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A Noninvasive Measure of the P300 in Rats Selectively Bred for Disparate Alcohol Preference During an Auditory Oddball Tas

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A NONINVASIVE MEASURE OF THE P300 IN RATS SELECTIVELY BRED FOR DISPARATE ALCOHOL PREFERENCE DURING AN AUDITORY ODDBALL TASK

by

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Abstract

The utility of genetics for predicting alcoholism and alcohol-related disorders is limited given environmental variance and a finite understanding of all genetic contributors. This has led to interest in phenotypic markers that can be used for classifying individuals at heightened risk for developing alcoholism and alcohol-related disorders. One such marker is the P300, an event-related potential (ERP) observed to have an attenuated amplitude and increased latency in both humans and animals who have a genetic predisposition to alcohol use. To study the utility of the P300 as a biomarker for alcohol use disorders (AUDs), we examined its characteristic in alcohol-preferring (P) and non-preferring (NP) rats naïve to alcohol using an auditory oddball task. Electroencephalography (EEG) was measured using a novel, noninvasive method after rats were trained to press a lever for food in response to the rare "target" tone, but not after the more frequent "standard" tone. The amplitude of the N2-P3 complex revealed a significant line x tone interaction ($F(1,37)=4.365$, $p=.044$, $\eta^2_p=.106$). Post-hoc analysis revealed an approaching significant attenuation in the N2-P3 amplitude for the P (versus NP) rats only for the target tone ($p=.077$, $\eta^2_p= .078$). These results support the previous findings reporting a decrease in P300 amplitude in those with a genetic predisposition to alcohol and adds support to the utility of the P300 as an endophenotypic marker of alcoholism.

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Abbreviations

age at first drink (AFD)

alcohol dehydrogenase (ADH1B)

alcohol-non-preferring rat (NP)

alcohol-preferring rat (P)

alcohol use disorder (AUD)

aldehyde dehydrogenase (ALDH2)

blood-alcohol concentrations (BACs)

electroencephalography (EEG)

ethanol (EtOH)

event-related oscillations (EROs)

event-related potentials (ERPs)

fixed-ratio (FR)

high-alcohol-drinking rat (HAD)

milliseconds (ms)

minutes (min)

postnatal day (PND)

single nucleotide polymorphisms (SNPs)

transistor-transistor logic **(**TTL)

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CHAPTER 1: INTRODUCTION

Preference: Impact of Genetics and Acute Alcohol Exposure

Much research has been conducted to better understand the mechanisms and moderators that lead to alcohol abuse and alcoholism (Apostolopoulos, Lemke, Barry, & Lich, 2018; Caneto, Pautassi, & Pilatti, 2018; Emmers, Bekkering, & Hannes, 2015). Having a better understanding of individuals who may be more susceptible to these debilitating conditions could aid in preventative care, which could reduce the economic burden of this disease. Currently, one of the most documented indicators for risk of alcoholism or alcohol-related disorders is a family history of alcohol-related problems (Cloninger, Bohman, & Sigvardsson, 1981). Twin studies have shown a significant heritability, with genetics contributing to approximately 50-60% of the variation of alcoholism for both men and women with the remainder being presumably attributed by environmental influences, such as sociocultural factors (Heath et al., 1997; Prescott, Aggen, & Kendler, 1999). These warrants a need to better understand the genetic risk factors of this disorder.

While some genes are well documented to be strongly associated with the risk of alcoholism, such as ADH1B (Alcohol Dehydrogenase) and ALDH2 (Aldehyde dehydrogenase) (Edenberg & Foroud, 2013), modern genetic studies still demonstrate only limited use in determining risk for subsequent alcoholism. For instance, using genetic techniques such as aggregated single nucleotide polymorphisms (SNPs) analyses to detect the genetic sum score of known risk alleles has demonstrated limited clinical utility, with family history still providing more predictive accuracy for alcoholism (Yan et al., 2014). This is due to remaining unknown variance contributed by other possible

risk alleles, and to a greater degree, the lack of understanding of how the environment (culture, childhood adversity, family environment, and religion) and individual characteristics (antisocial behavior, behavioral undercontrol, and ethnicity) impact known and unknown risk alleles (Enoch, 2006; Wall, Luczak, & Hiller-Sturmhöfel, 2016).

Event-Related Potentials as a Phenotype

Because of these continued shortcomings regarding the genetic contribution to AUD and related alcohol use disorders, interest in understanding the intermediate physiological phenotypes connected to this understood genetic contribution is warranted. More easily observable phenotypes can provide a clearer understanding of what genetic variations may be predictive of or related to certain aspects of alcoholism (Enoch & Goldman, 2001). Over the past few decades, electrophysiological measures, such as electroencephalography (EEG), event-related oscillations (EROs), and event-related potentials (ERPs), have shown promise as possible psychophysiological markers of a genetic risk for alcoholism. Of these, ERPs have received the most attention in terms of their potential ability to identify individuals demonstrating an alcohol-related phenotype (i.e., alcoholism or another alcohol-related disorder) as well as those with a family history of the disease (Porjesz et al., 2005).

ERPs are voltage deflections that are time-locked to a stimulus, and often with a subsequent response, and can be used to evaluate both sensory perception and cognitive functioning. They are commonly recorded using EEG and examined in terms of voltage and latency patterns (Picton et al., 2000). Raw EEG recordings are then filtered and averaged to produce ERP patterns that are then classified into specific components that correspond to the polarity of the deflection, latencies, scalp distribution, and sensitivity to

a selected task (Woodman, 2010). While early waves demonstrate more sensitivity to exogenous factors, such as the intensity of the stimuli, the later waves (typically those that peak after 100 ms) are considered more endogenous in that they are more so influenced by one's current cognitive functioning or physical state during stimulus processing (Kuperberg, 2008). Of these later ERPs, the P300 has an easily distinguishable amplitude suitable for EEG acquisition, and evidence that it may possess clinical utility as a an endogenous measure of neuronal activity and information processing has warranted extensive examination (Polich, 1998, 2007)..

Cortical Event-related Potential: P300

The P300, also referred to as the P3b or classic P3, is a late, parietally maximal positive deflection that has a peak latency around 300 – 450 ms after presentation of the eliciting stimulus, and has been shown to be involved with conscious attention, particularly in terms of the predictability of an expected stimuli or event (for review see Picton, 1992). This has most commonly been seen in studies with humans using an active oddball task, in which the participant is instructed to attend to a rare, "target", stimulus while ignoring a more frequent, "standard", stimulus, as the decreased probability of the rare "target" tone produces a distinguishable P300 comparatively to the standard (Polich & Margala, 1997).

The P300 in both visual and auditory discrimination tasks has been found to be attenuated in individuals with a genetic predisposition for alcoholism and alcohol abuse (i.e., positive family history) (Porjesz & Begleiter, 1998). These persistent deficits seem to indicate that the P300 amplitude could function as a potential trait marker for those with a propensity for alcoholism or alcohol abuse disorder (AUD). This is further

supported by findings that even those abstained from alcohol consumption for prolonged periods of time still demonstrate a lower P300 amplitude (Hill, 2000; Pfefferbaum, Ford, White, & Mathalon, 1991). This indicates a need to better understand how positive family history, a well-documented risk factor, influences the P300.

Family History of Alcohol Misuse and the P300

As previously noted, a reduction in P300 amplitude in both visual and auditory tasks has been seen in adult samples with a family history of alcoholism (defined as at least one first degree family member with alcoholism) in comparison to low risk and control samples (Patterson, Williams, McLean, Smith, & Schaeffer, 1987; Ramachandran, Porjesz, Begleiter, & Litke, 1996). Similar findings have also been found in children naïve to alcohol (Ehlers, Wall, Garcia-Andrade, & Phillips, 2001; Steinhauer & Hill, 1993), which implies that these reduced amplitudes in the P300 are apparent across developmental periods and even before, at least presumably, the consumption of alcohol. In addition, just as a high degree of genetic contribution has been seen for alcoholism (Heath, et al., 1997), the P300 has also shown to be highly heritable, with one sample of male twins demonstrated an estimated genetic contribution of 79% to P300 reduction (Katsanis, Iacono, McGue, & Carlson, 1997). However, the specific genetic influence of family history of alcoholism (irrespective of intake) on the P300, particularly its utility as a biomarker for alcoholism, is not entirely clear.

Some predictive ability of later alcohol misuse has been seen regarding an initially lower P300 amplitudes in comparison to controlled samples. For example, a reduced P300 amplitude was measured in hereditarily predisposed populations, those with a family history of alcohol, along with subsequent disinhibited or antisocial behavior

and increased alcohol intake and/or substance use (Berman, Whipple, Fitch, & Noble, 1993; McGue, lacono, Legrand, Malone, & Elkins, 2001). In a longitudinal study, 36 boys who were alcohol-naïve were divided into three distinct groups of family history: (1) sons of recovering alcoholics with a positive family history of alcohol, (2) sons of nonalcoholics with a positive family history of alcohol, (3) and sons of nonalcoholics with no family history of alcohol. A reduced P300 amplitude was seen initially in those with a positive family history, and after a 4-year follow up, those who had demonstrated the lowest P300 amplitudes continued to possess the highest substance use scores adjusted for age (Berman, Whipple, Fitch, & Noble, 1993).

A subsequent study examined age at the first drink (AFD), another well documented risk factor of future AUD (Dawson, Goldstein, Patricia Chou, June Ruan, & Grant, 2008), in two twin cohorts, one 10-12 year and one 16-18 year, to determine the likelihood of risk for future alcoholism and substance abuse based on initial P300 characteristics at the first assessment compared to the 3-year follow-up assessment (McGue et al., 2001). Individuals in the 16-18-year-old cohort demonstrated a reduction in the P300 amplitude regarding AFD, but these differences did not appear that were specifically associated with alcoholism. Rather, AFD was correlated across multiple indicators of disinhibited behavior and psychopathology. This supports the notion that a smaller P300 may be indicative of a decrease in disinhibitory behavior rather than consumption history (Pfefferbaum et al., 1991), as an inability to regulate the intake of substances, like alcohol, is higly characterisitc of AUD and other substance use disorders (Taylor, Carlson, Iacono, Lykken, & McGue, 1999).

While these studies support the P300 as a generalized biomarker of disinhibitory behavior (McGue et al., 2001) and alcohol misuse (Berman et al., 1993), the impact of genetics (i.e., family history) on the P300 is not fully consistent or understood. For instance, eight-year old children of alcoholic fathers with paternal delinquency (defined as at least one previous criminal conviction) were found to have a significant difference in P300 amplitude only in comparison to children of alcoholic fathers without paternal delinquency (Viana-Wackermann, Furtado, Esser, & SchmidtMan, 2007). This implies that other moderating environmental factors throughout development likely contribute to the attenuation of the P300 or even possibly diminish the impact of attenuation from hereditary factors. This is not surprising given the well-known but minimally understood gene-environment interaction related to substance use disorders like AUD (Merikangas & Avenevoli, 2000). Given these unknown factors contributed by environmental influences, the utility of controlled laboratory studies employing animal models of AUDs present a viable solution that can control for these moderating, environmental variations while still exploring the genetic contribution to changes in the characteristics of the P300.

Animal Models and the P300

Animal studies provide an effective means to compare specific genotypic and phenotypic characteristics related to alcohol intake while controlling for environmental influences. Through selective breeding, it is possible to produce specific rat lines with genotypic and phenotypic characteristics by selectively mating individuals from both ends of the genetic extremes via genetic drift. Through this process, the Indiana selection studies have produced lines of rats with and without a preference for ethanol (EtOH) that

can function as a model for alcoholism as well as a countermodel that does not demonstrate alcoholic tendencies (Murphy et al., 2002).

The alcohol-preferring (P) line and the high-alcohol-drinking (HAD) line of rats are both lines that have demonstrated characteristics and behaviors that are comparable to human alcoholics. However, the P line has been found to satisfy all originally proposed criteria for an animal model for alcoholism, while the HAD line has only meet 4 of 6 (McBride, Rodd, Bell, Lumeng, & Li, 2014). Specifically, P rats have been shown to be capable of developing metabolic tolerance (Lumeng & Ting-Kai, 1986) and show withdrawal effects from EtOH after extended free-choice consumption (Kampov-Polevoy, Matthews, Gause, Morrow, & Overstreet, 2000). Regarding the remaining criteria, P rats will orally self-administer EtOH when provided free-choice conditions to the point of pharmacologically significant blood-alcohol concentrations (BACs) and have a high tolerance to the acute effects of EtOH. In addition, EtOH is highly preferred by P rats, even before initial consumption, is positively reinforcing, and is consumed primarily for its pharmacological effects (Bell, Rodd, Lumeng, Murphy, & McBride, 2006; McBride et al., 2014).

The counterpart line to the P rats, non-alcohol-preferring (NP) rats, do not demonstrate these characteristics. EtOH is not reinforcing like it is in P rats and they will demonstrate a preference for water and behavioral inactivity in the presence of EtOH – neither of which are seen in P rats (Murphy, Gatto, McBride, Lumeng, & Li, 1989). These behavioral differences have also been correlated with electrophysiology, with P rats exhibiting an increase in EEG power in the 8-16 Hz frequency in the first 10 minutes of 10% EtOH availability (Robledo, Lumeng, Li, & Ehlers, 1993). This appears to

indicate that alcohol-related cues are less reinforcing or salient for NP rats in comparison to P rats.

Similar to differences found in human studies that evaluate the impact of family history of alcoholism on the P300, P rats have demonstrated attenuated P300 amplitudes compared to NP rates while still naïve to ethanol (Criado & Ehlers, 2010; Ehlers, Somes, Lumeng, & Li, 1999). So far, rat studies examining the P300 in P and NP rats have only utilized a passive form of the auditory oddball paradigm (i.e., does not require the subject to respond to the presentation of the tone) via either a three-tone (Criado & Ehlers, 2010; Ehlers, Somes, Lumeng, & Li, 1999) or two-tone task (Ehlers, Chaplin, Lumeng, & Li, 1991). The three-tone task differs from the common oddball at it adds a third "noise" tone designed to elicit the P3a response, a possible subcomponent of the P300, while still eliciting the classic P3 via presentation of the frequent standard tones and rare target tones (Polich, 2007). However, research has shown a greater P300 amplitude and clearer peak distinctiveness for target stimuli during an active auditory oddball task compared to a passive paradigm in both humans (Bennington & Polich, 1999) and rats (Ehlers, Kaneko, Robledo, & Lopez, 1994; Sambeth et al., 2003; Shinba, 1997). This indicates that an active oddball task would allow for a more robust examination of the P300 in animal models than a passive task model. However, no active task to this authors knowledge has been used to examine the P300 in P and NP lines.

Another issue is that these electrophysiological studies with animals (Criado & Ehlers, 2010; Ehlers et al., 1991, 1994, 1999; Robledo, Lumeng, Li, & Ehlers, 1993, Sambeth et al., 2003; Shinba, 1997) and many other similar studies not referenced in this work use surgically implanted and or invasive electrodes for electrophysiological

recordings. These include but not limited to screw electrodes (Ehlers et al., 1994; Robledo et al., 1993; Shinba, 1997), stainless steel single-wire (Criado & Ehlers, 2010; Ehlers et al., 1991, 1999), and implatned tripolar EEG electrodes (Sambeth, et al., 2003). While invasive methods are common practice in animal studies, they are not without drawbacks. While these more invasive methods often provide accurate physiological recording (less noise, can measure specific brain regions, etc.), subject attrition over time is relatively common often due to loss of function in the electrode. Study duration is also often limited by the lifespan of the electrode(s). Implementing less invasive approaches or noninvasive methods akin to those used in human studies could help alleviate these issues and make the findings more translational to human research.

Purpose of this Study

This study was designed with three objectives in mind. First, to test whether we could successfully use a novel, noninvasive methodology incorporating scalp Ag/AgCl cup electrodes (instead of in-dwelling, surgically implanted electrodes) to demonstrate that reliable results could be obtained using noninvasive electrodes. This approach would be more translatable to human EEG research, and it would also address the three R's of animal research (replacement, reduction, and refinement) as described by Burch, Russell, and Hume (1959). Second, to examine whether attenuation of the amplitude of the P300 would be seen in alcohol-P and NP rats naïve to alcohol. Third, to evaluate that P and NP subjects could successfully learn to perform an active auditory oddball task. Given that previous studies have found a diminished P300 amplitude and increased latency in P rats naïve to alcohol using a passive auditory oddball task (Criado & Ehlers, 2010; Ehlers et al., 1999) we anticipated similar electrophysiological findings. Specifically, we

anticipated that (1) subjects would successfully be able to perform the active auditory oddball task, (2) the P300 amplitude would be attenuated in alcohol-naïve P rats in comparison to alcohol-naïve NP rats to the target tone, and (3) that our noninvasive recording apparatus would prove to be a reliable and more efficient method for EEG acquisition in rats.

CHAPTER 2: METHOD

Subjects

Forty-six rats were delivered from Indiana University-Purdue University's (IUPUI) animal colony. They consisted of rats from the alcohol preferring (P) and alcohol non-preferring referring (NP) lines (23 rats per line), with an equal number of male and female for each line (12 males and 11 females per line). They arrived on post-natal day (PND) 45 and were pair housed in standard shoe-box size cages. Each cage included Teklad Laboratory-Grade Sani-Chips bedding. All subjects were fed a diet of Harlan Teklad 2018 rat chow and water *ad libitum.* The colony room remained on a 12-hour light/dark cycle with lights on starting at 0730 hour. At PND 50, subjects were placed on an IACUC-approved food restriction schedule and maintained at 85% of their individualized free-feeding weight until the end of experimentation. Each rat was weighed, fed, and tested during the same period of the lights-on phase of the cycle. All 46 rats were used for behavioral testing, but two rats were lost before EEG recordings due to physical complications that made them ill-suited for testing, leaving the number of rats used for electrophysiology at 44 (22 per line).

Apparatus

Ten identical sound-insulated and ventilated operant chambers were used for behavioral operant testing with six being equipped for EEG data acquisition. Each chamber contained two symmetrically retractable levers, both on the same wall, located 7 cm from the floor and 5.7 cm from a food magazine located in-between. A cue light was located another 5.7 cm above each lever. The house light was positioned on the wall opposite from the levers. The chambers also included two speakers located on the same

wall as the house light. One was used to play the standard (500 ms, 2.5 kHz, 80 dB) tone and the other to play the target (250 ms, 5 kHz, 80 dB) tone presented during operant tasks. All operant chambers were connected to a PC equipped with Med-PC IV software (Med Associates; St. Albans, VT), which was used to conduct all behavioral programs. This PC was also connected to a TTL (transistor-transistor logic) relay interface (Med Associates, model SG-726-TTL) to present a TTL pulse to a DataWave system (16-Bit High-Speed Multifunction DAQ Device, model #USB-1680G, Measurement Computing, Norton, MA) at the start of each trial, which was used for EEG data acquisition. A separate laptop running the SciWorks Express program (DataWave Technologies Corporation, Loveland, CO) recorded the electrophysiological data after amplification via Grass 8-16 E amplifiers (Grass Medical Instruments, Quincy, MA). EEG acquisition parameters were set to a high pass setting of 0.1 Hz, a low pass of 35 Hz, and a digitization rate of 500 Hz.

Operant Testing

Auto-shaping and fixed-ratio (FR) training. Auto-shaping and FR training commenced once subjects reached approximately 60 days of age. The specific details of these procedures are described in previous works (Sable et. al, 2006, 2009). Briefly, the target tone was presented each time a 45-mg dustless precision reward pellet (Bio Serv; Frenchtown, NJ) was delivered. This food pellet was used as a reinforcer across all behavioral tasks. Rats were required to press either response lever and earn 100 "non-free" food reinforcers during auto-shaping before proceeding to FR training. The goal was to train the rats to associate the pressing of either lever with presentation of the target tone and food delivery.

After the rats had successfully met the criteria for auto-shaping, they proceeded to FR training. The response requirement to earn a food reinforcer was gradually increased from FR1 (i.e., 1 lever press per reinforcer) to FR3 (i.e., 3 lever presses one reinforcer) and then FR5 (i.e., 5 lever presses per reinforcer). This furthered allowed the subjects to associate the reinforcer with the target tone, as well as indicated that lever presses not associated with the food reinforcers were not associated with the target tone. A session was considered completed after 100 successful reinforcers were delivered. All rats completed approximately 2-3 sessions for each FR schedule.

Target-detection training. After subjects completed all FR schedules, they proceeded to target-detection training. Only the right lever was used. Subject were tasked with pressing the lever after the presentation of the target tone within a set amount of time. They went through three different target-detection phases, with each subsequent phase shortening the amount of time allowed for a reinforced lever press (3000 ms, then 2000 ms, and finally 1500 ms). The goal was to train the rat to respond rapidly after the presentation of the target tone. If the subject failed to respond in time, the lever was retracted, the house light was extinguished until the start of the next trial, and no reinforcer was delivered. Each target-detection training session consisted of 200 trials with 6-s intervals between trials. Each subject was required to reach 90% accuracy for a given phase before moving on to the next phase.

Signal discrimination training and auditory oddball task. After target-detection training, rats moved on to signal-discrimination training. Again, only the right lever was used. For these phases, the rats had to learn to only press the lever after hearing the target tone (reinforcing stimulus) but now also had to withhold responding after

hearing the standard tone (non-reinforcing stimulus). The initial phase consisted of sessions with a ratio of 60% target and 40% standard tones (60T-40S). After reaching a range from 80-90% accuracy or greater to the target and 75% inhibition to the standard tone, the targets to standards ratio was adjusted in 10% intervals, with target tones decreasing by 10% and standard tones increasing by 10% for the proceeding schedules (60T-40S, 50T-50S, etc.). The final phase consisted of 20% targets and 80% standard tones (20-80) and was designated the "oddball task". As the number of targets decreased, the number of trials increased, such that 200 target trials were included in every phase.

For all signal-discrimination trials, including the oddball task, the start of each trial was signaled by the right lever extending and the house light illuminating. After 1000 ms, the rare or standard tone was presented, and the rat had 1500 ms to appropriately respond. Trials were allotted into four distinct bins in the Med-PC data file. A trial was classified as a "hit" if a target tone sounded, and the rat pressed the lever within the allotted time. If the rat failed to respond to the target tone, the trial was classified as a "miss". If a rat appropriately withheld responding after the standard tone was presented, the trial was classified as a "correct rejection". If the rat inappropriately pressed the lever after the standard tone was presented, the trial was classified as a "false alarm." Only hits were rewarded with a food pellet. For a "miss", the lever retracted, and the cue light shut off until the next trial. For "false alarms" the house light shut off along with retraction of the lever and shutting off of the cue light. The house light turned on again with the start of a new trial to allow subjects to differentiate between a "miss" and a "false alarm." No consequence was administered for any incorrect response. For all trials, a 50000 ms delay after the presentation of the tone before starting the next trial. This

allowed the rats to have time to consume a reward pellet (if delivered) and keep all trials at 6000 ms. See Table 1 for summary of behavioral tasks.

Electrode Placement and Measurement of P300 ERP

After meeting the necessary performance criteria on the oddball task, EEG recording sessions commenced. Each rat was anesthetized with a continuous flow of 1-2% isoflurane in pure oxygen delivered via a nose cone. After proper sedation, the fur was shaved from the base of the neck to the top of the head and a depilatory of potassium thioglycolate was applied for approximately 45 seconds to remove any residual stubble. The skin was then washed thoroughly with water, swabbed with betadine, and then wiped with 70% isopropyl alcohol. Two 6 mm cup Ag/AgCl electrodes (Spes Medica, Italy, GE) were attached using Collodion; one recording and one reference. The recording electrode was placed at the midline point above the parietal cortical regions, just posterior to bregma of the rat cranium. This placement is akin to the area between the Cz and Pz of the international 10-20 system for humans (Jasper, 1958), and was assumed to provide the greatest sensitivity to the P300 (Picton, 1992). The reference electrode was secured caudal to the right ear. The lead wire and connectors were fastened to a jacket worn by each rat (Instech Laboratories Incorporated, Plymouth Meeting, PA), while the remaining exposed lead wire was covered using Tegaderm HP transparent. Each jacket contained a port that provided access to the exposed lead wire to be connected for EEG data recordings. This procedure took approximately 15 min per rat, and subjects were provided a recovery period of 24 hours before resuming testing.

After recovery, from anesthesia, rats underwent EEG recordings with the oddball task. Each rat was placed into an operant chamber equipped for EEG acquisition. Rats

were genteelly restrained, and using a syringed with a blunt tip needle, electrode gel was placed under each electrode. The electrode wires were then connected to the lead wires hanging from the top of the chamber connected to the DataWave system. The lead wires were also secured to a counterweight pulley system to prevent the rats from having access to them during testing and to avoid them obstructing the subject's movement. Impedance was required to be below 10 kΩ and was checked on an individual basis before testing. If impedance was too high, the electrode was removed and reattached until adequate impedance was obtained. From there, a TTL pulse occurred to signal the start of each EEG acquisition session. Due to the weight of the EEG recording apparatus, rats were not required to respond and were not reinforced during the recording sessions. Rats were required to complete two sessions of EEG recordings. These sessions preceded over two subsequent days with each rat completing one session on one given day.

ERP Analysis

A custom MATLAB script (MathWorks; Natick, MA) was used to generate the average ERP waveform for each subject from the raw electrophysiological data. Each epoch consisted of a time window of 600 ms, with a 100 ms baseline period prior to the start of the tone. This was used to generate the corrected averages by subtracting the mean amplitude of this pre-stimulus baseline from each data point in the waveform. Epochs that included any of the following were excluded: amplitude variation of less than 1 μV across 50 consecutive data points (100 ms), amplitude greater than ±500 μV relative to baseline, and adjacent data points varying by more than 100 μV. Data from three rats (1 P and 2 NPs) were excluded due to an excessive number of rejected trials.

The P300 was considered as the mean voltage occurring 300-450 ms after onset of the tone. The N2-P3 peak-to-peak complex was also measured by subtracting the P300 voltage from the N200. The N200 was the mean voltage occurring 250-300 ms after tone onset.

Statistical Analyses

For the behavioral data, the independent variables were the genetic line of the subjects (P and NP), sex (male and female), and phase of signal discrimination training (i.e., 60-40, 50-50, 40-60, 30-70, and 20-80). The dependent variable was the percent of correct responses averaged across all days for each phase calculated via the following equation:

$$\% \text{ correct} = (\#\text{ hits} + \#\text{ correct rejections} / \#\text{total trials}) \times 100$$

Thus, the behavioral data were analyzed with a 2 x 2 x 5 mixed ANOVA, with line and sex as between-subjects factors and phase as a repeated-measures factor. For the electrophysiological data, the independent variables again included the between-subject factors of line and sex, but instead of phase included the repeated-measures factor of tone (target versus standard). The dependent variable was the amplitude (μV) of the N2-P3 complex, which was analyzed via a 2 x 2 x 2 mixed ANOVA. Post-hoc *t*-tests or simple contrasts were conducted after the omnibus analyses for any significant results. All analyses were conducted using IBM SPSS Statistics for Windows (version 25).

CHAPTER 3: RESULTS

Signal Discrimination and Auditory Oddball Performance

The omnibus mixed ANOVA on percent correct for the various signal discrimination phases revealed a main effect of phase [$F(4,168) = 60.668$, $p<.001$, $\eta^2_p=.591$]. Accuracy was significantly lower for the 60T-40S and 50T-50S phases than the proceeding phases ($p<.01$ and $\eta^2_p >.151$ for all comparisons, Figure 1). Additionally, a significant main effect of line was seen [$F(1,42)=5.489$, $p=.024$, $\eta^2_p=.116$]. P rats demonstrated 3.7% greater accuracy than NP rats (Figure 2). The main effect of sex was not significant.

The line x sex interaction approached significance but had a medium effect size [$F(1,42)= 3.656$, $p=.063$, $\eta^2_p=.080$]. Post-hoc analyses revealed a significant difference across lines for the males ($p = .003$, $\eta^2_p=.338$) but not for the females ($p= .579$, $\eta^2_p=.002$). Specifically, the male NP rats demonstrated decreased performance compared to male P rats (Figure 3). The line x phase, sex x phase, and line x sex x phase interactions were not significant, nor did they demonstrate an appreciable (i.e., medium or greater) effect size (Figure 4).

Effects of Line and Tone on N2-P3 Complex

The grand averaged ERP waveforms for both the P and NP lines to the target and standard tones are depicted in Figure 5. A clear N2-P3 complex amplitude can be seen in both P and NP lines for both the standard and target tone. The omnibus mixed ANOVA did not reveal a significant main effect of line [$F(1,37)= 2.130$, $p=.153$, $\eta^2_p =.054$], sex [$F(1,37)= 0.032$, $p=.859$, $\eta^2_p=.001$], or tone [$F(1,37)= 0.050$, $p=.824$, $\eta^2_p=.001$]. However, a significant line x tone interaction was found [$F(1,37)= 4.365$, $p=.044$, η^2_p

=.106]. The amplitude of the N2-P3 complex for the target tone appeared to be attenuated in the P rats compared to the NP rats (p=.077, η^2_p=.078), while no significant difference was seen between the lines for the standard tone (p=.987, η^2_p<.001; Figure 6). No significant line x sex x tone interaction was seen, with only a small effect size present [F(1,37)= 1.566, p=.219, η^2_p=.041].

CHAPTER 4: DISCUSSION

Accuracy of Auditory Signal Discrimination and Oddball Task

As anticipated, both the P and NP rat lines successfully learned to discriminate the target tone from the standard tone and complete the active auditory oddball task with high accuracy (Figure 1). These findings are consistent with previous studies that have investigated active oddball tasks in Wistar rats (Ehlers et al. 1994; Sambeth et al., 2003), but novel for these two genetic lines of alcoholism who previously were tested on only a passive auditory oddball task (Criado & Ehlers, 2010; Ehlers, Chaplin, Lumeng, & Li, 1991; Ehlers, Somes, Lumeng, & Li, 1999). Thus, the current findings present evidence that the methods used herein were effective for training rats selectively bred for alcohol preference to perform an active auditory oddball task analogous to tasks used in humans with a family history of alcoholism or AUD. This is advantageous not only because an active task has been shown to produce a clearer and more discernable waveform (Comerchero & Polich, 1999), but it allows for a more comparable comparison of the P300 across human and rat studies.

Somewhat surprisingly, P rats performed significantly better overall (i.e., across all training phases) than their NP counterparts (Figure 2). One possible explanation for the decreased performance in the NP rats is heightened anxiety during testing, which is a common inhibiting factor of behavioral performance. However, previous studies have shown that P rats, not NP rats, demonstrate greater anxiety-like behavior (Hwang, Stewart, Zhang, Lumeng, & Li, 2004; Stewart, Gatto, Lumeng, & Murphy, 1993; Zhang et al., 2010), but these findings lack consistency with some reporting no discernable differences in anxiety between P and NP lines (Badishtov et al., 1995; Roman, et al.,

2012; Viglinskaya et al., 1995). Sex differences in operant task performance have often been reported, with male rats typically having poorer performance than females on more difficult tasks (Dalla & Shors, 2009). While no overall sex difference was found, the difference between the lines appears to be moderated by sex (at least in part), as NP males were less accurate than P males (Figure 3), with no difference among P and NP females. Despite this, P and NP rats of both sexes successfully managed to learn and perform the active auditory oddball task. Both lines clearly exhibited the ability to distinguish the target from the standard tones. This was important to demonstrate so that any electrophysiological differences could be attributed to genetic history and not an obvious difference in ability to perform the oddball task.

N2-P3 for the Auditory Oddball Task

Although the rats were not required to respond during EEG recordings, a distinct ERP waveform was produced (Figure 5). Examining the difference in N2-P3 amplitude between lines to the target tone, a clear difference was seen between P and NP rats (Figure 6), with the P rats' amplitude being attenuated to the target tone compared to the NP rats. This difference cannot be attributed to a deficit in being able to distinguish between the tones, as P rats performed better than NP rats on the active auditory oddball task (Figure 1). This finding is consistent with previous studies that have found a reduced P300 amplitude to the target tone using a passive auditory discrimination task in P rats naïve to alcohol (Criado & Ehlers, 2010; Ehlers, Somes, Lumeng, & Li, 1999). Overall, there is good evidence that rats that are selectively bred for high alcohol preference demonstrate a phenotypic characteristic of reduced P300 amplitudes to salient stimuli.

As seen in Figure 5, the NP rats appeared to demonstrate the expected pattern of an increased amplitude to the target in comparison to the standard – an outcome previously seen in human and non-selectively bred rats (Sambeth et al., 2003; Shinba, 1997), but this difference between the standard and target was not statically significant. On the other hand, there was very little amplitude difference between the target and standard in the P rats. In fact, looking at the waveforms (Figure 5), the amplitude of the target appears to be below that of the standard tone (Figure 6) for the P rats, albeit this difference lacked statistical significance as it did in the NPs. During the oddball task, the P300 is primarily elicited to rare, meaningful stimuli – in this case, the target tones which were previously associated with food availability. In the P rats the amplitude to the target was reduced (compared to the NP rats) suggesting the salience of the target may be attenuated in the P rats.

Lessons Learned and Suggestions for Future Research

Despite our best efforts to develop and demonstrate that a noninvasive approach to EEG recording could produce similar results to more invasive methods, our method was prone to increased background noise. While precautions and steps were taken to reduce noise through proper filtering, this was not optimal given a few limitations of the current EEG apparatus. Often to reduce noise, a ground electrode is conventionally used, but due to the limited scalp space on the rats for the electrodes, the limited number of ports provided on the EEG recording system used, and the need to reduce the amount of wiring around the rat, we opted to eliminate the ground. Future research will need to revisit how to incorporate a ground electrode to obtain EEG recordings with less background noise.

In addition, the jacket worn by the rats was also more restrictive than anticipated and we settled to eliminate the need for rats to respond during the auditory oddball task. This turned are originally trained active task to a passive one. While rats did spend considerable time actively responding to the tones prior to recording, unlike what has been done in studies of these lines with a passive task (Criado & Ehlers, 2010; Ehlers et al., 1999), this switch to a passive task during EEG recording likely reduced the distinctiveness of the waveform peaks and amplitude of the P300 (Ehlers et al. 1994; Sambeth et al., 2003; Shinba, 1997). In future implementations of this noninvasive EEG setup, researchers will need to take steps to reduce the restrictiveness and discomfort of the jackets. At present we are considering the use of a wireless setup that uses telemetry (Cotugno, Mandile, D'Angiolillo, Montagnese, & Giuditta, 1996) and providing the rats adequate time to habituate to the jackets prior to placing the electrodes. Doing these things should decrease distraction and discomfort and allow the rats to freely move in the chamber while performing the active auditory oddball task. This in turn, should increase amplitude of the P300.

Despite these shortcomings, there was a relative difference in the amplitude of the P300 to the target tone between P and NP rats. While this provides further support for the use of the P300 as a functional biomarker for a genetic predisposition to alcoholism or AUD, further investigations are needed to understand what is driving this difference. Previous studies in humans have noted an *increase* in P300 amplitude to alcohol-related cues in individuals with low sensitivity (i.e., low acute response to alcohol) (Bartholow, Henry, & Lust, 2007; Bartholow, Lust, & Tragesser, 2010), in those who consume greater sums of alcohol habitually (Herrmann, Weijers, Wiesbeck, Böning, & Fallgatter,

2001) and in binge drinkers (Petit, Kornreich, Verbanck, & Campanella, 2013). Reduced sensitivity to the sedative effects of alcohol and a need to consume alcohol are characteristics clearly seen in P rats (McBride et al., 2014), yet differences related to alcohol cues have yet to be examined in the P and NP rat lines. While these current findings show that the N2-P3 amplitude was decreased in P rats, the target tone was associated with a nonalcohol-related reinforcer. Examining differences in P300 reactivity to alcohol-related cues (i.e., target tone predicts alcohol delivery) versus nonalcohol-related cues (i.e., target tone predicts food reward) in these genetic lines would allow for a better understanding of how genetics may influence reward salience and associated P300 amplitude to associated cues like the target tone.

Conclusions

With this study we confirmed that a noninvasive method can be used for examining the P300 in rats. Our findings were consistent with those found in previous human studies (Berman et al., 1993; Ehlers et al., 2001) and invasive animal studies (Criado & Ehlers, 2010; Ehlers et al., 1999). This study also succeeds in demonstrating that the P and NP rats were capable of learning and accurately responding to an active auditory oddball task, though measures need to be taken to improve the recording apparatus to allow for the active task to continue to be used during the EEG recording period. Lastly, the anticipated attenuation of the P300 in the P rats in comparison to the NP rats to the target tone was seen. This is consistent with the hypothesis that a reduced P300 could function as a possible biomarker for alcoholism, given findings from previous human studies that found a reduced P300 in those with a family history of alcoholism compared to family history negative individuals (Ehlers et al., 2001; Patterson et al.,

1987; Ramachandran et al., 1996; Steinhauer & Hill, 1993). Our findings point to the need to continue focusing on improving noninvasive methods for examining the P300 in animal models and forming a better understanding of the relationship between amplitude of the P300 and reward salience in P rats and those with a genetic predisposition to alcoholism and alcohol misuse.

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Appendix

Table 1. Summary of behavioral testing phases for training of the auditory oddball task.

Testing Phase	**Description of Task**
Autoshaping	Rat trained to press response lever
FR Training	Association between lever press, presentation of target tone, and food delivery strengthened
StimDetect_3000	Rat had to press lever within 3000 ms of hearing target tone
StimDetect_2000	Rat had to press lever within 2000 ms of hearing target tone
StimDetect_1500	Rat had to press lever within 1500 ms of hearing target tone
60T_40S	Trials consisted of 60% target tones and 40% standards (only presses after target were reinforced)
50T_50S	Trials consisted of 50% target tones and 50% standards (only presses after target were reinforced)
40T_60S	Trials consisted of 30% target tones and 60% standards (only presses after target were reinforced)
30T_70S	Trials consisted of 30% target tones and 70% standards (only presses after target were reinforced)
20T_80S*	Trials consisted of 20% target tones and 80% standards (only presses after target were reinforced)

Note: FR = fixed ratio, * “ODDBALL” TASK

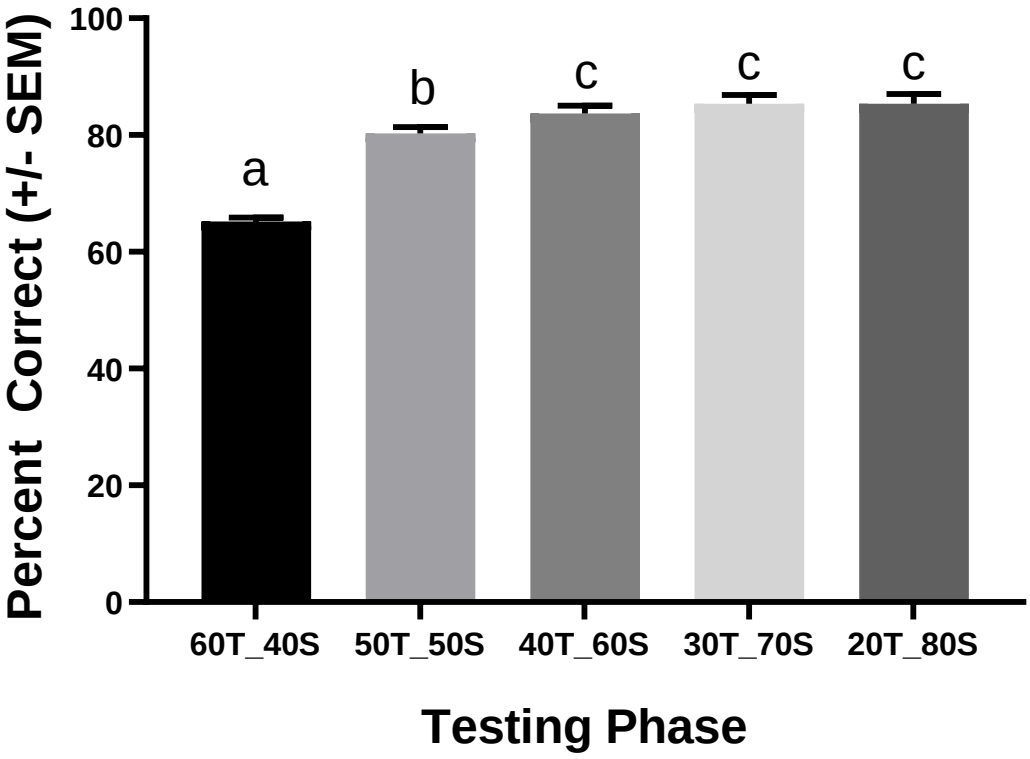

Figure 1**.** Response accuracy across all signal discrimination phases (20T-80S being oddball task). Different letters indicate a significant difference (p <.01 and η^2_p >.151 for all comparisons).

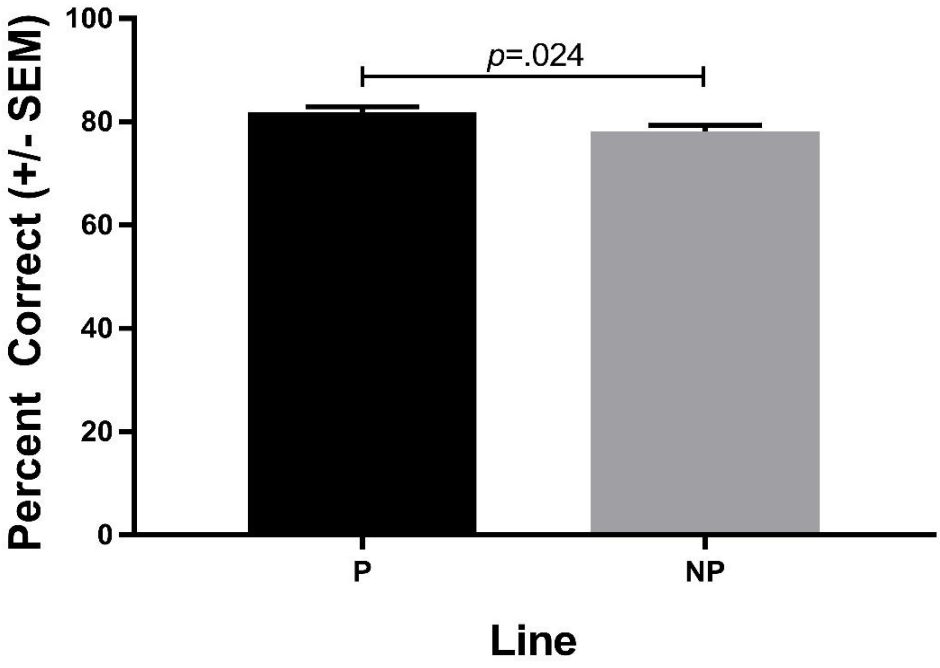

Figure 2. Response accuracy between genetic lines collapsed across sex and all phases. Overall, P rats were 3.7% more accurate than NP rats (p=.024, η^2_p=.116).

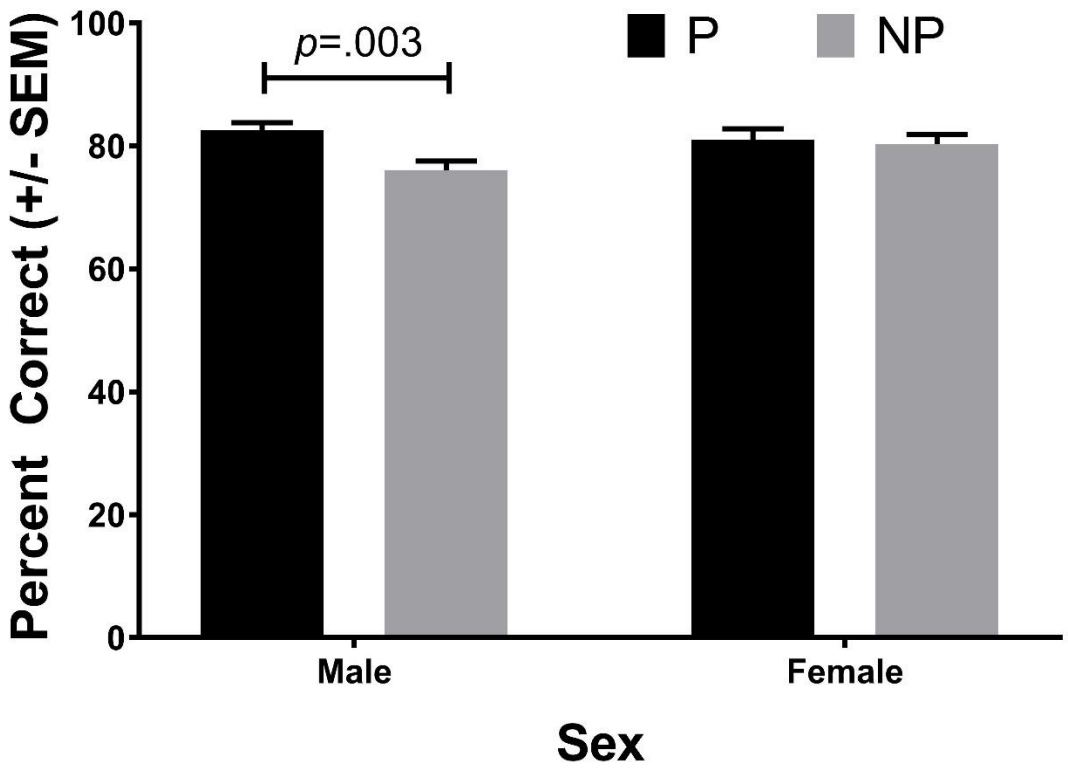

Figure 3. Response accuracy collapsed across all phases for each sex within each line. Male NP rats were less accurate than P males (p=.003, η^2_p=.338). There was no difference between P and NP females.

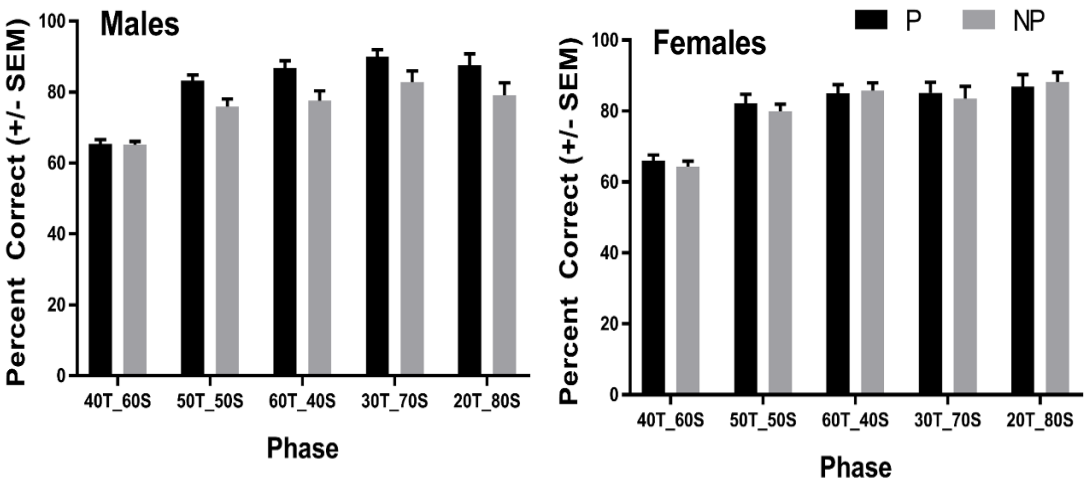

Figure 4. Response accuracy across all phases for both sex and line. While there was no significant line-related interaction, NP rats exhibited reduced accuracy across all phases excluding the first phase (40T-60S).

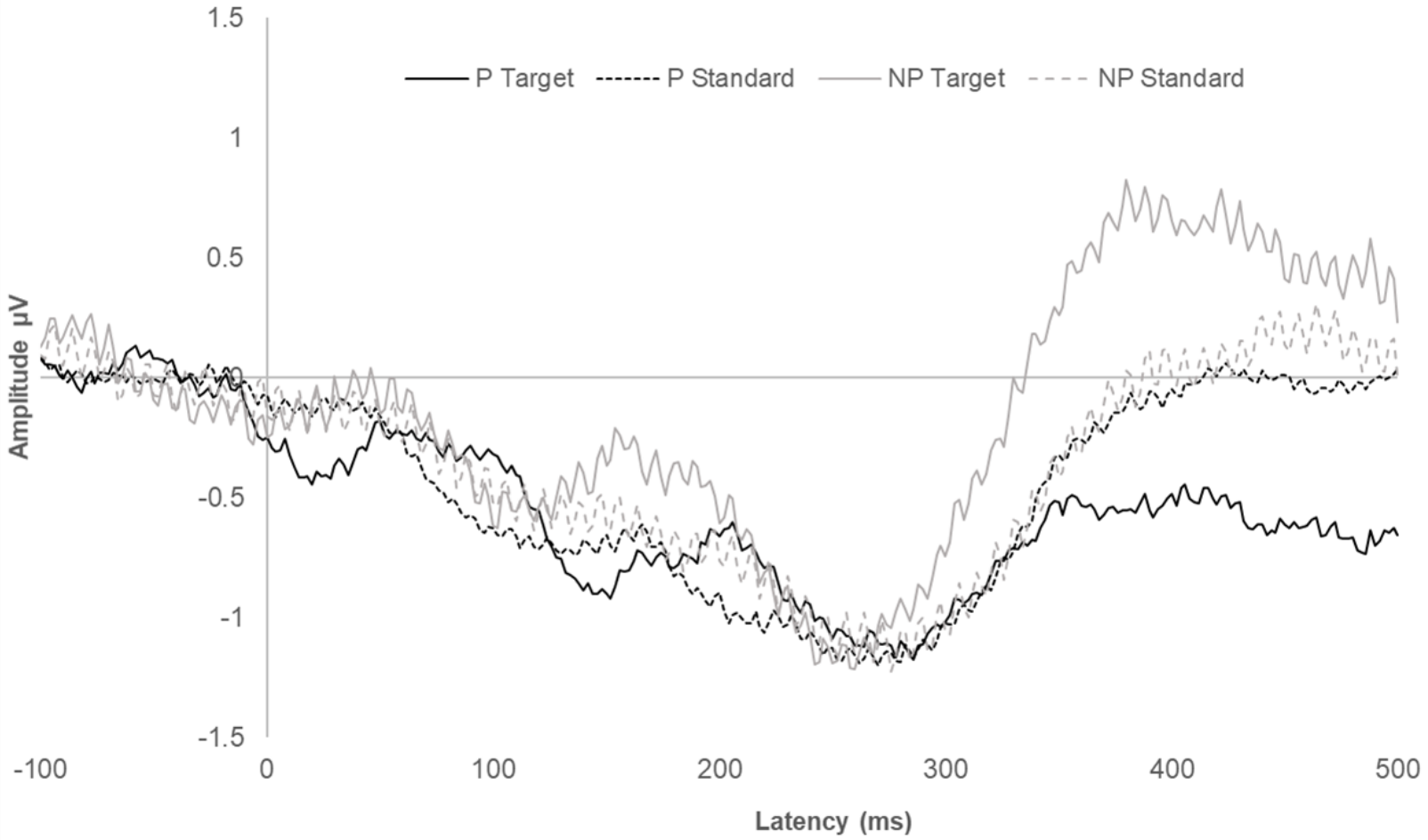

Figure 5. Average ERP waveform for both P and NP rats to the standard and target tone. The P300 (300-450 ms) to the target tone appears to be attenuated in the P rats in comparison to the NP rats.

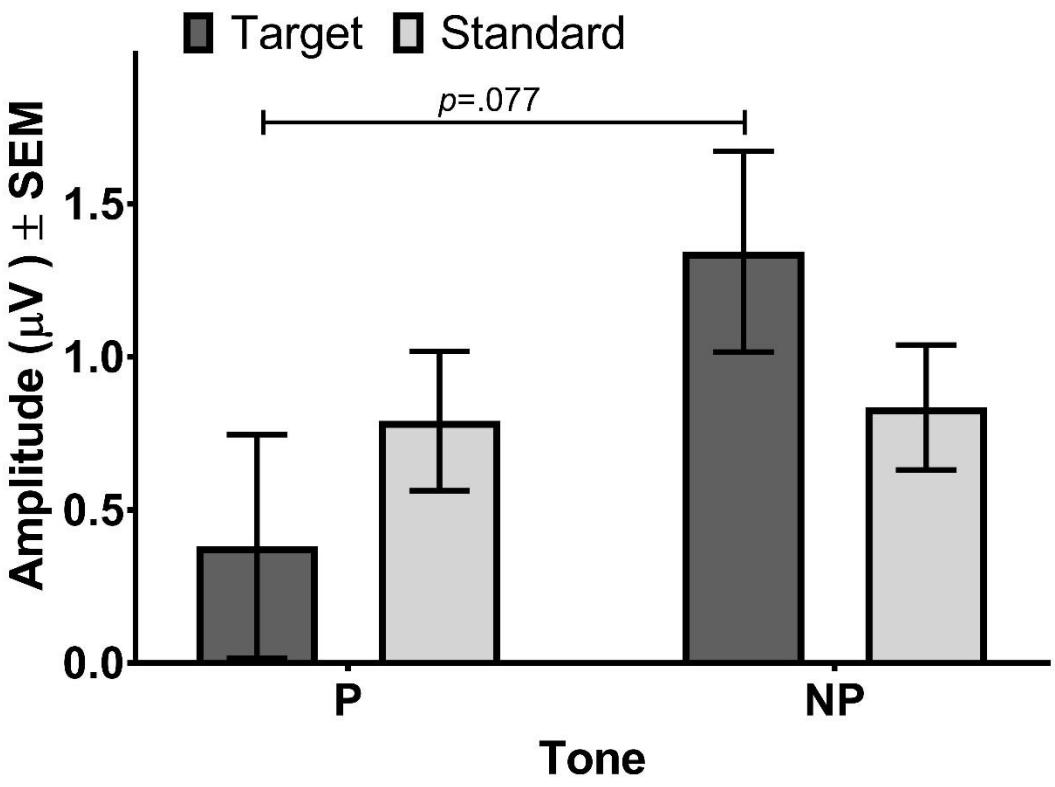

Figure 6. N2-P3 complex amplitude for P and NP rats to the target and standard tones during the oddball task (20T-80S). The difference in amplitude to the target tone between P and NP rats only approached significance (p =.077), but a medium effect size was present (η^2_p=.078). The amplitude of the N2-P3 complex for the target tone was attenuated in the P rats. No significant difference was seen across line for the standard tone (p = .989, η^2_p<.001).

THE UNIVERSITY OF MEMPHIS

IACUC PROTOCOL ACTION FORM

To:	Helen Sable
From	Institutional Animal Care and Use Committee
Subject	Animal Research Protocol
Date	April 14, 2015

The institutional Animal Care and Use Committee (IACUC) has taken the following action concerning your Animal Research Protocol No. **0766** Psychophysiological and Behavioral Measures in P and NP Rats during a Signal Detection Task

☒ Your protocol is approved for the following period:

From: April 14, 2015 To: April 13, 2018

☐ Your protocol is not approved for the following reasons (see attached memo).

☐ Your protocol is renewed without changes for the following period:

From: _____ To: _____

☐ Your protocol is renewed with the changes described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated _____ for the following period:

From: _____ To: _____

☐ Your protocol is not renewed and the animals have been properly disposed of as described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated _____