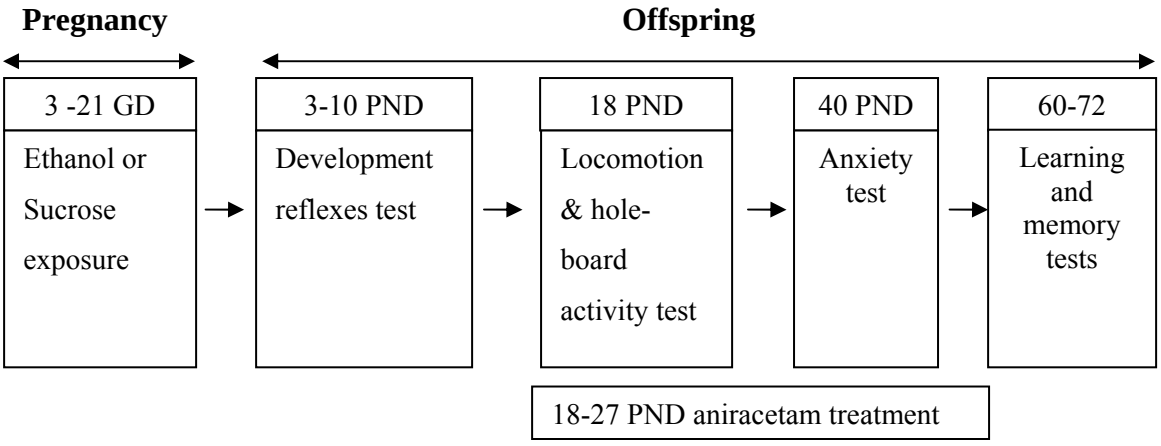


Table 3.2. Plus maze test performance: the effect of treatments on number and time spent in the open arm.

	Control	**Ethanol**	**Control + Aniracetam**	**Ethanol + Aniracetam**
Male	**n = 25**	**n = 33**	**n = 9**	**n = 14**
Number of entries into open arms	2.5±0.3	2.4±0.3	4.6±0.7 *#	5.5±0.6 *#
Time spent in open arms	31.8±5.4	26.9±3.2	54.2±8.2 *#	59.1±5.8 *#
Female	**n = 19**	**n = 29**	**n = 7**	**n = 17**
Number of entries into open arms	3.4±0.3	2.1±0.2 *	5.4±1.2 *#	4.8±0.5 *#
Time spent in open arms	41.2±2.9	23.9±2.7 *	70.5±15.6 *#	58.3±7.1 *#

Number of entries into open arm and duration of time spent in the open arm of the plus maze for each of the treatment group (PND 40) for comparison.
Values are given as mean ± S.E.M
* Significantly different from control group at $P < 0.0001$ by ANOVA
# Significantly different from prenatal ethanol exposed group at $P < 0.001$ by ANOVA

Table 3.3. Changes in percentage of helpless learners and those with >15 escape failures during learning and memory test.

Treatment Groups		Helpless Learners (%)	Animals with >15 escape failures (%)
Ethanol	M (n=20)	45	45
	F (n=20)	25	10
Control	M (n=20)	9	9
	F (n=20)	4	4
Control + Aniracetam	M (n=20)	24	24
	F (n=20)	5	5
Ethanol + Aniracetam	M (n=20)	20	5
	F (n=20)	0	0

4 POSTNATAL ANIRACETAM TREATMENT IMPROVES PRENATAL ETHANOL INDUCED ATTENUATION OF AMPA RECEPTOR MEDIATED SYNAPTIC TRANSMISSION

Abstract

Aniracetam is a nootropic compound and an allosteric modulator of AMPA receptors (AMPARs) which mediate synaptic mechanisms of learning and memory. Here we analyzed impairments in AMPAR-mediated synaptic transmission caused by moderate prenatal ethanol exposure, and investigated the effects of postnatal aniracetam treatment on these abnormalities. Pregnant Sprague Dawley rats were gavaged with ethanol or isocaloric sucrose throughout pregnancy, and subsequently the offspring were treated with aniracetam on postnatal days (PND) 18 to 27. Hippocampal slices prepared from these pups on PND 28 to 34 were used for the whole-cell patch-clamp recordings of AMPAR-mediated spontaneous and miniature excitatory postsynaptic currents in CA1 pyramidal cells. Our results indicate that moderate ethanol exposure during pregnancy results in impaired hippocampal AMPAR-mediated neurotransmission and critically timed aniracetam treatment can abrogate this deficiency. These results highlight the possibility that aniracetam treatment can restore synaptic transmission and ameliorate cognitive deficits associated with the fetal alcohol syndrome.

Introduction

Exposure to ethanol during pregnancy can have deleterious effects on the immature nervous system. Fetal alcohol syndrome (FAS) is the extreme end of a spectrum of ethanol related teratogenic effects and is characterized by morphological malformations, behavioral problems, and deficits in learning and memory (Bagheri et al., 1998). Previous studies have demonstrated that ethanol exposure causes developmental abnormalities of hippocampal granule cells which give rise to mossy fibers (Wigal and Amsel, 1990) and significant decrease in pyramidal cell density in the CA1 region of the hippocampus(McGoey et al., 2003). Furthermore, *in utero* ethanol exposed animals show marked impairment of spatial memory (Pauli et al., 1995), along with decreased long-term potentiation (LTP) in the CA1 (Morrisett et al., 1989; Tan et al., 1990; Richardson et al., 2002).

The mechanism by which ethanol impairs synaptic transmission and LTP in the hippocampus is not clearly understood (Goodlett and Horn, 2001). However, several studies have demonstrated that ethanol can modulate N-methyl-D-aspartate receptors (NMDARs) (Swartzwelder et al., 1995; Naassila and Daoust, 2002; Nixon et al., 2004). It is well known that offspring exposed to ethanol during pregnancy show reduced NMDAR sensitivity to exogenously applied NMDA and an enhancement of the Mg^{2+} block of the NMDA receptors at hyperpolarized potentials (Morrisett et al., 1989). Although one study reported that alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) are relatively insensitive to ethanol in the CA3 of hippocampal slices (Weiner et al., 1999), acute ethanol exposure has resulted in inhibition of AMPARs

in the nucleus accumbens, the central amygdala, and the somato-sensory cortex (Nie et al., 1994; Lu and Yeh, 1999; Roberto et al., 2004), indicating a potential role for modulation of AMPARs in FAS.

The activity of AMPARs is known to provide major source of neuronal depolarization, which is necessary to trigger Ca^{2+} influx and induction of synaptic plasticity. Furthermore, changes in activity and trafficking of AMPARs underlie maintenance of synaptic plasticity. Experiments in transgenic mice (Tsien et al., 1996), as well as pharmacological studies in rodents and humans (Rammsayer, 2001) also highlight the importance of AMPARs in learning and memory. It is noteworthy that memory enhancing nootropic molecules like aniracetam [1-(4-methoxybenzoyl)-2-pyrrolidinone] enhance cognitive function by potentiating AMPARs (Staubli et al., 1994a; Staubli et al., 1994b; Zivkovic et al., 1995). Aniracetam, a positive allosteric modulator, stabilizes the glutamate-bound state and slows the AMPAR deactivation rate by strengthening the association between glutamate and the ligand-binding domain, thus, increasing the channel open time as a result of longer activation times (Vyklicky et al., 1991; Partin et al., 1996; Arai et al., 2000; Lawrence et al., 2003). In addition, aniracetam has been shown to reduce the desensitization of AMPAR (Isaacson and Nicoll, 1991; Tang et al., 1991; Arai et al., 2000; Sun et al., 2002). Consequently, this pyrrolidinone nootropic compound enhances cognitive function by modulating glutamatergic transmission in the hippocampus and may potentially be an effective treatment in cognitive dysfunction (O'Neill et al., 2004) associated with FAS (Vaglenova and Vesselinov Petkov, 2001).

There are currently no clinical remedies available for either specific or global fetal alcohol effects (Hannigan and Armant, 2000). If AMPAR dysfunction contributes to the

observed cognitive impairments in prenatal ethanol exposure, a treatment that maintains AMPAR function would be beneficial. In the present study, we used the whole-cell patch-clamp technique to study glutamatergic transmission in the CA1 region of the hippocampus, and address the hypothesis that prenatal ethanol exposure leads to AMPARs dysfunction which can be alleviated by postnatal aniracetam treatment.

Materials and Methods

Chemicals

Unless indicated, all chemicals were from Sigma (St. Louis, MO) or Tocris Cookson (Bristol, UK).

Chronic prenatal ethanol exposure paradigm

Two-month old, pregnant Sprague Dawley rats (Zivic-Miller Laboratories, Pittsburgh, PA) were purchased on their gestation day (GD) 2 and housed in a pathogen free USDA and AAALAC approved facility at Auburn University. Beginning on GD 3, rats were randomly assigned to one of two treatment groups: ethanol or isocaloric sucrose. Each pregnant animal in the ethanol group received a moderate dose of ethanol (4g/kg of body weight/day) as an aqueous ethanol solution (38% v/v). This ethanol regimen mimics a moderate drinking pattern and had been used in previous studies without adverse effects (Keshavarzian et al., 2001). In the isocaloric-sucrose group, each pregnant rat received same volume of isocaloric sucrose solution. Both groups had ad libitum access to water and food. Each treatment was given daily from GD 3 to 20 (term,

about 20) by gavage, once a day (to avoid additional stress effects of repeated gavage). Daily ethanol administration was chosen to avoid repeated withdrawal episodes (Maier and West, 2001). On GD 14 blood samples were taken from the tail vein 1 hour after ethanol administration, when the apparent maximal maternal blood ethanol concentration (BEC = 185 ± 0.3 mg/dl; n = 6) was achieved. At birth, all litters were weighed and culled to a final number of 10 pups/litter. The day after parturition was considered postnatal day (PND) 1. The progeny was kept with dams until PND 25. In order to control litter effects, no more than 1 mate per gender was placed into a particular group (Wainwright, 1998). Commercially available Ampamet (aniracetam) (A.Menarini Ind. s.r.l, France) tablets were broken and powdered in a glass mortar and the resulting powder stirred in dichloromethane for 24 hours. The suspension was then filtered and the filtrate evaporated to dryness under reduced pressure. The resulting white powder was recrystallized from a mixture of n-heptane and benzene to yield white crystals. The purity and identity of the material was confirmed by GC-MS analysis. Randomly selected pups from both groups were treated with aniracetam at a single dose of 2 ml/kg at 50 mg/kg, dissolved in distilled water, by gavage for 10 days (PND 18-27), during their preadolescent period (Spear, 2000). To avoid the acute effects of aniracetam, animals were tested one to seven days after the last dose of the drug. This dose and route was selected upon previous studies by numerous authors (Giurgea, 1980; Coper and Herrmann, 1988; Petkov et al., 1991; Gouliaev and Senning, 1994; Vaglenova and Vesselinov Petkov, 2001). The age of studied rats (males and females) varied between 28 and 34 days, average ages of rats in all compared groups were between 30.8 and 31 days. There were no significant differences in age or gender between groups. Inclusion of age

or gender as a covariate in ANOVA did not affect any reported conclusions regarding the significance of effects of aniracetam and ethanol treatments on all measured parameters, thus, justifying consideration of samples in which all data derived from 28- to 34-day-old male and female rats were pooled together.

Transverse hippocampal slice preparation

Hippocampal slices (400 μm) were prepared from prenatal ethanol, isocaloric sucrose, and aniracetam treated rats at PND 28 to 34, as previously described (Zeng et al., 1995; Zeng and Tietz, 1999). Briefly, rats were sedated with $C0_2$, decapitated and transverse hippocampal slices were prepared on a vibratome (Warner instruments, Hamden, CT) in ice-cold, pregassed ($95\%O_2$:$5\%CO_2$) ACSF containing (in mM): NaCl 119, KCl 2.5, $MgSO_4$ 1.3, $CaCl_2$ 2.5, NaH_2PO_4 1.0, $NaHCO_3$ 26.0 and dextrose 11.0. Slices were maintained for ≥ 1 h in gassed ACSF and then transferred to a chamber perfused with ACSF at a rate of 2 ml/min.

Electrophysiological recording

Whole-cell patch-clamp electrophysiological recordings from CA1 pyramidal neurons were performed on the stage of a microscope (Olympus BX51WI; Olympus America Inc., Center Valley, PA) with water immersion differential interphase contrast objectives, at 32^0C with an Axoptach 200B amplifier (Molecular Devices Corp., Sunnyvale, CA). Whole-cell recordings from CA1 pyramidal neurons were made with a pipette (5-7 MΩ resistance) containing (in mM): K-gluconate 100, EGTA 0.6, $MgCl_2$ 5.0, Na-ATP 2.0, Na-GTP 0.3 and HEPES 40. The serial resistance (R_s, defined here as the

total resistance between the amplifier and the cell interior) during recordings was in the range of 10-15 MΩ and there was no difference in this parameter between experimental groups. To obtain low noise recordings R_s was not compensated, but was monitored throughout the experiment. Recordings were terminated when R_s was ≥ 15 MΩ or a significant increase occurred. Tight seals (≥ 2 GΩ before breaking into whole cell mode) were achieved by applying negative pressure to the pipette during approach to the cell. AMPAR-mediated action potential dependent spontaneous EPSCs (sEPSCs) were first recorded in the presence of picrotoxin (PTX, 50 μM) and DL-2-amino-5-phosphonopentanoic acid (APV, 100 μM) to suppress the $GABA_A$ receptor and NMDAR mediated currents, respectively. Five-seven minutes after addition of 1 μM, TTX, to suppress the action-potential-dependent transmitter release, recordings of AMPAR-mediated miniature EPSCs (mEPSCs) were performed at a holding potential of -80 mV. At the end of all experiments 30 μM CNQX was added to the bath solution. Complete blockage of all synaptic activity was taken as evidence that the sEPSCs and mEPSCs were mediated by AMPARs. Current output was low-pass filtered (2 kHz), DC-offset and amplified 10 000 fold. The signal was continuously monitored on-line (pClamp 8.0 Software, Molecular Devices Corp., Sunnyvale, CA), digitized (Digidata 1200, Axon) and stored for later off-line analysis. Baseline mEPSC activity was recorded in each neuron at least for 15 min.

Data analysis

Acquired data were analyzed with Mini Analysis program (Synaptosoft Inc., Fort Lee, NJ), only if there was an adequate number of clearly identifiable sEPSCs and mEPSCs; i.e. events with the time course broadly resembling that of the "ideal sEPSC/mEPSC" (the rise time shorter than the decay time). Peak sEPSC/mEPSC amplitude was measured from the baseline. The time constant of decay (τ_d) was defined as the time interval needed for a sEPSC/mEPSC to decline to 1/e of the maximal value, and was calculated from the time needed for a sEPSC/mEPSC to decrease from 90-30%. Decay kinetics and amplitudes (*A*) were also estimated using a single exponential function: [$y(t) = A*\exp(-t/\tau_d)$]. The rise time (τ_r) was defined as the time needed for the sEPSC/mEPSC to reach from 10-90% of the maximal value. The coefficient of variation, which served as an indicator of variability, of each parameter was calculated as the ratio of the standard deviation (SD) to the respective mean value.

To evaluate the statistical significance of the linear effects of aniracetam and ethanol (and their interaction) on the correlation between rise time and amplitude (and decay time and amplitude), the General Linear Model was used (Statistical Analysis System, SAS Inc., Cary, NC). For example, the relationship between rise time (τ_r) and amplitude (*A*) was described examined by fitting different intercepts/different slopes regression model.

$$\text{Rise} = \beta_0 + \beta_1 \text{A} + \beta_2 \text{Drug} + \beta_3 \text{Drug} * \text{A} + \beta_4 \text{Alc} + \beta_5 \text{Alc} * \text{A} + \beta_6 \text{Drug} * \text{Alc} * \text{A} + \varepsilon$$

where β_0 and β_1 represent the intercept and slope for the control animals, β_2 and β_3 represent the change in intercept and slope in the aniracetam treated group of

animals, β_4 and β_5 represent the change in intercept and slope in the ethanol treated group, β_6 represents the interaction effect between aniracetam and ethanol, and ε is the random error (assumed to be normal with mean zero and variance σ^2). The exact same model was built for the relationship between decay time and amplitude. The variable Drug is coded as 0 for no aniracetam and 1 for aniracetam treatment and the variable Alc is coded as 0 for no ethanol and 1 for ethanol. Note that when both Alc and Drug are zero this represents the control animals, and when both are equal to one, it represents the animals that were treated with both ethanol and aniracetam. As stated previously, age or gender did not significantly change the interpretations or conclusions derived from the above model.

In addition, data was compared statistically by two-way ANOVA using general linear model procedure in SAS (Statistical Analysis System, SAS Inc., Cary, NC) with ethanol and aniracetam as factors and the nonparametric Kolmogorov-Smirnov test (K-S test). In all cases statistical significance was reported when $P < 0.05$.

Results

Changes in the amplitude and frequency of miniature and spontaneous EPSCs by prenatal ethanol exposure and postnatal aniracetam treatment

Regulation of synaptic AMPAR-mediated mEPSCs could arise from modifications in the channel properties of synaptic AMPARs as well as from changes in the number of functional receptors present in CA1 synapses. To investigate the effects of ethanol and aniracetam on number and properties of AMPARs activated by a single

vesicle release, we first compared the average amplitudes of mEPSCs recorded in the presence of 1 μM tetrodotoxin (Fig. 4.1A). Significant changes in amplitude were found across groups (control: 15.09 ± 0.54 pA; ethanol: 11.84 ± 0.47 pA; control+aniracetam: 18.32 ± 0.65 pA; ethanol+aniracetam 16.91 ± 0.63 pA; N = 10-15 rats per group; *N* = 2-4 neurons per rat; two-way ANOVA: $P < 0.0001$, for both ethanol and aniracetam treatments) (Fig. 4.1B). The effect of aniracetam on mEPSC amplitude of the prenatally exposed ethanol group was not significantly different from its effect on control group ($P > 0.05$). In other words, there was no significant interaction effect between aniracetam and ethanol. Consistent with analysis of mean values, analysis of cumulative probability distributions of mEPSC amplitudes (Fig. 4.1C) compared by K-S test showed that the prenatal ethanol exposed group had a leftward shift as compared to controls, control+aniracetam and prenatal ethanol+aniracetam groups ($P < 0.001$). Both control+aniracetam, and prenatal ethanol+aniracetam groups showed a rightward shift when compared with the control ($P = 0.027$, 0.035 respectively). These results indicate that postnatal aniracetam treatment restores impaired AMPAR function in prenatally ethanol exposed rats.

To determine the presynaptic effects of treatments on glutamate release, we compared the frequency of mEPSCs among the four groups (Ghamari-Langroudi and Glavinovic, 1998; Ling et al., 2006). Prenatal ethanol exposure decreased the mean frequency. In contrast, there was a considerable increase in mEPSC frequency in the postnatal aniracetam treated group exposed to ethanol *in utero*. Basal mEPSC frequencies were as follows: control: 0.34 ± 0.04 Hz; ethanol: 0.19 ± 0.02 Hz; control+aniracetam: 0.39 ± 0.05 Hz; ethanol+aniracetam 0.42 ± 0.05 Hz ($P < 0.0001$ for both ethanol and

aniracetam treatments, two-way ANOVA) (Fig. 4.1D). For mEPSC frequency, there was a significantly stronger effect of aniracetam on the prenatally ethanol exposed group ($P < 0.05$ for interaction between ethanol and aniracetam treatments; two-way ANOVA). Based on K-S test for cumulative distributions of inter-event interval (Fig. 4.1E), the only significant difference that was observed was for the prenatally ethanol exposed group ($P < 0.002$) indicating a rightward shift (longer intervals). Because the mEPSC frequency is a measure of presynaptic function, these results indicate that prenatal ethanol exposure leads to a decrease in synaptic glutamate release. Furthermore, the increased mEPSC frequency in aniracetam treated groups suggests that aniracetam may increase glutamate release. This altered glutamate release may be due to the changes in the number of presynaptic terminals, or modifications in the probability of release.

Similar effects of ethanol and aniracetam treatments were observed on action potential-evoked spontaneous AMPAR-mediated synaptic transmission recorded in the absence of tetrodotoxin. Table 4.1 shows average synaptic parameters of sEPSCs recorded in the four experimental groups. Prenatal exposure to ethanol significantly decreased the mean amplitude, frequency, rise time and decay time ($P < 0.005$*;* two-way ANOVA, N = 10-20 rats per group; N = 2-4 neurons per rat). After treatment with aniracetam, the above four parameters increased significantly ($P < 0.001$). Aniracetam treatment for the prenatally ethanol exposed group also significantly increased these parameters compared with controls ($P < 0.05$). These results suggest that prenatal ethanol exposure results in decreased AMPAR function and synaptic glutamate release. However, in the ethanol + aniracetam treated group, the ethanol induced decreases in AMPAR-mediated synaptic transmission was ameliorated.

Changes in the temporal parameters and variability of mEPSCs induced by prenatal ethanol exposure and postnatal aniracetam treatment

To study the time course of mEPSCs among the four groups, we compared their rise time and decay time constants. Ethanol and aniracetam treatments significantly affected the rise time (Fig. 4.3A, $P < 0.01$) and the decay time (Figs. 4.2 and 4.3C, $P < 0.001$). There was also a significantly stronger effect of aniracetam on the ethanol exposed group decay time ($P < 0.005$) compared to control. There was only a tendency for significant interaction between effects of aniracetam and ethanol on the rise time ($P = 0.052$). Relative cumulative probability distributions of rise time (Fig. 4.3B) and decay time constants (Fig. 4.3D) were constructed for all recorded neurons to assess changes in temporal parameters of mEPSCs. There were significant differences in the rise time (K-S test, $P < 0.002$) and decay time (K-S test, $P < 0.001$) in neurons of aniracetam treated groups and control group compared to ethanol exposed group. These findings demonstrate that the time courses of mEPSCs in ethanol exposed animals are faster than in control and postnatally aniracetam treated pups.

To compare the variability of mEPSCs following ethanol exposure or aniracetam treatment, the coefficient of variance (CV; defined as SD/mean) was calculated for amplitude (Fig. 4.4A), rise time (Fig. 4.4B), and decay time (Fig. 4.4C) across groups. Prenatal ethanol exposure alone did not lead to a significant changes in the CV of amplitude, rise time, and decay time ($P > 0.05$), whereas aniracetam significantly increased the CV of these values ($P < 0.05$) and there were significant interactions between effects of ethanol and aniracetam on CV of amplitude and rise time ($P < 0.05$). The baseline noise did not contribute significantly to the CV of mEPSCs, since it was

consistently low in all four compared groups.

Amplitude dependence of rise and decay times: changes induced by postnatal aniracetam treatment

Since the relationship between amplitude and time course of mEPSCs recorded from pyramidal neurons can be differentially affected by dendritic filtering (Wyllie et al., 1994), and changes in AMPAR desensitization (Atassi and Glavinovic, 1999), plots of rise time and decay time constants versus amplitude were constructed using mean values of each experiment and used to investigate interdependence between these parameters (Fig. 4.5). There was no correlation between rise times and amplitudes of events recorded in neurons from the control group ($R^2 = 0.02$, $P > 0.7$) (Fig. 4.5A, filled circles). There was a weak correlation between rise time constants of ethanol exposed groups ($R^2 = 0.14$, $P < 0.05$) (Fig. 4.5B, filled circles). Similarly, there was no correlation between decay time constants and amplitudes from controls ($R^2 = 0.05$, $P > 0.3$) (Fig. 4.5C, filled circles) and a modest correlation between these parameters for ethanol exposed rats ($R^2 = 0.26$, $P < 0.005$) (Fig. 4.5D, filled circles). Interestingly, a strong interdependence of rise time was observed for aniracetam treated control ($R^2 = 0.48$, $P < 0.001$) (Fig. 4.5A, open circles) and prenatal ethanol exposed aniracetam treated groups ($R^2 = 0.66$, $P < 0.001$) (Fig. 4.5B, open circles). A strong correlation was also found for amplitude and decay time in aniracetam treated control group ($R^2 = 0.46$, $P < 0.001$) (Fig. 4.5C, open circles) and for aniracetam treated ethanol exposed group ($R^2 = 0.66$, $P < 0.001$) (Fig. 4.5D, open circles). Thus, aniracetam treatment strongly increases dependence between temporal parameters and amplitude.

Next we used the different intercepts/slopes model (see Materials and Methods) to determine if the above regression models were significantly different between treatment groups and to analyze the dependence of rise and decay times on the amplitude and aniracetam and ethanol treatments, and interactions between these factors. This analysis also revealed no significant linear dependence (slope) between rise and amplitude for the control animals ($P > 0.7$) (Table 4.2), in agreement with the outcome of correlation analysis described above (Fig. 4.5). There were significant amplitude-independent average effects (intercepts) on rise time for aniracetam and ethanol treatments ($P < 0.0001$ and $P < 0.01$, respectively), and significant positive linear amplitude-dependent effects (slopes) for both aniracetam and ethanol treatments ($P < 0.0001$ and $P < 0.05$, respectively). The combined aniracetam and ethanol effect (interaction) was also significant ($P < 0.05$). Since the term describing interaction is negative, the combined effect of ethanol and aniracetam on the rise time is considered to be "less than additive."

For decay time, there was a significant amplitude-independent effect (intercept) of ethanol treatment ($P < 0.01$), a significant positive linear amplitude-dependent effect (slope) of aniracetam treatment ($P < 0.001$), and interaction between aniracetam and ethanol effects ($P < 0.05$). Since the term describing interaction is positive, the combined effect of ethanol and aniracetam on decay time is considered to be "more than additive". In summary, this analysis dissected effects of ethanol and aniracetam treatments on temporal parameters in amplitude-dependent and independent components and showed differential contributions of these components to variability of rise versus decay times. Also, the mode of interaction between ethanol and aniracetam effects appeared to be different for rise versus decay times.

Discussion

In this study we used a sensitive animal model that exhibits lower birth weights, developmental retardation and learning and memory deficits (observed in the two-way active avoidance test) similar to that seen in FAS (Pandiella et al., 2005). Our behavioral analysis has revealed that the observed cognitive deficits can be ameliorated by postnatal treatment with aniracetam (Pandiella et al., 2005). Here we investigated the AMPAR-mediated synaptic transmission using this animal model, since these receptors are critical for certain forms of learning and memory.

We report here several novel findings. First, AMPAR-mediated synaptic transmission in CA1 pyramidal neurons is impaired by ethanol exposure *in utero*. Second, this study provides mechanistic evidence for the consideration of aniracetam as an effective therapy for reducing the risk of cognitive impairments in FAS, by enhancing the AMPAR-mediated synaptic transmission. We demonstrate that AMPAR-mediated miniature and spontaneous current frequencies are dramatically reduced in fetal alcohol exposure. This reduction in frequency may reflect an increase in postsynaptically silent synapses or be due to presynaptic deficits. The latter hypothesis is consistent with the previous findings which indicate that ethanol exposure altered spontaneous glutamate vesicle release (Bolshakov and Siegelbaum, 1995; Wasling et al., 2004; Mameli et al., 2005), impairs axonal development (Davies and Smith, 1981), decreases the number of hippocampal presynaptic nerve terminals (Hoff, 1988), and synaptic density (Kuge et al., 1993; Gibson et al., 2000). Thus, the combined evidence suggests that prenatal ethanol exposure modifies AMPAR function by decreasing the basal glutamate release and thereby possibly contributes to cognitive impairments in our animal model of FAS.

We also noted a significant reduction in AMPAR-mediated mEPSC amplitude, rise time and decay time constants in ethanol exposure indicating a role for postsynaptic mechanism in decreasing synaptic transmission. These findings are in agreement with those of Moykkynen et al., (2003) and Mameli et al., (2005) who demonstrated that exogenously applied ethanol inhibits steady-state currents activated by AMPA in hippocampal slices. Therefore, it is conceivable that both presynaptic and postsynaptic effects of ethanol on hippocampal neurons are likely to contribute to impaired AMPAR-mediated synaptic transmission.

In addition to a direct effect of ethanol on development of pups, there could be indirect influences via changes in mother-pup interactions in the ethanol exposed group (Chen et al., 1982; Rockwood and Riley, 1986; Murillo-Fuentes et al., 2001). In this respect it is noteworthy that although there are reductions in birth weight and developmental reflexes in the ethanol exposed pups, they compensate for these deficiencies by PND 5 (unpublished data). Since our experiments were conducted at PND 28-34, differences in mother-pup interactions would presumably not have any gross effect on physiological status of pups at this age. However, we can not fully exclude more delicate effects of abnormal mother-pup interactions.

In the present study, postnatal aniracetam treatment significantly increased the mEPSC amplitude (increase in larger amplitude events), frequency, decay time and rise time. Therefore, it is evident that aniracetam acts by reversing the presynaptic and postsynaptic alterations caused by prenatal ethanol exposure. It is well established that aniracetam exerts its acute effect on AMPARs by slowing the rate of deactivation of the receptor, which has a subsequent effect on desensitization (Ito et al., 1990; Isaacson and

Nicoll, 1991). However, because our experiments were carried out 1-7 days following termination of aniracetam treatment, the modifications we observed should reflect long-term aniracetam-induced alterations of synaptic transmission.

The mechanisms responsible for these long-term modifications in AMPAR function may be related to up-regulation of the expression of the flip isoform of GluR subunits, which desensitize and deactivate slower than the flop versions (Partin et al., 1996). In addition, aniracetam has been shown to activate protein kinases such as PKC–γ in the hippocampus, thus, enhancing hippocampal learning and memory (Lucchi et al., 1993; Lu and Wehner, 1997; Smith and Wehner, 2002). A sustained increased ratio of membrane:cytosolic hippocampal PKC-γ following termination of aniracetam treatment has been demonstrated, indicating a long-term effect of aniracetam (Smith and Wehner, 2002). It is reasonable to speculate that AMPAR activity is enhanced after aniracetam treatment due to phosphorylation of GluR2 by PKC (McDonald et al., 2000) or PKC-dependent changes in expression of other molecules engaged in the control of AMPAR desensitization. These changes suggest that there is a long-term effect of preadolescent aniracetam treatment in improving the impaired synaptic transmission in prenatal ethanol exposure via modulating the AMPARs in conditions such as FAS.

It is interesting to note that the long-term effects of aniracetam share some features with the acute effects of the drug (Ghamari-Langroudi and Glavinovic, 1998; Isaacson and Nicoll, 1991; Tang et al., 1991; Arai et al., 2000; Sun et al., 2002), which involve a reduction in the desensitization and deactivation of AMPARs. Specifically we show an increase in mEPSC decay time which corresponds to the reduced deactivation and desensitization of postsynaptic AMPARs. We also observed several other long-term

changes such as an increase in the Coefficient of Variance (a measure of variability) of amplitude, decay time, and rise time; furthermore, there was an upward shift in the ratio of τ_d:*A* and τ_r:*A* in aniracetam treated groups which were similarly observed in acute studies using aniracetam (Ghamari-Langroudi and Glavinovic, 1998) and another inhibitor of AMPAR desensitization, cyclothiazide (Atassi and Glavinovic, 1999).

In conclusion, the findings of the present study are significant because they provide, at least in part, a plausible explanation for the alterations in the behavioral responses due to modifications in AMPAR-mediated synaptic transmission in FAS. Furthermore, the postnatal aniracetam treatment ameliorates the cognitive impairments by modulating the hippocampal synaptic AMPAR function. Therefore, this study has addressed a challenging task of understanding the precise contribution of AMPAR-mediated synaptic transmission in the pathophysiology associated with FAS and the pharmacological intervention by aniracetam.

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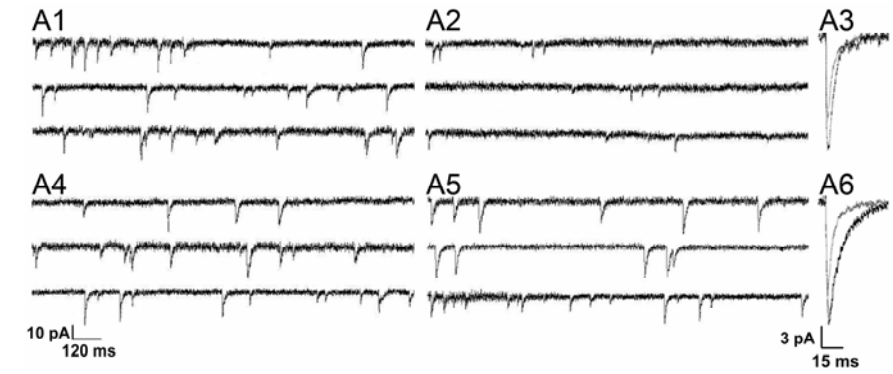

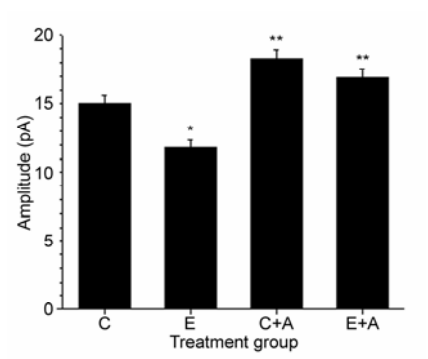

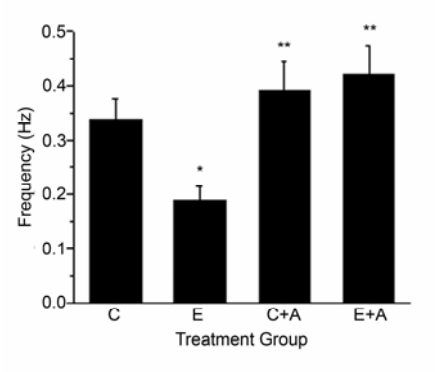

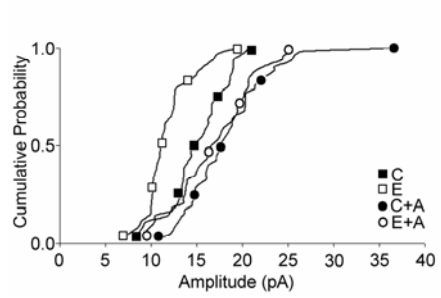

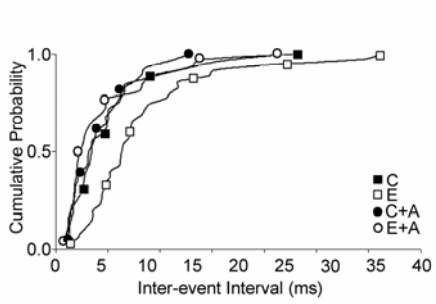

Figure 4.1. AMPAR-mediated mEPSCs of prenatally ethanol exposed group is improved by postnatal aniracetam treatment.(A) Left: Sample traces of mEPSCs from an individual neuron from each of the four groups. Neurons from prenatal ethanol exposed rats (A2) showed a decreased mean mEPSC amplitude and frequency when compared to neurons from control rats (A1). Each panel represents 1.5-s traces. (A3): Representative average mEPSCs from a control CA1 neuron (dashed line trace, n = 147 events), and a neuron from a prenatally ethanol exposed rat (gray trace, n = 83 events) different from those shown in traces. Following 10-day postnatal aniracetam treatment, there was a significant increase in average mEPSC amplitude and frequency in CA1 pyramidal neurons relative

to prenatal ethanol exposed neurons. (A6): Representative average mEPSCs from a neuron from an ethanol exposed animal (gray trace, n = 83 events) and a neuron from aniracetam treated, ethanol exposed rat (black trace, n = 123 events) (A1: control, A2: prenatal ethanol, A4: control, postnatally treated with aniracetam, A5: postnatally aniracetam treated, prenatal ethanol exposed rat). (B) Bar graph illustrating the sensitivity of AMPAR-mediated mEPSCs amplitude to chronic prenatal ethanol exposure and postnatal aniracetam treatment. (C) Relative cumulative probability distribution of mEPSC amplitudes in CA1 neurons from all 4 groups (N = 10-15 rats per group; *N* = 2-4 neurons per rat). The distribution of mEPSC amplitudes in CA1 neurons from ethanol exposed rats was significantly shifted to the left indicating a significant decrease in the proportion of larger amplitude events (K-S test, $P < 0.001$). There was a significant increase in the proportion of larger amplitude events in aniracetam treated groups (corresponding to the right shift of the cumulative probability distribution).(D) Bar graph illustrating the decrease in mEPSC frequency in ethanol exposed group, where as a ~2 fold increase in the aniracetam treated group. (E): Cumulative frequency (interevent interval) histograms depicting the distributions of mEPSC data for all the cells tested in E (K-S test, $P < 0.002$). * $P < 0.0001$ indicates significant differences compared with the isocaloric sucrose (control) group. ** $P < 0.0001$ indicates significant differences in the ethanol exposed group versus ethanol aniracetam treated group. Groups: C, control; E, prenatally ethanol exposed; C+A, control group treated with aniracetam postnatally; E+A, prenatal ethanol exposed group treated with aniracetam postnatally.

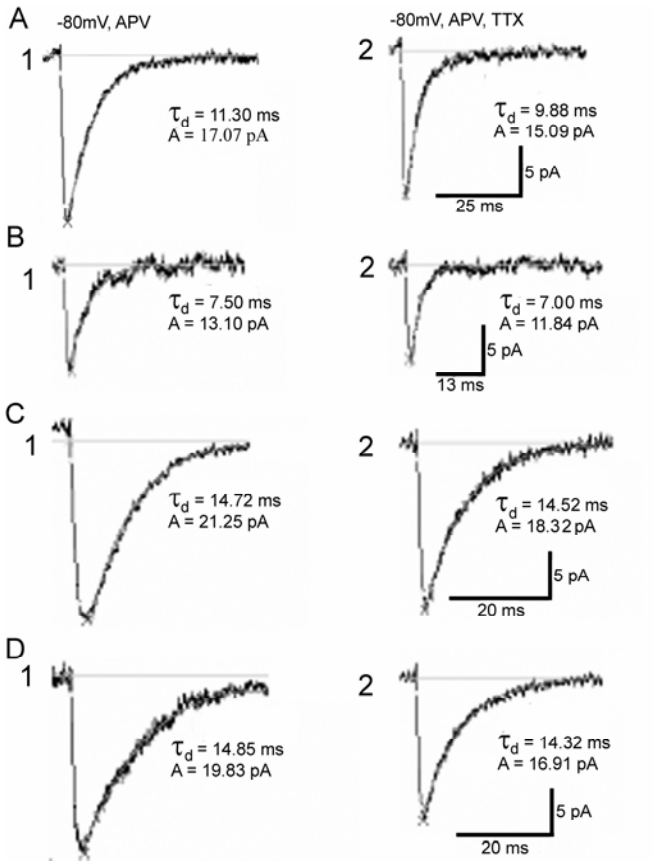

Figure 4.2. Basic properties of spontaneous and miniature excitatory postsynaptic currents (sEPSCs and mEPSCs) in hippocampal CA1 pyramidal neurons recorded at -80 mV. A1: averaged sEPSCs recorded from a control in the absence of tetrodotoxin (TTX). A2: Bath application of 1 μM TTX did not significantly alter averaged mEPSC wave forms. B1: Averaged sEPSCs and B2: mEPSCs of prenatally ethanol exposed animals had a significantly altered averaged amplitude and decay time constants, compared to control. C: Averaged sEPSCs and mEPSCs recorded from control group treated with aniracetam postnatally. Note this sEPSCs and mEPSCs had slow decay time and increased mean amplitude compared to the control and the ethanol exposed group. D: Postnatal aniracetam treatment prolonged the averaged sEPSCs and mEPSCs waves. Records in A-D were taken from single CA1 hippocampal pyramidal cells from 4 different groups. Traces are averages of 44-164 individual mEPSCs. Decays were fitted with a single-exponential function. Fits are shown superimposed and time constants and mean amplitudes are given below each trace. τ_d, decay time constant; *A*, mean peak amplitude.

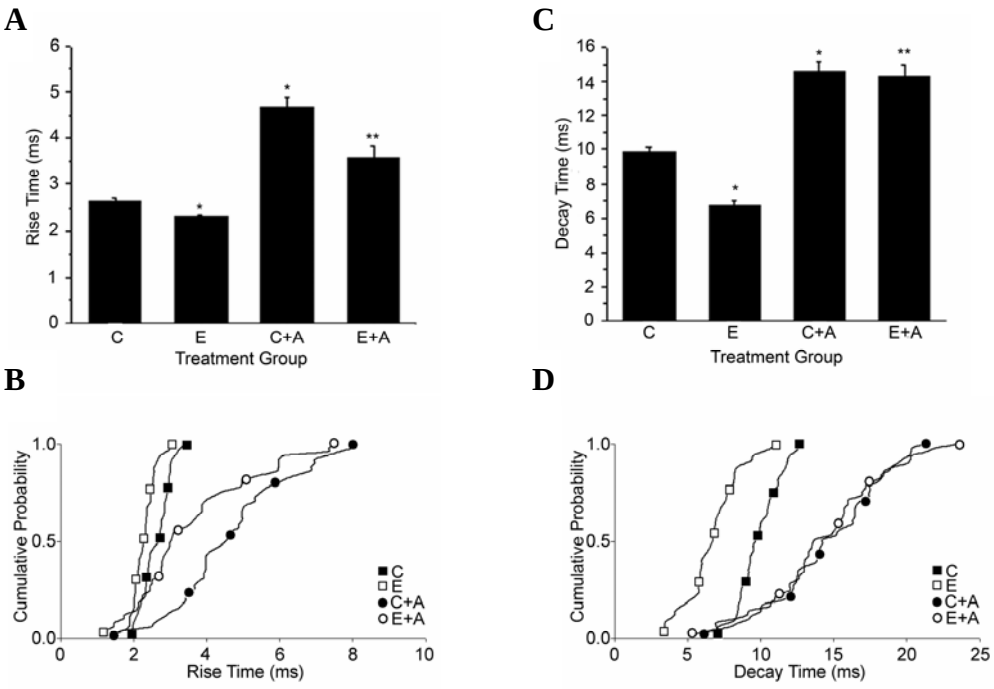

Figure 4.3. Effects of chronic prenatal ethanol and postnatal aniracetam treatment on mEPSC rise time and decay time constants in CA1 pyramidal neurons from rats sacrificed 1 day after 10-day oral aniracetam treatment. Mean rise time (A) and decay time (C) constants had a significant difference across groups. Relative cumulative frequency distributions of mEPSC rise time (B) and decay time (D) constants in CA1 pyramidal neurons from 4 groups. There were significant differences in the rise time and decay time constants in ethanol exposed group relative to control as well as ethanol exposed, aniracetam treated group. However, the interaction term of drug only on the prenatal ethanol exposure for the rise time was not significant; whereas, a significant interaction was noted for decay time. * $P < 0.01$ indicates significant differences compared with control group. ** $P < 0.001$ indicates that postnatal aniracetam treatment increased both rise and decay time compared with ethanol exposed group. Groups: C, control; E, prenatally ethanol exposed; C+A, control group treated with aniracetam postnatally; E+A, prenatal ethanol exposed group treated with aniracetam postnatally.

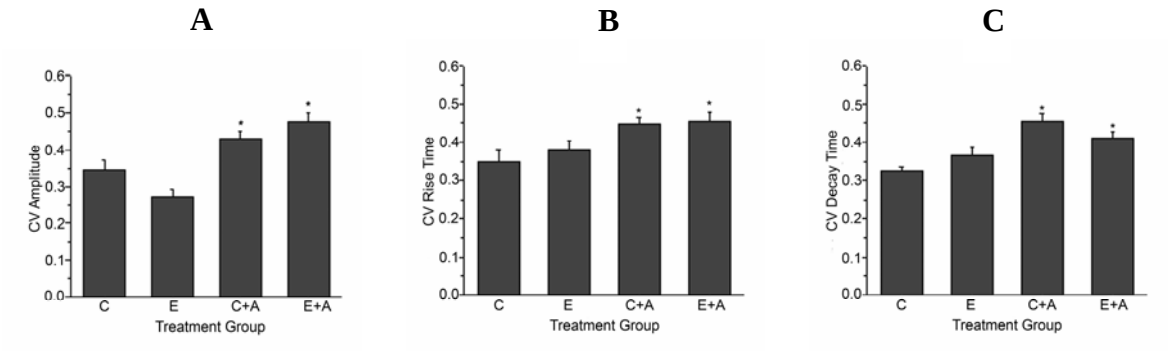

Figure 4.4. Coefficient of variance (CV = SD/mean) of mEPSC amplitudes (A), rise time (B) and decay time (C) constants. * $P < 0.05$, CV of amplitude, rise time and decay time is larger in aniracetam treated animals than in control and ethanol exposed animals. Groups: C, control; E, prenatally ethanol exposed; C+A, control group treated with aniracetam postnatally; E+A, prenatal ethanol exposed group treated with aniracetam postnatally.

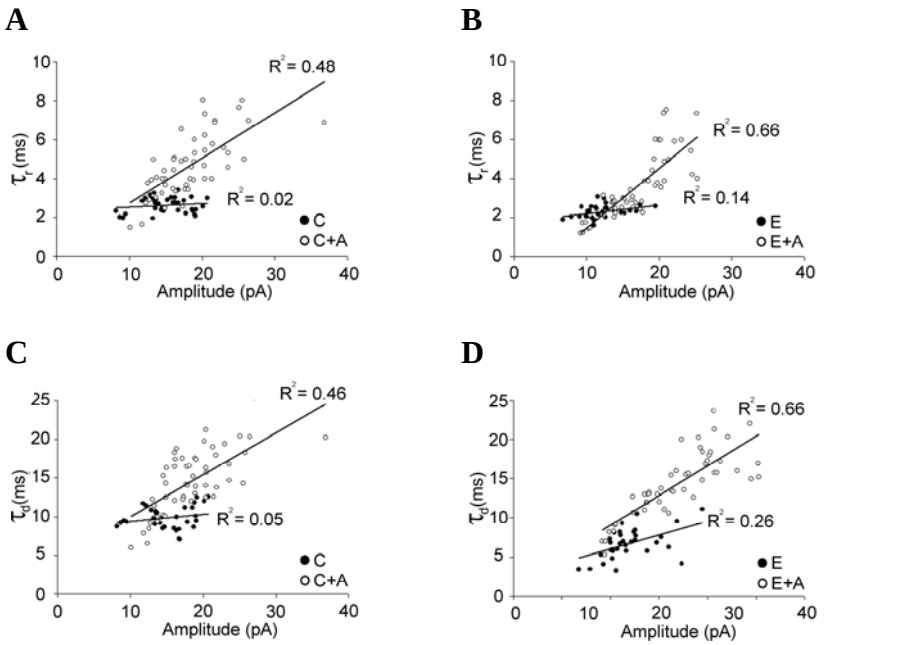

Figure 4.5. Amplitude dependence of both decay and rise times of mEPSCs increases following treatment with aniracetam. The relationship between the amplitudes and the time constants of rise of mEPSCs (A and B) or the time constants of decay (C and D) in the absence (filled circles) and presence (open circles) of aniracetam treatment. In all cases the lines were fitted using the least-squares method. There were no significant correlations in the rise time (A) and decay time constants (C) versus amplitude recorded in all control neurons (n = 36). There was a strong correlation between the rise time versus amplitude of each individual experiments of aniracetam treated control (A) and aniracetam treated ethanol exposed group (B). The correlation between the decay time constant and amplitude of aniracetam treated control (C) and aniracetam treated ethanol exposed group (D) was also strong. There was a weaker correlation in rise time (B) or decay time (D) versus amplitude in neurons from ethanol exposed animals in the absence of aniracetam treatment. Groups: C, control; E, prenatally ethanol exposed; C+A, control group treated with aniracetam postnatally; E+A, prenatal ethanol exposed group treated with aniracetam postnatally.

Table 4.1. sEPSC properties in hippocampal neurons from rats exposed to ethanol, isocaloric-sucrose (control) and aniracetam.

Parameter	Control (N=10)	Ethanol (N=18)	Control+Aniracetam (N=15)	Ethanol+Aniracetam (N=20)
Amplitude*: A* (pA)	17.07±0.67	13.10±0.91*	21.25±1.61^{+}	19.83±1.25$^{\#}$
Frequency*: F (* Hz)	0.44±0.10	0.24±0.02*	0.45±0.10	0.48±0.06$^{\#}$
Rise time: τ_r (msec)	2.50±0.07	2.14±0.07*	4.34±0.47^{+}	3.39±0.36$^{\#}$
Decay*:* τ_d (msec)	11.30±0.47	7.50±0.33*	14.72±1.20^{+}	14.85±1.01$^{\#}$

Notes: Values represent mean ± S.E.M which were calculated from amplitudes (*A*), frequencies (*F*), time constants of rise (τ_r) and decay (τ_d) determined for individual cells (total 30-50 cells from each group). N provides the number of rats and *N* (number of neurons per rat) is 2-4.

* Statistical difference ($P < 0.005$) between ethanol treated group and control group by ANOVA.

+ Statistical difference ($P < 0.001$) between control and control + aniracetam treated group by ANOVA**.**

Statistical difference ($P < 0.001$) between ethanol exposed group and ethanol + aniracetam treated group by ANOVA**.**

Table 4.2. Slopes and *y*-intercepts to assess the linear effects of prenatal ethanol exposure and postnatal aniracetam treatment on rise time (τ_r) vs. amplitude (A) and decay times (τ_d) vs. amplitude (A), as well as coefficient of determination (R^2).

Parameter	Estimate ± S.E.M	*P*-value
Slope τ_r:A (ms/pA): Control	-0.01 ± 0.04	0.728
Slope τ_r:A (ms/pA): Aniracetam	0.26 ± 0.04	<0.0001
Slope τ_r:A (ms/pA): Ethanol	0.09 ± 0.04	0.034
Slope Ethanol*Aniracetam interaction	-0.05 ± 0.02	0.039
y-intercept τ_r (ms): Control	2.83 ± 0.55	<0.001
y-intercept τ_r (ms): Aniracetam	-2.64 ± 0.58	<0.0001
y-intercept τ_r (ms): Ethanol	-1.51 ± 0.55	0.007
Coefficient of determination (R^2)	0.72	
Slope τ_d:A (ms/pA): Control	0.16 ± 0.1	0.104
Slope τ_d:A (ms/pA): Aniracetam	0.36 ± 0.09	<0.001
Slope τ_d:A (ms/pA): Ethanol	0.12 ± 0.11	0.294
Slope Ethanol*Aniracetam interaction	0.14 ± 0.06	0.016
y-intercept τ_d (ms): Control	7.42 ± 1.49	<0.001
y-intercept τ_d (ms): Aniracetam	-2.34 ± 1.57	0.138
y-intercept τ_d (ms): Ethanol	-4.02 ± 1.49	0.008
Coefficient of determination (R^2)	0.76	

To assess the linear effects of prenatal ethanol exposure and aniracetam treatment (and their interaction) on the correlation between rise time and amplitude (and decay and amplitude), a different slopes/different intercept model was applied using the General Linear Model (GLM) procedure in SAS. (N=10-15 animals).

5 AMELIORATING EFFECTS OF PREADOLESCENT ANIRACETAM TREATMENT ON PRENATAL ETHANOL-INDUCED IMPAIRMENT IN ACTIVITY OF AMPA RECEPTORS

Abstract

Ethanol-induced damage in the developing hippocampus may result in cognitive deficits commonly observed in fetal alcohol spectrum disorder (FASD). Cognitive deficits in FASD are partially mediated by alterations in glutamatergic synaptic transmission. Recently we reported that the function of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) glutamate receptor subtype is impaired following fetal ethanol exposure. This finding led us to develop a rational approach for the treatment of alcohol-related cognitive deficits using aniracetam, an allosteric modulator of AMPA receptors (AMPARs). Here, we hypothesize that aniracetam ameliorates prenatal ethanol-induced deficits through long lasting potentiation of AMPAR-mediated synaptic transmission, as a result of the modification of AMPAR single channels. Analysis of miniature excitatory postsynaptic currents (mEPSCs) in hippocampal slices from 28 to 34 day-old rats revealed persistent improvement in amplitude, frequency and time courses of mEPSCs after aniracetam treatment in ethanol-exposed animals. In addition, single channel properties of synaptic AMPARs of ethanol-exposed pups treated with aniracetam exhibited increase in channel open probability, conductance, mean open times, number and duration of bursts and decreased closed times. Our findings emphasize the

importance of targeting AMPARs using compounds like aniracetam, and suggest a potential strategy for an effective therapeutic approach which would address a major public health problem.

Introduction

Consumption of ethanol during pregnancy can have severe teratogenic effects on the developing fetus leading to fetal alcohol spectrum disorder (FASD); specifically, the most devastating of these is neurological dysfunction. The neurotoxic effects of ethanol on the developing central nervous system are not uniform, and are carried out by different mechanisms of multiple cellular and molecular pathways (Breese et al., 1993; Charness et al., 1994; Deltour et al., 1996; Perrone-Bizzozero et al., 1998; Bearer et al., 1999). The severity of the neurotoxic insult varies among different regions of the brain, and depends on the consumption amount, duration, and developmental timing of the prenatal ethanol exposure (Coles et al., 1991).

The hippocampus is one of the structures most vulnerable to FASD (Guerri, 1998); moreover, the alterations in the hippocampal tissue may include reduced pyramidal cell density (Barnes and Walker, 1981), decreased presynaptic glutamate release, and impaired glutamate binding (Farr et al., 1988). The consequences of these neurophysiological changes in FASD are deficits in learning and memory along with the behavioral disorders due to hippocampal damage (Heaton et al., 1995). Numerous experimental evidence indicates that fetal and/or neonatal ethanol exposure alters normal function of ligand-gated NMDA receptors (NMDAR) by decreasing the NMDAR

sensitivity to glutamate, which increases the inhibition of the Mg^{+2} block of NMDARs (Weaver et al., 1993; Gruol et al., 1998; Costa et al., 2000). We have recently shown that prenatal ethanol exposure impairs AMPAR-mediated synaptic transmission in hippocampal slices by decreasing the glutamate release and altering the amplitude and time courses of synaptic AMPAR currents (Wijayawardhane et al., 2007).

Aniracetam [1-(4-methyoxybenzoyl)-2-pyrrolidinone] is a nootropic compound which has been the subject of extensive animal research and has been shown to improve learning and memory (Staubli et al., 1994; Zivkovic et al., 1995) by acting as a positive allosteric modulator of the AMPAR. Furthermore, aniracetam has been shown to improve memory in humans (Ingvar et al., 1997), and has been successfully used in the treatment of depression and other psycho-neurological disorders (O'Neill et al., 2004). Studies using acute hippocampal slices and rapid perfusion patch-clamp electrophysiology experiments have demonstrated that aniracetam slowed deactivation (Vyklicky et al., 1991; Partin et al., 1996; Arai et al., 2000; Lawrence et al., 2003) and desensitization of AMPARs (Isaacson and Nicoll, 1991; Tang et al., 1991; Arai et al., 2000; Sun et al., 2002). Aniracetam enhances hippocampal learning and memory (Cumin et al., 1982; Pontecorvo and Evans, 1985; Vincent et al., 1985; Spignoli and Pepeu, 1987; Toide, 1989; Bartolini et al., 1996) by activating hippocampal PKC and maintaining an increased membrane:cytosolic PKCγ ratio (Lucchi et al., 1993; Lu and Wehner, 1997). The aniracetam enhanced PKCγ, PKC activity and subsequent increased phosphorylation of glutamate receptor subunits (Smith and Wehner, 2002; Gomes et al., 2003) have been suggested to alter single channel kinetics of AMPARs.

Currently there is no specific treatment for the complex neurological dysfunctions such as deficits in learning and memory associated with FASD. However, a previous study using a rat model (Vaglenova and Vesselinov Petkov, 2001), has shown that postnatal aniracetam treatment during adolescence could ameliorate the behavioral disorders associated with mild doses of *in utero* ethanol exposure. Furthermore, we have recently demonstrated that postnatal aniracetam treatment during preadolescence could also reverse the complex behavioral alterations observed in offspring chronically exposed to moderate levels of ethanol by modulating the AMPAR-mediated synaptic transmission at cellular level (Pandiella et al., 2005; Wijayawardhane et al., 2007).

In view of the above findings, we hypothesize that aniracetam-induced behavioral and cellular modifications are due to long lasting effects of this drug on single channel properties of synaptic AMPARs. Thus, the current study investigated the effects of prenatal ethanol exposure and postnatal preadolescent aniracetam treatment on the functional properties of hippocampal synaptic AMPARs. We have utilized a combination of electrophysiological analyses measuring whole cell synaptic currents of hippocampal slices and single channel currents from isolated synaptosomes reconstituted in lipid bilayers to determine how changes in specific channel properties of synaptic receptors contribute to altered synaptic transmission. The findings of this study demonstrate for the first time that the behavioral characteristics of FASD, including memory impairment (Pandiella et al., 2005), are associated with modulation of AMPARs at the single ion channel level, which can be ameliorated by aniracetam treatment. Thus, understanding more about the modulation of AMPAR single channel properties is essential for designing new therapeutic strategy for learning and memory deficits associated with

FASD.

Materials and method

Solutions, drugs and chemicals

For slicing, we used an ice-cold, pregassed (95%O2/5%CO2) ACSF with the following composition (mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1 NaH_2PO_4, 26 $NaHCO_3$ and 11 dextrose; at pH 7.4. AMPAR-mediated miniature currents were recorded in the presence of picrotoxin (PTX, 50 μM) and DL-2-amino-5-phosphonopentanoic acid (APV, 100 μM) to suppress the $GABA_A$ receptor and NMDAR-mediated currents, respectively and 1 μM TTX, to suppress the action-potential-dependent transmitter release. At the end of all experiments 30 μM CNQX was added to the bath solution. Whole cell recording pipette contained (in mM): K-gluconate 100, EGTA 0.6, $MgCl_2$ 5.0, Na-ATP 2.0, Na-GTP 0.3 and HEPES 40.

In synaptosome preparation, ice-cold modified Krebs-Henseleit buffer (mKRBS) containing in mM: 118.5 NaCl, 4.7 KCl, 1.18 $MgSO_4$, 2.5 $CaCl_2$, 1.18 KH_2PO_4, 24.9 $NaHCO_3$, 10 dextrose, 10 μg/ml adenosine deaminase, pH adjusted to 7.4 by bubbling with 95%O_2/5%CO_2, was used to homogenize the isolated hippocampi. To minimize proteolysis, protease inhibitors (0.01 μg/ml leupeptin, 0.005 mg/ml pepstatin A, 0.10 mg/ml aprotinin, 5 mM benzamide) were included in the buffer. The glass pipettes were filled with intracellular solution containing (mM): 110 KCl, 4 NaCl, 2 $NaHCO_3$, 0.1 $CaCl_2$, 1 $MgCl_2$ and 2, 3-N-morpholino popane sulfonic acid. Extracellular bath solution (ECF) contained (mM): 125 NaCl, 5 KCl, 1.25 NaH_2PO_4, and 5 Tris-HCl. Furthermore,

the NMDA receptor antagonist DL-2-amino-5-phosphonopentanoic acid (50 μM), kainate receptor antagonist methyl glutamate analog (2S,4R)-4-methylglutamate (SYM 2081; 1 μM), GABA receptor antagonist picrotoxin (100 μM), sodium channel blocker, tetrodotoxin (1 μM) and potassium channel blocker, tetraethylammonium chloride (2 μM), were added to the ECF. Channels were activated by application of AMPA (290 nM). At the end of each experiment AMPAR antagonist CNQX (1 μM) was added to confirm the recorded single channels were AMPA currents. All chemicals unless stated, are from Tocris, Cookson, Bristol, UK, and Sigma, St Louis, MO.

Experimental animals

Two-months old, pregnant Sprague Dawley rats (Zivic-Miller Laboratories, Pittsburgh, PA) were purchased on their gestational day (GD) 2 and housed in a pathogen-free USDA and AAALAC approved facility at Auburn University. Animals were kept under constant temperature (22 ^{0}C) and fixed 12:12-h light-dark cycle.

Chronic prenatal ethanol exposure paradigm

On GD 3, each pregnant animal was randomly assigned to receive one of two treatment regimens: ethanol or isocaloric sucrose. Each pregnant animal in the ethanol group was administered a moderate dose of aqueous ethanol solution (4 g/kg of body weight/day, 38% v/v). This ethanol regimen had been shown to mimic a moderate-type drinking pattern and had been used in previous studies without adverse effects (Keshavarzian et al., 2001). For the isocaloric-sucrose group each pregnant rat was assigned to receive the same volume of isocaloric sucrose solution. Both groups had ad

libitum access to water and food. Each animal was given the daily treatment by gavage, from GD 3 to 20 (term, about 20), once a day (to avoid additional stress effects of repeated gavage). Daily ethanol administration was chosen to avoid repeated withdrawal episodes (Maier and West, 2001). On GD 14, maternal blood samples were taken from the tail vein 1 hour after the ethanol dose, when the apparent maximal maternal blood ethanol concentration (BEC = 185 ± 0.3 mg/dl; n = 6) was achieved. The day that pups were born was noted as postnatal day (PND) 1 and litter size was recorded. All litters were weighed and culled to a maximum of 10 pups/litter. Pups were weaned on PND 25, housed according to treatment and sex and to control litter effects, no more than 1 mate per gender was placed into a particular group (Wainwright, 1998). Randomly selected pups from both groups were treated with aniracetam (commercially available as Ampamet) at a single dose of 2 ml/kg at 50 mg/kg, dissolved in distilled water, by gavage for 10 days (PND 18-27) (Spear, 2000). This dose and route was selected upon previous studies by our laboratory and numerous other authors (Giurgea, 1980; Coper and Herrmann, 1988; Petkov et al., 1991; Gouliaev and Senning, 1994; Vaglenova and Vesselinov Petkov, 2001). To avoid the acute effects of aniracetam, brain samples were collected at least one day after the last dose of the drug.

Transverse hippocampal slice preparation and whole cell recording

Hippocampal slices (400 μm) were prepared from prenatal ethanol, isocaloric sucrose, and aniracetam treated rats at PND 28 to 35, as previously described (Zeng et al., 1995; Zeng and Tietz, 1999). Briefly, rats were sedated with $C0_2$, decapitated and transverse hippocampal slices were prepared on a vibratome (Warner instruments,

Hamden, CT) in ice-cold, pregassed (95%O_2:5%CO_2) ACSF. After 1 h incubation period the slices were transferred to a chamber perfused with ACSF at a rate of 2 ml/min.

Individual hippocampal slices were transferred to the recording chamber fitted on the stage of a microscope (Olympus BX51WI; Olympus America Inc., Center Valley, PA) with water immersion differential interphase contrast objectives. Whole-cell patch-clamp electrophysiological recordings were performed at 32°C with an Axoptach 200B amplifier (Molecular Devices Corp., Sunnyvale, CA). Recordings from CA1 pyramidal neurons were made with a pipette of 5-7 MΩ resistance. The serial resistance (R_s, defined here as the total resistance between the amplifier and the cell interior) during recordings was in the range of 10-15 MΩ and there was no difference in this parameter between experimental groups. To obtain low noise recordings R_s was not compensated, but was monitored throughout the experiment. When R_s was ≥ 15 MΩ or a significant increase occurred, recordings were terminated. Tight seals (≥ 2GΩ before breaking into whole cell mode) were achieved by applying negative pressure to the pipette during approach to the cell. AMPAR-mediated action potential independent miniature EPSCs (mEPSCs) were recorded in the presence of channel blockers. AMPAR-mediated mEPSCs were recorded at a holding potential of -80 mV. Complete blockage of all synaptic activity after addition of 30 μM CNQX was taken as evidence that the mEPSCs were mediated by AMPA receptors. Current output was low-pass filtered (2 kHz), DC-offset and amplified 10 000 fold. The signal was continuously monitored on-line (pClamp 8.0 Software, Molecular Devices Corp., Sunnyvale, CA), digitized (Digidata 1200, Axon) and stored for later off-line analysis. Baseline mEPSC activity was recorded in each neuron at least for 15 min.

Synaptosome preparation

Isolation of synaptosomes was carried out as described by Johnson and co-workers (Johnson et al., 1997) with some modifications. Briefly, rats (34 days old) were sedated with CO_2, decapitated, and the brains were removed and placed in ice-cold pregassed ACSF, and the hippocampi were isolated and stored at -80^0 C. Isolated hippocampi were then homogenized in homogenization buffer using a potter homogenizer by applying 10 strokes. All steps were carried out at 4^0 C to minimize proteolysis. The homogenate was diluted with 350 µl of additional ice-cold mKRBS buffer. This mixture was loaded into a 1 cc syringe and was filtered through a pre-wetted (150 µl mKRBS), 13 mm diameter Millipore syringe filter holder. This diluted filtrate was forced through a 100 µm pore cell strainer (BD Falcon, Bedford, MA) pre-wetted with 150 µl of mKRBS, and collected in a 1.5 ml Eppendorf tube. This filtrate was loaded into another 1 cc syringe and forced over a pre-wetted 5 µm pore low protein binding filter (Lillex-SV; Millipore Corp., Bedford, MA). The filtered homogenate was then spun at 1000 x g for 15 min in a micro centrifuge at 4^0 C. The supernatant was removed and the pellet rich in synaptosomes were resuspended in 20 µl mKRBS and stored for further use. Western immunoblot analysis confirmed that the harvested synaptosomal preparations used for the experiments were enriched in both postsynaptic density components and AMPAR subunits, as compared to the whole brain preparation (data not shown).

Reconstitution of synaptosomal AMPARs in lipid bilayers

Experimental procedures for recording single channel AMPAR currents from synaptosomes were carried out as described previously (Vaithianathan et al., 2005). The P-2000 laser puller (Sutter Instrument Company, Novato, CA) was used to pull pipette with 100 MΩ resistance. The phospholipids were prepared by dissolving 1, 2-diphytanoil, -sn-glycero-3-phosphocholine (Avanti Polar-Lipids Inc., Alabaste, AL) in hexane (Aldrich Chemical Co., Milwaukee, WI) to achieve a concentration of 1 mg/ml. After addition of 3-5 µl from this synthetic phospholipids into 300 µl of bath solution (ECF), the artificial bilayer was formed by successive transfer of two monolayers onto the tip of the glass pipettes using "tip-dip" method, in an asymmetric saline condition with "outside-out" configuration. After forming a stable membrane, addition of ~ 3-5 µl suspension of synaptosomes into the ECF and gentle stirring facilitated in fusion of synaptosomal fragments into the lipid bilayer.

Single channel recording

Single synaptosomal AMPAR channel currents were elicited by application of 290 nM of AMPA, in the presence of NMDA, kainate, GABA receptor and sodium and potassium channel blockers. The single channel currents were amplified (Axopatch 200 B, Molecular Devices., Foster city, CA), filtered at 2 kHz, digitized between 5-25 kHz using a PCM interface (VR-10B Digital Data Recorder, Instrutech Corp., Elmont, NY) and stored in VHS tape. The pClamp 9 software was used for both online data acquisition and offline analysis. The channel currents which were sensitive to AMPAR antagonist CNQX were used for analysis.

Data analysis

Acquired whole-cell data were analyzed with Mini Analysis program (Synaptosoft Inc., Fort Lee, NJ), only if there was an adequate number of clearly identifiable mEPSCs. Peak mEPSC amplitude was measured from the baseline. The time constant of decay (τ_d) was defined as the time interval needed for a mEPSC to decline to 1/e of the maximal value, and was calculated from the time needed for a mEPSC to decrease from 90-30%. Decay kinetics and amplitudes (*A*) were also estimated using a single exponential function: [$y(t) = A*\exp(-t/\tau_d)$]. The rise time (τ_r) was defined as the time needed for the mEPSC to reach from 10-90% of the maximal value.

Only the data exhibiting long stretches of single channel current transition without baseline shifts were chosen for single channel analysis. The single channel open probability was estimated as $P_o = R_o / (R_c+R_o)$, where R_c and R_o stand for the areas under the current-amplitude histogram corresponding to close and open states respectively, fitted with a sum of two Gaussians using the Microcal Origin program. The single channel conductance of AMPARs were obtained by plotting current as a function of membrane voltage, according to the equation $g = I / (V-V_o)$, where I is the single channel current, V is the voltage and V_o is the reversal potential (Vaithianathan et al., 2005). Log transformed open and close time histograms were fitted best with the exponential log probability variable method. The data are reported as means ± S.E.M. One-way and three-way analysis of variance (ANOVA) was used to test for the statistical significance. Statistical significance is defined as $P < 0.05$.

Results

Long term beneficial effects of aniracetam on AMPAR-mediated mEPSCs in prenatal ethanol exposed rats

As per our previous report, the basic properties of AMPAR-mediated mEPSCs of the CA1 pyramidal neurons of the hippocampus are modulated by postnatal, preadolescence aniracetam treatment (Wijayawardhane et al., 2007). To determine whether these effects are long lasting, we compared the average amplitude, frequency, rise time, and decay time of mEPSCs collected during seven days after the last dose of aniracetam. The AMPAR-mediated mEPSCs were recorded in the presence of 1 μM tetrodotoxin, 50 μM picrotoxin and 100 μM APV (Fig. 5.1A). To analyze changes in mEPSC amplitude (Fig. 5.1B), frequency (Fig. 5.1C), rise time (Fig. 5.1D) and decay time (Fig. 5.1E) at various time points after the end of aniracetam treatment, we used three-way ANOVA, considering aniracetam, ethanol, and time after the end of aniracetam treatment as three factors underlying variability of the measured parameters in four groups of animals (control group, prenatal ethanol exposed group, control group treated with postnatal aniracetam, prenatal ethanol exposed group treated with postnatal aniracetam). We revealed a significant negative effect of ethanol and positive effects of aniracetam on all parameters of mEPSCs, as previously described (Wijayawardhane et al., 2007). Time interval after the end of aniracetam treatment also significantly influenced amplitude, decay time and frequency of mEPSCs. There was a progressive increase in amplitude and frequency in time, as shown in figure 5.1. However, there was no interaction between the time and other factors, indicating that the time courses of changes in amplitude and other parameters are similar in all four compared groups. Thus,

aniracetam and ethanol treatments led to persistent changes in frequency of occurrence, amplitude and duration of mEPSCs.

Single channel conductance states of synaptosomal AMPARs after treatments with ethanol and aniracetam

To understand the mechanisms involved in the changes in AMPAR-mediated mEPSCs at a single receptor level, we investigated the channel properties of synaptic AMPARs utilizing isolated synaptosomes from the four treatment groups. Single channel recordings of synaptosomal AMPARs were carried out as described elsewhere (Vaithianathan et al., 2005). The figure 5.2 shows representative single channel responses elicited by 290 nM AMPA in the four experimental groups (at a membrane potential of + 90 mV) and the blockade of channel activity by 1 μM CNQX. Channel activity is evident by upward transitions of the current, representing the open state.

From visual inspection of the traces, it was immediately apparent that prenatal ethanol exposure produced less frequent and briefer openings (Fig. 5.2B1) than the control (Fig. 5.2A1). We therefore reasoned that, because of prenatal ethanol exposure, AMPARs are less sensitive to AMPA and may result in less frequent, briefer openings, thus causing AMPAR-mediated synaptic impairment. Conversely, from a cursory examination it is apparent that following postnatal aniracetam treatment, the probability of opening, conductance and mean open times, increased significantly (Fig.5.2C1 and 5.2D1).

The mean single channel open probability (Po) for control group was 0.29 ± 0.02 (n = 15, Fig. 5.2A2). Prenatal ethanol exposure resulted in a significant reduction in Po

(0.11 ± 0.01, n = 20, $P < 0.00001$, Fig. 5.2B2). However, postnatal aniracetam treatment significantly increased the Po (control + aniracetam group 0.40 ± 0.01, n = 20, $P < 0.00001$, Fig. 5.2C2 and in ethanol +aniracetam group 0.40 ± 0.02, n = 20, $P < 0.00001$, Fig. 5.2D2).

We investigated whether the conductance of AMPAR channels was influenced by prenatal ethanol exposure and postnatal aniracetam treatment. Figure 5.3 shows channel activity in the four treatment groups (at a membrane potential of + 90 mV) with the corresponding amplitude distributions. Some channel openings underwent transitions to several different conductance states before finally closing. Therefore, it was difficult to decide whether an increase in amplitude (during a channel opening) arose from a transition to a new conductance state or from a second channel that had simultaneously opened. To avoid artifacts that might result from the superimposition of channel openings, only the events that arose directly from the closed state, or returned directly to it, were included in amplitude histograms. As a result, the values we obtained may be slightly biased because the probability of superimposition of openings is greater when events are longer lasting as occurred for the higher conductance openings in aniracetam treated groups.

Amplitude distributions were best fitted with multiple Gaussians. In the control group (Fig. 5.3A), and prenatal ethanol exposed group (Fig. 5.3B), we achieved a reasonable fit with three Gaussian components; however, the histograms obtained for postnatal aniracetam treatments (Fig. 5.3C and 5.3D) required three rightward shifted Gaussians to provide an adequate fit. When we examined the data from all the events recorded from four treatment groups, it was possible to identify two main conductance

levels (see below) for each group, however the postnatally aniracetam treated groups had two clearly distinct levels of conductance.

Our main single channel conductance estimates (mean ± S.E.M.) for the four different groups were 10.12 ± 0.47 and 20.60 ± 1.50 pS (n = 16), 6.09 ± 0.86 and 12.48 ± 2.97 pS (n = 16), 19.21 ± 1.71 and 37.64 ± 5.33 pS (n = 12), and 15.20 ± 0.69 pS and 22.91 ± 1.22 pS (n = 22) for control, prenatally ethanol exposed, control animals treated with aniracetam and prenatally ethanol exposed animals treated with aniracetam, respectively (Fig 5.3A-D, Table 5.1). Thus, we found an approximate 40% reduction in mean single channel conductance in AMPARs from prenatally ethanol exposed rats. In addition, we found an approximate doubling of the conductance when treated with aniracetam postnatally. These differences were statistically significant (one-way ANOVA, $P < 0.003$). Figure 5.3E summarizes the current-voltage relationship of the four treatment groups (five to seven individual experiments from each group). Each group revealed a linear current-voltage relationship, consistent with results from previous recording in isolated synaptosomes reconstituted in lipid bilayers (Vaithianathan et al., 2005).

Gating of single synaptosomal AMPARs after treatments with ethanol and aniracetam

By examining the single channel openings, there appeared to be a clear change in their kinetic properties in all treated groups (Fig. 5.4). We investigated this by constructing histograms of open periods and closed times. The distributions of open and closed times, as shown in figure 5.4-1 and 5.4-2 respectively, were best fitted with two, three or four exponential components when channels were activated by 290 nM of

AMPA. As the open period measured the total time a channel was open, the value obtained was independent of the conductance level(s) adopted during the opening.

The mean time constants for open periods for all four groups are given in the Table 5.1. The mean values of the fast components (τ_1 and τ_2) were significantly decreased in prenatal ethanol exposure as compared to control (one-way ANOVA, $P < 0.01$). Also, we detected additional longer open period components (τ_3 and τ_4) in aniracetam treated groups, which were rarely present in control and prenatal ethanol exposed groups. As apparent from figure 5.4-1, the overall effect of aniracetam treatment was to shift the open periods to the right.

As illustrated in figure 5.4-2, an adequate description of the closed time periods required distributions to be fitted with the sum of two exponential components in control group, three exponential fits in prenatal ethanol exposed group and four exponential fits in the aniracetam treated groups. The mean time constants for closed periods for all four groups are given in the Table 5.1. The mean values of the closed time components (τ_1, τ_2 and τ_3) were significantly increased in prenatal ethanol exposure as compared to control (one-way ANOVA, $P < 0.02$). Also, we detected additional longer open period component (τ_4) in aniracetam treated groups, which were rarely present in control and prenatal ethanol exposed groups.

We also estimated burst activity in the four treatment groups (Table 5.2). One-way ANOVA detected a significant decrease in number and duration of burst in the prenatal ethanol exposed group, an increase in duration and number of bursts in the group treated postnatally with aniracetam.

Ethanol and aniracetam-induced changes in activity of single synaptosomal AMPARs are positively correlated with changes in whole-cell recorded postsynaptic currents

Regulation of synaptic AMPAR-mediated mEPSCs could arise from modifications in the single channel properties of synaptic AMPARs. To investigate the relationship between activity of single synaptosomal AMPARs and mEPSCs, plots of mEPSC amplitude, decay time and rise time constants versus single channel open probability and major conductance were constructed using respective mean values of each group (Fig. 5.5). We found significant positive correlations between the open probability of AMPARs and amplitude and decay time of mEPSCs. Also the major conductance of AMPAR channels and the rise time of mEPSCs were positively correlated. These results support the view that ethanol-and aniracetam-induced changes in the magnitude and temporal parameters of postsynaptic AMPAR-mediated currents are due to changes in gating and conductance of AMPARs.

In summary, our data revealed a decrease in the mean open time, probability of channel opening, number of bursts, and mean burst duration after prenatal ethanol exposure; furthermore, this suggests that prenatal ethanol induces a long lasting impairment of AMPARs at the single channel level. Enhancements in the kinetic properties upon aniracetam treatment indicate that these alterations may be capable of influencing mEPSC time courses (Wijayawardhane et al., 2007) and animal behavior (Pandiella et al., 2005) by modifying channel properties of AMPARs.

Discussion

In this study we demonstrate that fetal alcohol exposure impairs synaptic AMPAR function. In addition, we show that postnatal aniracetam treatment alleviates these impairments even after discontinuation of aniracetam administration. We used a sensitive and reliable animal model, which exhibits the behavioral and cognitive impairments associated with FASD (Pandiella et al., 2005) to demonstrate the modifications in activity of AMPARs. Our experiments reveal two main findings: (1) Prenatal ethanol exposure resulted in impaired synaptic transmission by altering the single-channel properties of synaptic AMPARs. (2) Postnatal aniracetam treatment enhanced synaptic transmission by modulating single channel properties of AMPARs in animals exposed to ethanol. Therefore our results support the notion that AMPAR-mediated synaptic impairment may be involved in the pathophysiology of cognitive impairment associated with FASD, and timely postnatal intervention by aniracetam treatment (Wijayawardhane et al., 2007) would ameliorate these impairments by potentiating single channel activities of synaptic AMPARs.

Prenatal ethanol exposure attenuates single channel currents of synaptic AMPARs

We have recently shown that ethanol exposure during fetal development decreases the AMPAR-mediated miniature and spontaneous postsynaptic currents in the CA1 hippocampal pyramidal cells (Wijayawardhane et al., 2007). Analysis of single-channel data obtained in the present study indicates that prenatal ethanol exposure reduces the single-channel open probability, conductance, open time, number and duration of bursts while increasing the channel closed time. This further supports the

view that prenatal ethanol exposure significantly reduces the AMPAR-mediated synaptic transmission by modulating AMPARs.

The observed lower conductance obtained for events activated by 290 nM AMPA suggests that lower concentrations may not be sufficient to fully open the AMPAR channels of ethanol exposed animals. Previous studies have shown that LTP is decreased in rats exposed *in utero* to ethanol (Morrisett et al., 1989); moreover, our data shows a reduction in AMPAR conductance, which explains this phenomenon, as changes in AMPAR conductance are known to correlate with changes in LTP. Since LTP is one of the primary manifestations of plasticity in the hippocampus and has been correlated with enhanced learning (Berger and Thompson, 1982; Berger, 1984), this reduced conductance of AMPARs may partially explain underlying learning and memory deficits observed in FASD. The decrease in channel open probability may be due to the reduced sensitivity of AMPARs after *in utero* ethanol exposure. Whether this reduced sensitivity is due to alterations in AMPAR subunit expression remains to be determined. However, reduced sensitivity of NMDA receptors to glutamate due to prenatal ethanol exposure has been demonstrated previously. The reduced single channel conductance (Benke et al., 1998) and open probability of synaptic AMPARs results in a decreased synaptic efficacy, and thereby contributes to the impaired synaptic transmission in fetal alcohol exposure. The mean current amplitude of single channel currents is a product of single channel conductance and channel open probability. Therefore, a reduction in conductance and open probability will reduce the mean current amplitude of single synaptic receptors. The decrease in mean current amplitude and open probability of single synaptic AMPARs can decrease mEPSC amplitudes, and this is exactly what was observed in our experiments.

Moreover, we observed significant positive correlations between parameters of single AMPARs and mEPSCs collected for all four experimental groups. The decreased mean open time, number and duration of bursts, and increased channel closed time possibly resulted from the enhanced rate of entry into desensitized state in ethanol exposure (Wainwright, 1998). The increased single channel closed times and decreased open times of synaptic AMPARs can result in fast decay of synaptic currents. This was clearly observed in our mEPSC measurements using hippocampal slices (Wijayawardhane et al., 2007).

Impaired single-channel properties of synaptic AMPARs in fetal ethanol exposure are ameliorated by postnatal aniracetam treatment

We show here that even after seven days following the termination of aniracetam treatment, the enhancement of AMPAR-mediated mEPSC amplitude, frequency, rise and decay time constants in CA1 neurons were significantly higher compared to animals not treated with aniracetam. We report here that aniracetam treatment increases the AMPAR single channel conductance, open probability, open time, number and duration of burst while decreasing the closed time, suggesting that, aniracetam treatment enhances the AMPAR-mediated synaptic transmission by potentiating AMPAR function. To the best of our knowledge, this is the first direct demonstration of long term effects of aniracetam on AMPAR function in the hippocampus. The enhancement in AMPAR-mediated mEPSC responses observed in the aniracetam treated groups requires permanent modifications in factors that regulate synaptic transmission. Our results show increase in mEPSC frequency, amplitude, decay time constants indicating modifications of both pre

and post synaptic mechanisms. These results give further insight and clear mechanisms to our previous observations of aniracetam treatment in improving cognitive deficits caused by prenatal ethanol exposure (Wijayawardhane et al., 2007).

Studies have demonstrated that aniracetam is a positive allosteric modulator which slows deactivation (Vyklicky et al., 1991; Partin et al., 1996; Arai et al., 2000; Lawrence et al., 2003) and desensitization of AMPARs (Isaacson and Nicoll, 1991; Tang et al., 1991; Arai et al., 2000; Sun et al., 2002). The cognitive enhancing properties of aniracetam are primarily attributable to slowing of deactivation and desensitization (Arai and Lynch, 1998). However, in this study we demonstrate that the effects of aniracetam persist even after the discontinuation of this drug. Therefore, it was of interest to determine how the single channel properties are modified after the cessation of treatment with aniracetam.

In our experiments, preadolescent aniracetam treatment significantly increased the open channel probability and conductance which possibly resulted in increased mEPSC amplitudes. It is noteworthy that mean channel conductance of AMPARs roughly doubled in the preadolescent aniracetam treated group. This increase in channel conductance after aniracetam treatment is rather a new finding. In aniracetam treated groups, two major conductance values were observed for AMPARs (aniracetam, 19 pS & 37 pS; ethanol + aniracetam 15 pS & 22 pS), and these values were much higher than that of control groups without aniracetam (10 pS & 20 pS). The increases in conductance may be due to changes in surface expression of AMPARs with subunit composition that exhibits higher conductance, resulting from aniracetam treatment. The strong amplitude dependence of τ_d in the aniracetam treated animals (Wijayawardhane et al., 2007) may be

due to the opening of high conductance channels with longer mean open time. The correlation between increased mean open time in single channel measurements and increased decay time in whole-cell recordings further supports the idea that the mechanism of AMPAR-mediated synaptic enhancement by aniracetam treatment is similar in both preparations, indicating a modulation of a channel protein or a regulatory unit which is closely bound to the receptor-channel (Wainwright, 1998). This could have possibly resulted from the inhibition of desensitization/deactivation followed by some permanent modification occurred at the receptor level. Also, elevated synaptic activity in the presence of aniracetam may activate cascades of intracellular signaling events, such as in increase in activity of protein kinase C, resulting in changes in expression and posttranslational modification of AMPAR subunits.

In conclusion, our results indicate that AMPA glutamate receptors are vulnerable to prenatal ethanol exposure and it provides an important mechanistic basis of cognitive impairment associated with FASD. Therefore, targeting these receptors using cognitive enhancers such as aniracetam offer an exciting therapeutic measurement with great potential for treating cognitive impairments associated with prenatal alcohol exposure.

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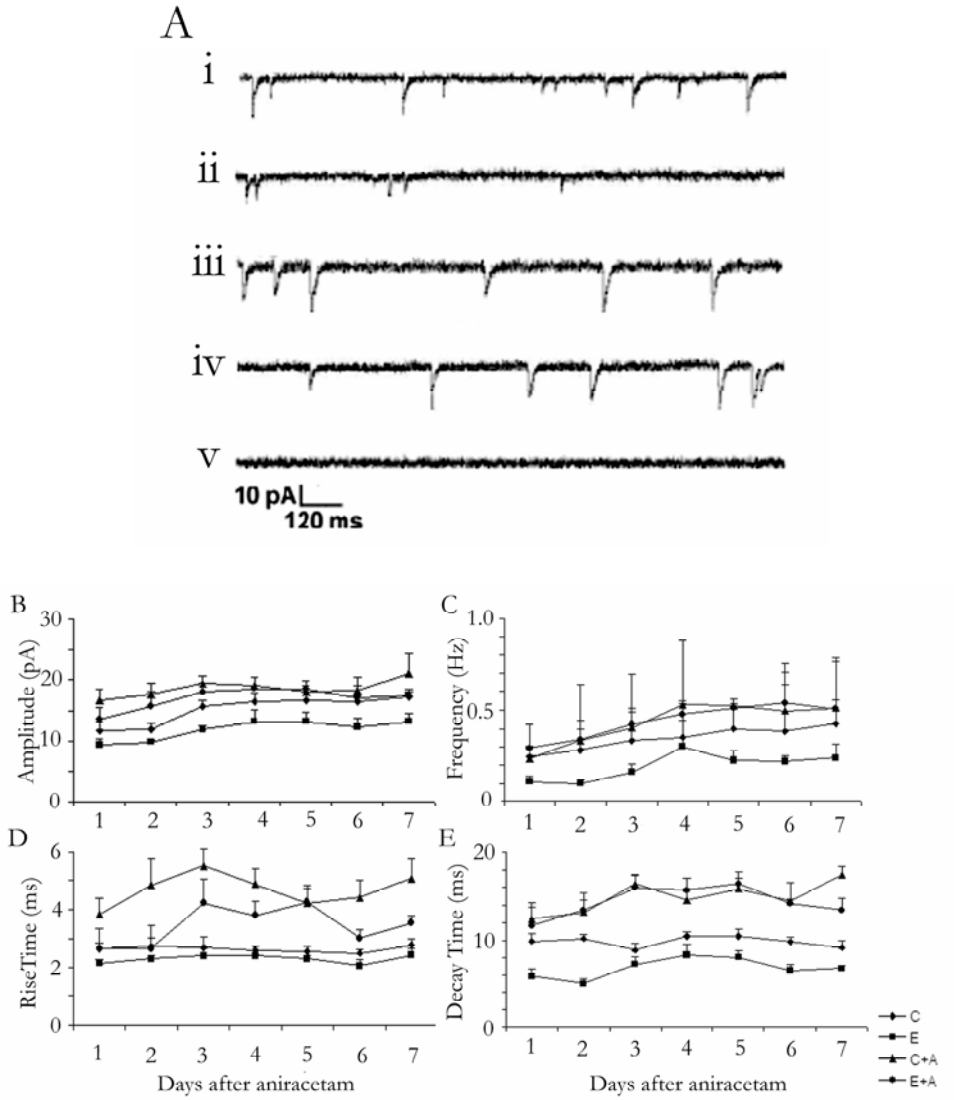


Figure 5.1. AMPAR-mediated miniature excitatory postsynaptic current (mEPSC) properties of the rat hippocampal slices depending on the time interval after aniracetam treatment. (A) Sample traces from control group (i), prenatal ethanol exposed group (ii), control group treated with postnatal aniracetam (iii), prenatal ethanol exposed group treated with postnatal aniracetam (iv), and in the presence of bath application of CNQX to completely abolish the AMPAR-mediated currents (v). Time course of mean amplitude (B), frequency (C), rise time (D) and decay time (E) of mEPSCs show that aniracetam treatment induces persistent changes in activity of AMPARs. (n=10-15 rats per group).

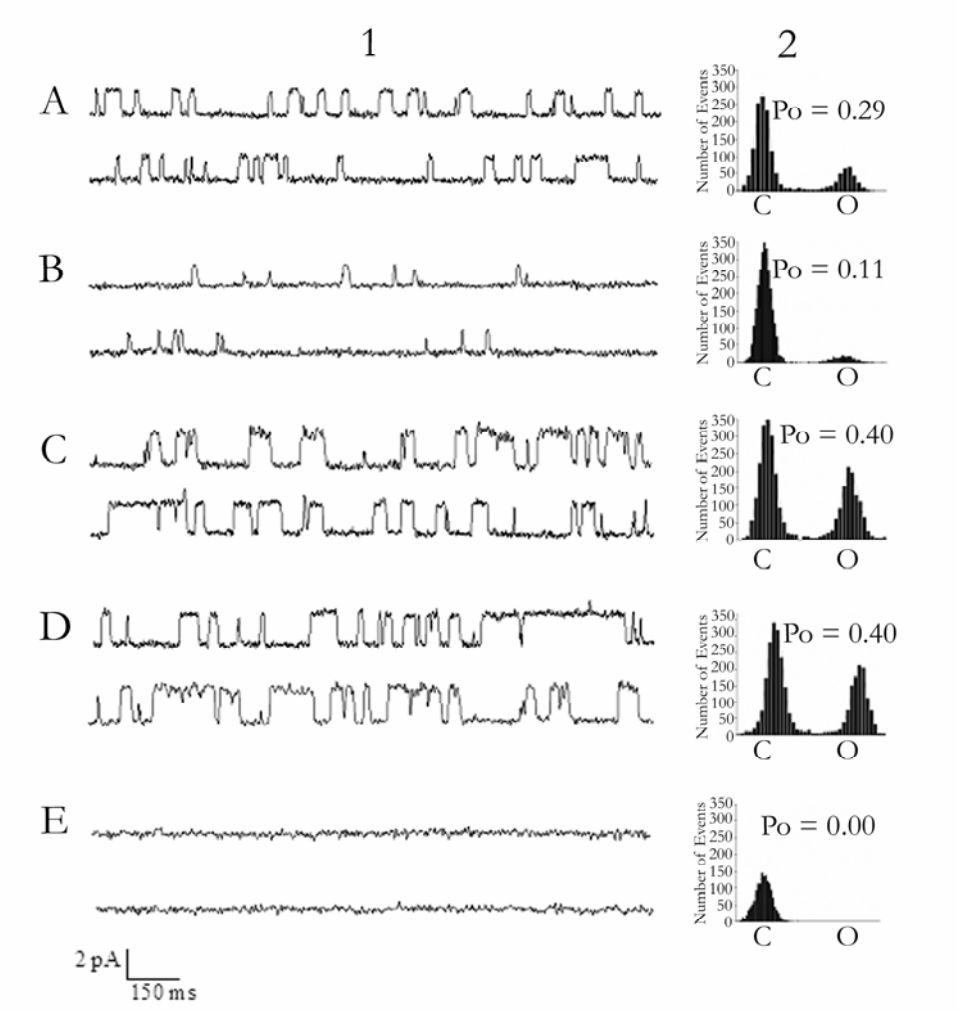
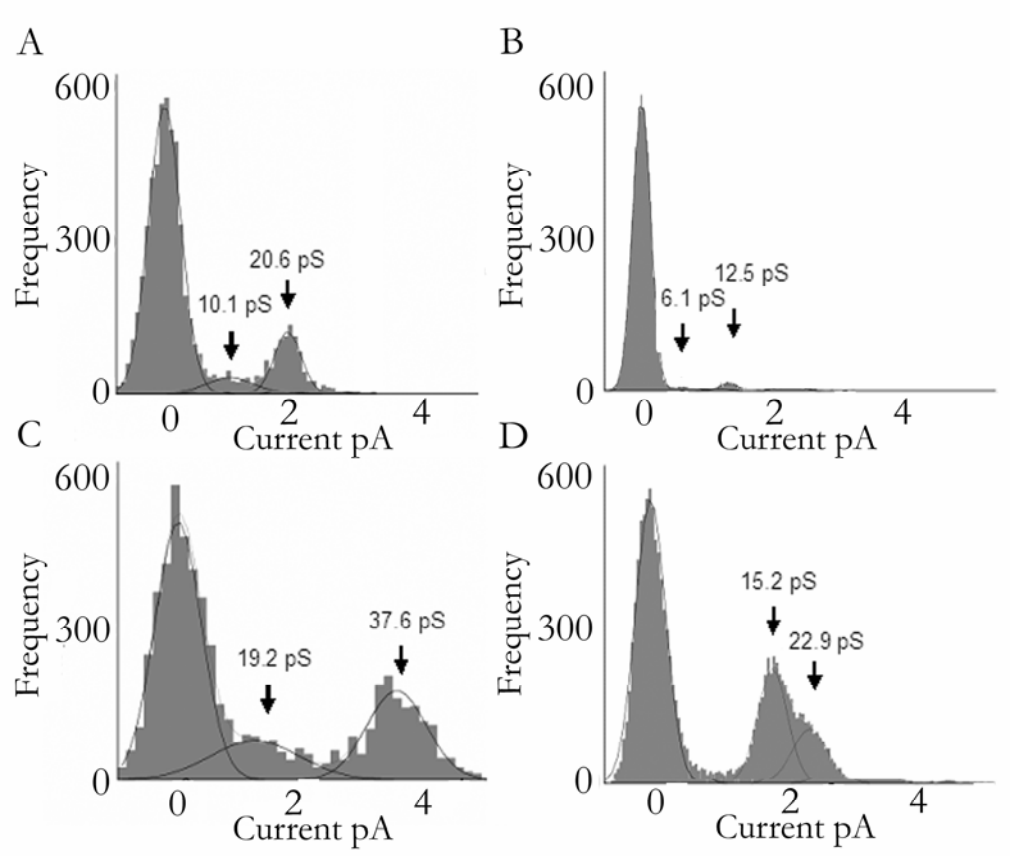


Figure 5.2. Single channel currents activated by 290 nM AMPA, in reconstituted AMPARs from hippocampal slices. 1 and 2: onset of response to application of 290 nM of AMPA (1A-1D), or 1 μM CNQX (1E) and their respective current amplitude frequency histograms (2) to show the open (O) and closed (C) channel probabilities, from four different treatment groups. (A - control, B - prenatal ethanol, C - control + postnatal aniracetam, D - prenatal ethanol + postnatal aniracetam and E - CNQX). Note that the events are markedly briefer and less frequent when activated in the prenatal ethanol exposed group (B1) compared with control (A1). When postnatal aniracetam treatment was given (D1), the single-channel currents were similar to those from control group treated with aniracetam (C1). Currents were examined at V_m = 90 mV. Bathing solution contained AP5 (50 μM), SYM 2081 (1 μM) to block NMDA receptors and kainate receptor currents respectively; Data were low-pass filtered at 2 kHz and digitized between 5-25 kHz.


A
600
300
0
Frequency
20.6 pS
10.1 pS
0
2
4
Current pA
B
600
300
0
Frequency
12.5 pS
6.1 pS
0
2
4
Current pA
C
600
300
0
Frequency
37.6 pS
19.2 pS
0
2
4
Current pA
D
600
300
0
Frequency
15.2 pS
22.9 pS
0
2
4
Current pA

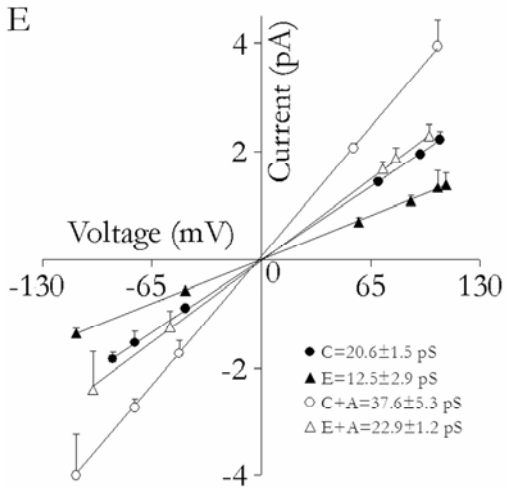

E
4
2
-2
-4
Current (pA)
Voltage (mV)
-130
-65
0
65
130
C=20.6±1.5 pS
E=12.5±2.9 pS
C+A=37.6±5.3 pS
E+A=22.9±1.2 pS

Figure 5.3. Single-channel events displayed a marked change in conductance in prenatal ethanol exposure and postnatal aniracetam treatment. Amplitude histograms of single-channel currents activated by 290 nM AMPA, in the four treatment groups. Amplitude distributions show multiple conductance levels in synaptosomal AMPAR channels. AMPAR channel amplitude distributions were fitted with three Gaussian components in the four groups. Arrow indicates mean conductance levels obtained from fitted Gaussians. (A - control, B - prenatal ethanol, C - control+ postnatal aniracetam, D - prenatal ethanol + postnatal aniracetam).
E: The current-voltage relationship for the observed major conductance levels of synaptosomal AMPARs of the four groups. The mean amplitudes of currents elicited by the addition of 290 nM of AMPA were plotted against membrane holding potentials (V_H was from -110 to +110 mV) and approximated using their linear regression. The open squares and the open triangles represent the higher current transition activity obtained from the aniracetam treated control and ethanol exposed groups respectively (R^2=0.99).

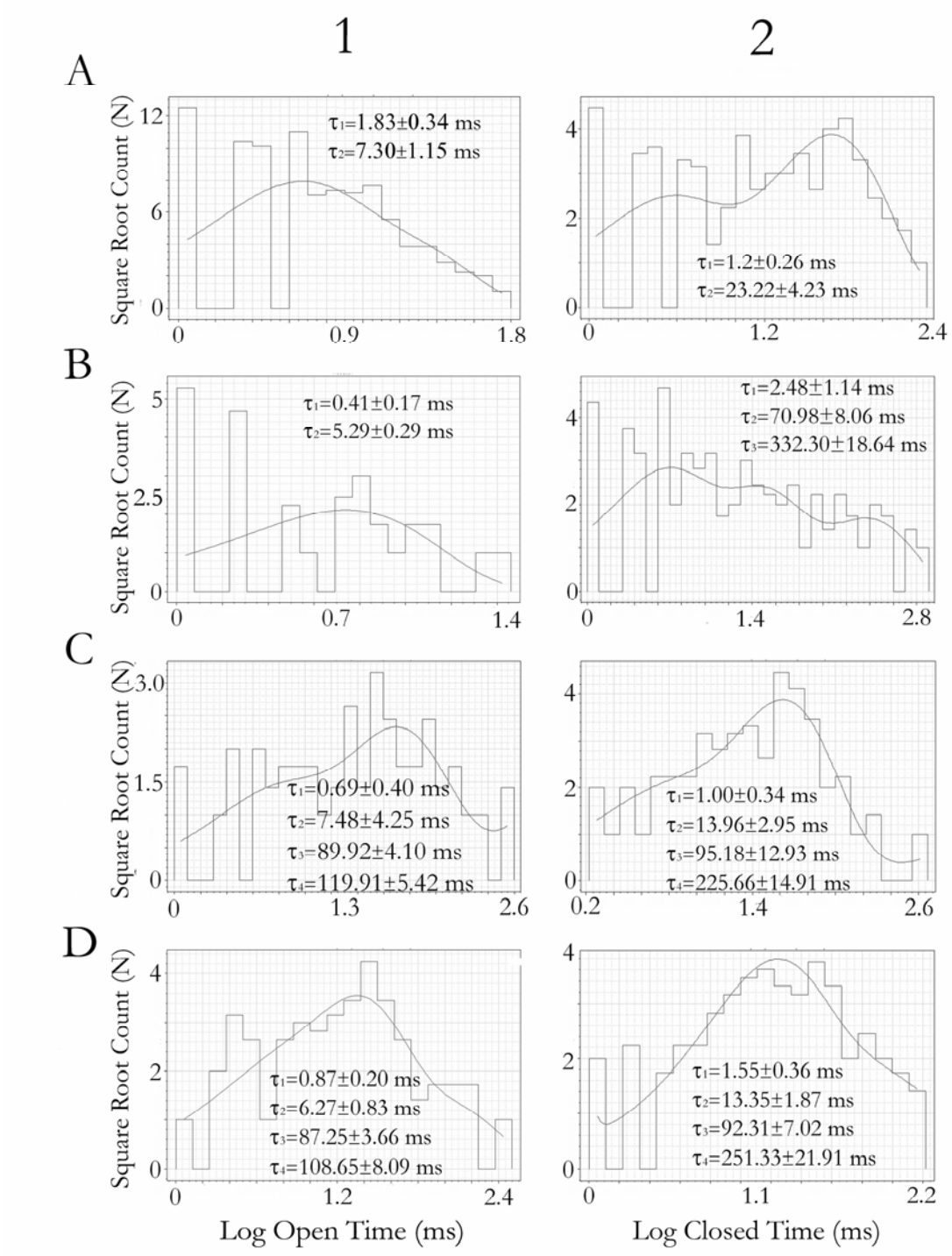

1
2
A
B
C
D
Square Root Count (N)
τ1=1.83±0.34 ms
τ2=7.30±1.15 ms
τ1=1.2±0.26 ms
τ2=23.22±4.23 ms
τ1=0.41±0.17 ms
τ2=5.29±0.29 ms
τ1=2.48±1.14 ms
τ2=70.98±8.06 ms
τ3=332.30±18.64 ms
τ1=0.69±0.40 ms
τ2=7.48±4.25 ms
τ3=89.92±4.10 ms
τ4=119.91±5.42 ms
τ1=1.00±0.34 ms
τ2=13.96±2.95 ms
τ3=95.18±12.93 ms
τ4=225.66±14.91 ms
τ1=0.87±0.20 ms
τ2=6.27±0.83 ms
τ3=87.25±3.66 ms
τ4=108.65±8.09 ms
τ1=1.55±0.36 ms
τ2=13.35±1.87 ms
τ3=92.31±7.02 ms
τ4=251.33±21.91 ms
Log Open Time (ms)
Log Closed Time (ms)

Figure 5.4. Distributions of open periods and closed times for AMPAR channels in four treatment groups. 1: Open period distributions for AMPAR single channels from control (A), prenatally ethanol exposed (B), control group treated with aniracetam (C), and prenatally ethanol exposed group treated with aniracetam (D). These distributions were fitted with two, three or four exponential components. Estimated time constants obtained from best fit of the distributions are indicated within the figure. Note the increase in τ_3 and τ_4 (the longer duration events) with the postnatal aniracetam treatment. 2: Close time distributions from the same four groups. The distributions were fitted with either two, three or four components. Note the increase in all three components (τ_1, τ_2 and τ_3) in the prenatal ethanol exposed group. All distributions were obtained from time-course fitting events. (V_m = 90 mV).

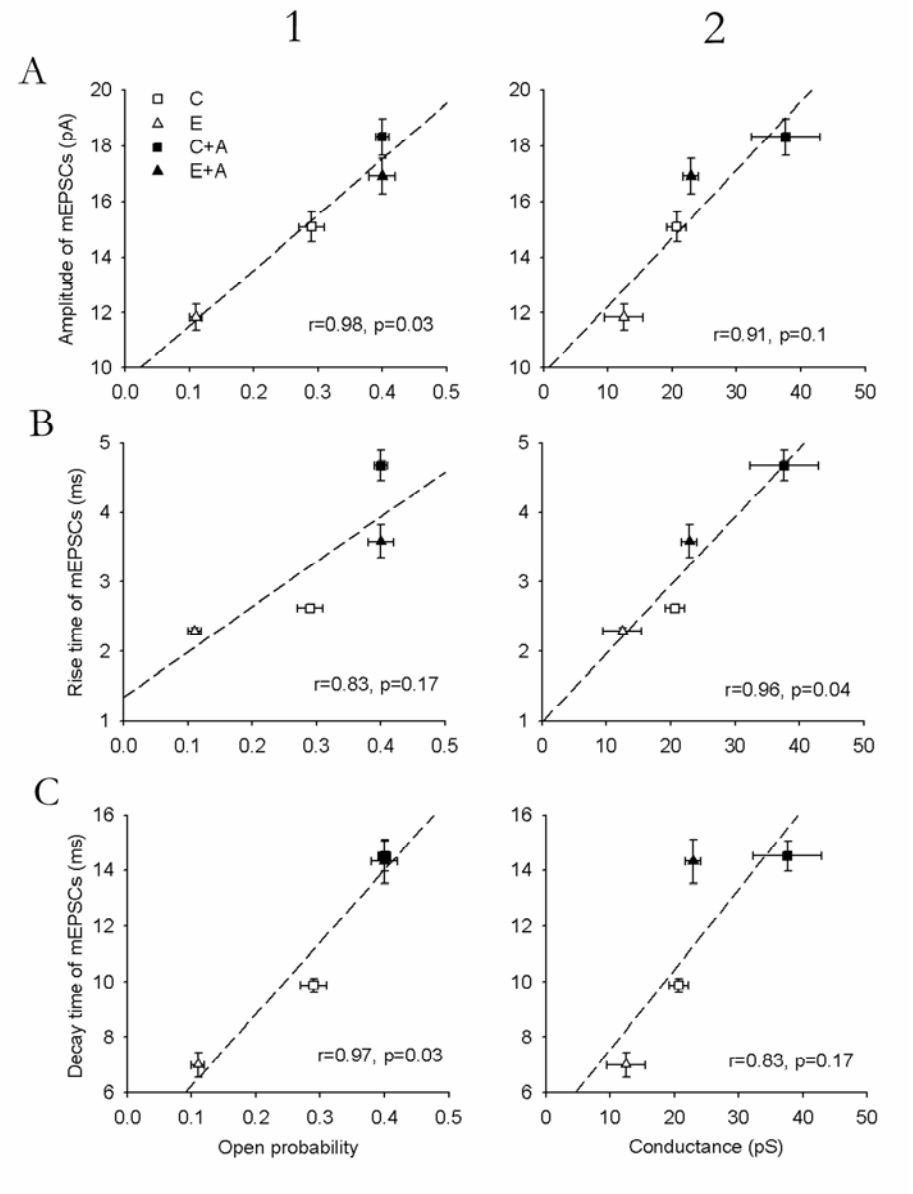


Figure 5.5. Relationships between single AMPAR channel open probability (1) and conductance (2) versus the amplitude (A), rise (B) and decay (C) time constants of AMPAR mEPSCs. Regression (dashed) lines were fitted using the least-squares method. Each point represents mean±S.E.M. corresponding to one of four treatment groups (C - control, E - prenatally ethanol exposed, C+A - control group treated with aniracetam postnatally, E+A - prenatal ethanol exposed group treated with aniracetam postnatally; n=10-15 animals per group), r is the coefficient of correlation, p is the level of significance that r is not equal to 0.

Table 5.1. Single channel properties of synaptic AMPARs of four different treatment groups.

Group	Po	Conductance (pS)		Open period (ms)				Closed time (ms)			
		$\gamma 1$	$\gamma 2$	τ_1	τ_2	τ_3	τ_4	τ_1	τ_2	τ_3	τ_4
C	0.29±0.02	10.12 ±0.47	20.68 ±1.50	1.83 ±0.34	7.30 ±1.15			1.2 ±0.26	23.22 ±4.23		
E	0.11±0.01*	6.09* ±0.86	12.48* ±2.97	0.41* ±0.17	5.29 ±0.92			2.48* ±1.14	70.98* ±8.06	332.30 ±18.64	
C + A	0.40±0.01*	19.21* ±1.71	37.64* ±5.33	0.69* ±0.40	7.48 ±4.25	89.92# ±4.10	119.91 ±5.42	1.00 ±0.18	13.96* ±2.95	95.18 ±12.93	225.66 ±14.91
E + A	0.40±0.02*#	15.19*# ±0.69	22.91# ±1.22	0.87 ±0.20	6.27 ±0.83	87.25# ±3.66	108.65 ±8.09	1.55# ±0.36	13.35*# ±1.87	92.31# ±7.02	251.33 ±21.91

Notes: Values represent mean ± SEM which were calculated from channel open probability (Po), conductance $\gamma 1$, $\gamma 2$, channel open periods (τ_1, τ_2, τ_3, τ_4) and channel closed times (τ_1, τ_2, τ_3, τ_4).
Asterisk indicate significant differences with the corresponding values in control (*) and prenatal ethanol exposed group (#). ($P < 0.02$; One-way ANOVA; n = 12-16).

Table 5.2. Distribution of burst kinetics in four experimental groups.

Parameter (± S.E.M.)	C (n = 14)	E (n = 20)	C + A (n = 21)	E + A (n = 21)
Number of bursts	35 ± 3	26 ± 3*	39 ± 4	48 ± 4*#
Mean burst duration (ms)	3.68 ± 0.36	2.66 ± 0.14*	6.25 ± 0.59*	3.38 ± 0.34

Notes: Values represent mean ± SEM which were calculated by burst analysis. Asterisk indicate significant differences with the corresponding values in control (*) and prenatal ethanol exposed group (#) ($P < 0.05$; One-way ANOVA).

6 SUMMARY AND CONCLUSIONS

Distinguishing features of prenatal ethanol exposure in humans as well as rodents are impaired cognitive and behavioral functions, resulting from damage to the CNS (Hamilton et al., 2003; Riley et al., 2004). CNS dysfunctions are the most severe and permanent consequences of maternal alcohol intake. The hippocampus is one of the important brain regions especially susceptible to ethanol and has been associated with cognitive and behavioral deficits (Guerri, 2002). Reports indicate that the hippocampal CA1 area is highly susceptible to prenatal ethanol exposure (Tran and Kelly, 2003).

In our work we showed that the rat offspring prenatally exposed to a moderate dose of ethanol presents significant neurobehavioral consequences. The ethanol exposed group showed retardation in growth, and a deficiency in developmental reflexes. Our results are indicative of poor novelty seeking behavior, poor adaptation with increased anxiety levels observed in the hole board and plus-maze tests. The level of anxiety was measured in the plus-maze test as the relative amount of time and number of entries made into the open arms compared to that in closed arms.

There is evidence indicating that prenatal ethanol exposure results in acquisition and memory deficits observed in active avoidance and condition reinforcement (shuttle-box). This test is considered to measure the associative learning which is the process of formation of new mental links among events in which most of the brain structures such as the hippocampus and amygdale are involved.

One step further:

Where as the role of ethanol's effects on the CNS and glutamatergic synaptic transmission has received a great deal of attention in the last few decades a few studies have further investigated whether AMPARs, one of the key elements involved in the fast excitatory neurotransmission in the brain, also contribute to the associated cognitive and behavioral deficits in ethanol exposure *in utero*. Our findings suggest that ethanol may actually damage CA1 pyramidal neurons by inhibiting postsynaptic AMPARs and decreasing the presynaptic glutamate release. Thus, we have identified a novel mechanism that may be involved in the pathophysiology of FAS.

The identification of new mechanisms and their respective contributions to ethanol induced fetal damage should accelerate the development of rational approaches to the diagnosis, treatment and prevention of alcohol related birth defects. This knowledge would also provide public education and counseling of alcohol-dependent women of childbearing age. Therefore, we used a rational approach to determine the use of a nootropic compound (aniracetam) that targets AMPARs to provide an effective therapeutic intervention against this devastating public health problem.

Interestingly, we have shown that 10 day aniracetam treatment for the offspring exposed to ethanol *in utero* could successfully reverse the neurobehavioral impairments associated with prenatal ethanol exposure. We showed that rat offspring treated with aniracetam, had significant increases not only in the number of entries to the open arm but also increased time spent in the open arms in the plus-maze test, indicating an anxiolytic effect of the drug. Our results also demonstrated that aniracetam treatment caused a significant increase in the rat's avoidance of the conditioned stimuli while

decreasing the poor and helpless learners in the ethanol exposed group, as determined by the two-way active avoidance test.

In addition to the cognitive enhancement observed after aniracetam treatment, AMPAR mediated synaptic transmission in the hippocampal pyramidal cells also showed an enhancement. The resulted synaptic enhancement of the hippocampal neuron, thus may have contributed to the observed behavior modifications related to learning and memory.

To date, no global mechanisms of the alcohol-induced damage to fetal brain development has been established, and a single mechanism is unlikely to account for the various components of the neurobehavioral deficits associated with FAS. Therefore, identifying AMPARs as one of the targets of ethanol teratogenicity has led us to define an effective treatment strategy.

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