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Can alpha 7 nicotinic agonists reduce the effects of 3rd trimester ethanol exposure?

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This summer I worked in Dr. Barron's laboratory studying the effects of fetal ethanol exposure using a rat model. The main focus of this laboratory is to try and understand some of the mechanisms that contribute to how prenatal ethanol exposure affects the brain and ways to try and reduce the damaging effects. Recent studies have shown that giving choline to rats exposed to ethanol prenatally can reduce some of the adverse effects of prenatal ethanol exposure. Choline is a full agonist at the alpha 7 nicotinic acetylcholine receptor subtype (α7nAChR) and so we hypothesized that α7nAChRs may be particularly sensitive to the effects of early ethanol exposure and that α7nAChR agonists might be neuroprotective to some of the damaging effects of ethanol during early development. One of the main areas affected by prenatal ethanol exposure is the hippocampus and the α7nAChRs play an important part in the development of this structure. This summer we have begun to address this hypothesis through the use of *in vivo* and *in vitro* rodent models to determine if early ethanol exposure alters this cholinergic receptor and to see if DMXB (3-(2,4)-Dimethoxybenzylidine anabaseine), an α7nAChR agonist, can reduce neurotoxicity and behavioral defects caused by prenatal ethanol exposure. This project is currently underway but began later than anticipated. DMXB is a novel drug (also currently in clinical trials) that we obtained from Dr. William Kem from the University of Florida and in order to obtain this, we had to have a Materials Transfer Agreement (MTA) approved by both universities. The process for this was started in February and not completed until mid July.

We have now obtained the DMXB and are in the process of conducting the studies proposed in my application and these studies will continue through this fall. After communication with Evie Russell, she recommended that I write this report on the project I worked on this summer. I will follow up with a report of the project originally proposed when it is completed.

In the meantime, I have been studying the interaction between prenatal ethanol exposure and hypoxia. This study employs many of the same paradigms I proposed and has therefore allowed me to learn many of the techniques that I will continue using with DMXB.

Prenatal ethanol exposure can produce life-long damaging effects for the offspring although the severity of these effects can vary between individuals. Many factors have been proposed to explain this variation including genetics, dose and timing effects, and early postnatal environmental factors. One theory suggests that "provocative factors" can increase the severity of the effects of prenatal ethanol exposure (Abel & Hannigan, 1995). In other words, prenatal ethanol exposure reduces the ability of the brain and/or body to respond to additional minor stressors. We hypothesized that mild, brief periods of hypoxia (reduced oxygen flow) is one of these "provocative factors" and that if brief episodes occur, it can increase the severity of the effects of prenatal ethanol exposure. More specifically, our hypothesis is that that ethanol withdrawal, followed by hypoxia will produce more damaging effects than either insult alone. We have developed this hypothesis on the basis that ethanol exposure and hypoxia share similar cellular mechanisms and produce some of the same damaging effects, therefore making the interaction increasingly damaging.

Hypoxia is a reduction in oxygen levels that can be compromise the developing central nervous system (CNS). Normal labor often includes brief episodes of hypoxia that the healthy fetus is able to overcome using a variety of physiological responses (Huch A, Huch R, Schneider, & Rooth, 1977). Data suggests that prenatal ethanol exposure may disrupt the body's compensatory response to hypoxic conditions, further increasing the damaging effects of decreased oxygen levels. Even low doses of prenatal ethanol exposure can affect the contractility of the umbilical cord arteries and this inhibits oxygen flow to the fetus since the umbilical cord is the main source of oxygen to the fetus (Iveli et al., 2007). There is also evidence that prenatal ethanol exposure impairs placental function which is another way that the fetus receives essential nutrients and oxygen (Burd, Roberts, Olson & Odendaal, 2007).

Both prenatal ethanol exposure and hypoxia produce damaging effects to the CNS (Mitchell, Paiva, Moore, Walker & Heaton, 1998). Our hypothesis is that these damaging effects may become more severe when a fetus is experiencing both ethanol withdrawal and brief episodes of hypoxia as what might occur during labor and/or delivery. Once the underlying mechanisms that contribute to these deficits are better understood, pharmacological intervention could help to reduce and diminish these effects. We have been working to understand these mechanisms using *in vivo* and *in vitro* rat models.

One of our main aims has been developing an *in vitro* organotypic hippocampal slice culture model to assess the interaction between ethanol exposure, ethanol withdrawal, and hypoxia. However, my main task has been working with the *in vivo* model to explore the interaction between ethanol exposure, ethanol withdrawal, and hypoxia using various behavioral paradigms. The *in vivo* model studies the "human third trimester brain growth spurt" in which the CNS undergoes significant development. Rat pups were exposed to ethanol administered by an oral gavage on postnatal days 1-7. This model used a split litter design with one female and one male rodent designated to each of the following treatment groups of ethanol, an intubated control, and a nontreated control. Animals receiving ethanol were given 4.5g/kg of ethanol per day in a milk based diet. This dose of ethanol is relatively low and in our rodent model does not typically produce obvious behavioral deficits.

On PND 8 the pups were divided into hypoxia and control groups. Equal numbers of animals from the ethanol treatment group, milk, and nontreated control were put into either the hypoxia or control groups. Hypoxia was administered for 8.5 minutes, by pumping nitrogen into the hypoxia chamber at 5 liters per minute. The control chamber was pumped with 5 liters per minute of compressed air for the same amount of time. After the 8.5 minutes, hypoxia animals were removed from the chamber and placed on a clean cloth while the control animals were returned to their home cage. Both the cloth and cage sit on a heating pad to keep the pups warm. Hypoxia animals remain on the cloth until they regain their normal color and begin to walk around.

We employed several behavioral paradigms including an open field paradigm to assess locomotor activity as well as a water maze performance to test spatial learning and memory. For open field testing, both male and female preweanling rats (PND 20-21) were individually placed in a 55-cm round open field testing chamber. Activity was recorded for 30 minutes for two consecutive days using a camcorder that is part of the SMART real time video tracking system. The dependent variables included distance traveled (in 5 minute blocks) and entries and time spent in the center of the open field chamber (defined as the central 25% of the maze). Time spent in the center of the open field chamber is often considered a measure of anxiety although it can be used for inhibitory control. Rats usually do not like the center of an environment instead

staying near the walls (called thigmotaxis) and so entering the center could be an indicator of poor inhibitory control.
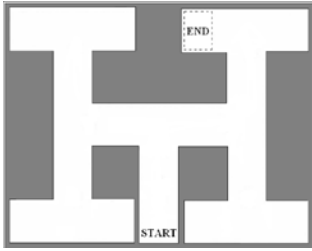
The water maze performance was assessed on two consecutive days between PND 40-45. Acquisition of the water maze was tested on Day 1 and retention of this task was tested on Day 2. The apparatus was a 130 x 90 x 40 cm black Plexiglas chamber, divided such that several divergent paths, each 18 cm wide, branched off from the central start area (Von Euler et al. 2006 (see Figure). Water temperature was maintained at 76° ±2 F. The rodents were required to make three successive right/left turns in order to reach a submerged platform. The water was made with non-toxic black paint so that the platform was not visible when it was submerged. A plastic sheet surrounded the maze which reduced extra-maze clues. The major advantage to this maze over more traditional mazes that test spatial learning was that the task could be learned in one day so that the retention of navigating the maze could be tested the next day.

The behavioral studies represented a 3 (neonatal treatment) x 2 (hypoxia treatment) x 2 (sex) factorial design. Control treatments were collapsed to make the study a 2 x 2 x 2 design because there were no differences between control groups. The data was analyzed using ANOVA with repeated measures. Significant interactions were broken down by simple main effects analyses and simple main effects were broken down by Duncan's post hoc tests. Behavioral studies required 10 – 12 subjects per cell of the experimental design.

In open field testing and in the water maze performance neither the low dose of ethanol or the brief episode of hypoxia produced significant behavioral deficits on their own. The combination on ethanol and hypoxia produced significant changes in locomotor activity as evident by a significant ETOH x OXYGEN interaction for total distance travelled on Days 1 and 2, $F (1,87) = 5.91$, $p=.017$ in the open field. Post hoc comparisons showed that ETOH/HYPOXIA exposed subjects had greater locomotor activity on Day 1 and Day 2 compared to all other treatment groups (Figure 1). Repeated measures ANOVA revealed a significant DAY x SEX x ETOH interaction $F (1,87) = 5.77$, $p<.05$ for time spent in the center. There was also a main effect of oxygen $F (1,87) = 4.47$, $p <.05$, such that hypoxia treated animals spent more time in the center. To better understand the data, separate analyses were run for Day 1 and 2. For Day 1, while the ETOH x HYPOXIA interaction was not significant $F (1,87) = 3.61$, $p=.061$ post hoc comparisons showed that ETOH/HYPOXIA exposed animals spent more time in the center compared to all other treatment groups (Figure 2). There were no significant differences between groups on Day 2 of testing.

Ethanol and hypoxia differentially affected males and females in the water maze task. ETOH/HYPOXIA exposed males had impaired acquisition compared to all other treatment groups, while there were no differences between treatment groups for females. The combination of ethanol and hypoxia also significantly increases the amount of time spent in the center. A repeated measures ANOVA on water maze performance revealed a significant DAY x OXYGEN x SEX interaction $F (1,93) = 6.978$, $p=.010$. To better understand the data, separate analyses were run for each sex. For males, there was a significant ETOH x OXYGEN interaction $F(1,51) = 6.24$, $p=.016$. Post hoc comparisons for each day revealed that ETOH/HYPOXIA exposed males required a greater number of trials to reach criterion on Day 1 compared to all other groups (Figure 3). For females, there were no differences between groups.

The results for open field testing supported our hypothesis that the interaction of ethanol exposure and hypoxia produced more damaging effects than either challenge did alone. As expected, the ETOH/HYPOXIA exposed subjects were hyperactive and stayed in the center for a greater duration of time during open field testing. Time spent in the center can indicate either increased anxiety levels or poor inhibitory control. Rats are generally not comfortable entering the center of a chamber. Therefore, more time spent in the center of the chamber could indicate poor inhibitory control. Additional studies need to be done to understand exactly what time spend in the center of a camber means.

The results for water maze performances were interesting because the interaction of ethanol and hypoxia produced the expected effects for the males but not for the females. It took the ETOH/HYPOXIA exposed males longer to learn the water maze on Day 1 this was not true for the ETOH/HYPOXIA exposed female. However, on Day 2 there were no significant difference between treatment groups or between sexes. Thus it appears that it takes the ETOH/HYPOXIA exposed males longer to learn the task but they were apparently able to retain it. This is a different pattern than what we have previously seen in the laboratory with higher doses of ETOH in which the subjects can acquire the task but are impaired at 24 hour retention. There is evidence that males are more sensitive to hypoxic challenges compared to females (Stupfel, Perramon, Gasc, Magnier & Duriez, 1978) which may help explain some of the differences in water maze performance. Further study will help us understand this affect (as well as the sex differences in sensitivity).

Being involved in this lab has been a great experience for me and I have grown a tremendous amount over the course of the summer. Research techniques that I was exposed to over the summer will help me throughout my career. I have also gained a much greater understanding of behavioral neuroscience and in particular the effects of prenatal ethanol exposure. I am thankful to be a part of research that has helped to better understand the factors that contribute to fetal ethanol exposure which in turn helps understanding how to control some of the damaging effects of fetal ethanol exposure.

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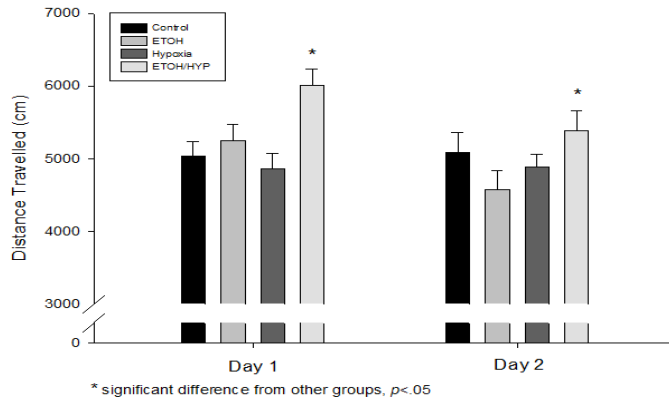
Figures

Figure 1. Post hoc comparisons show that ETOH/HYPOXIA animals had greater locomotor activity and traveled farther distances on Day 1 and Day 2 than any other treatment group.
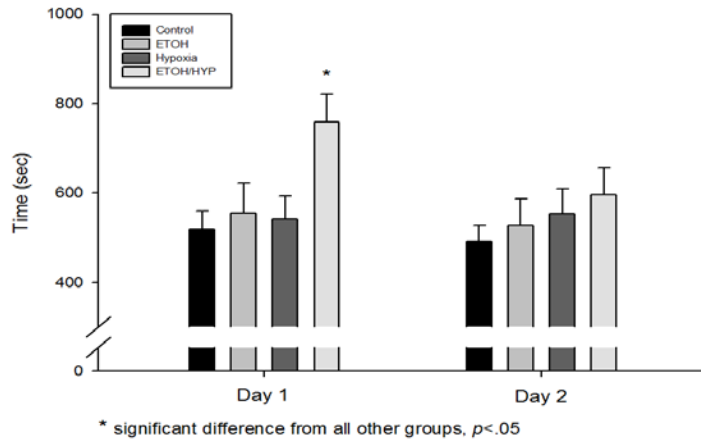

Figure 2. Post hoc comparisons showed that ETOH/HYPOXIA animals spent more time in the center on Day 1 than any other treatment group.
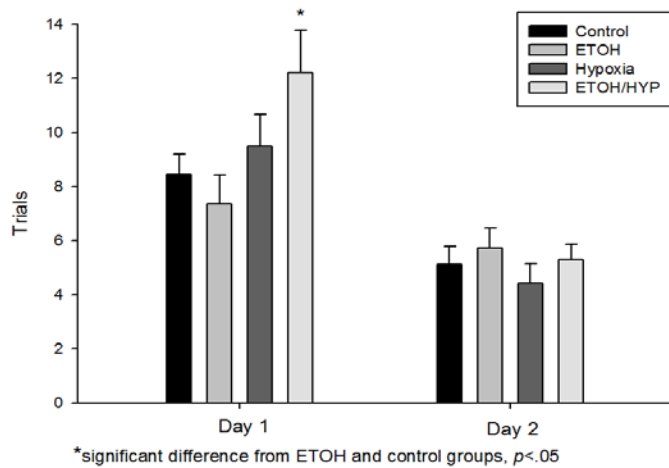

Figure 3. Post hoc comparisons on just male subjects showed that males in the ETOH/HYPOXIA treatment group took more trials to learn the water maze on Day 1 with no significant different in retention trials on Day 2.