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CORTICO-STRIATAL COORDINATION DISRUPTED IN BEHAVIORALLY INFLEXIBLE MODERATE PRENATAL ALCOHOL EXPOSED MICE

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BY

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B.S. Biology, University of Denver, 2012

PhD. Biomedical Sciences, University of New Mexico, 2017

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ABSTRACT

Up to 61% of adolescent school aged children with fetal alcohol spectrum disorder (FASD) have been suspended or expelled. Executive function deficits, like dis-inhibition and cognitive inflexibility, have been proposed to be better predictors of behavioral problems then IQ score, which qualifies these individuals for developmental disability and special school programs. Reversal learning, a widely used behavioral paradigm for assessing cognitive flexibility across species, has been shown to be impaired in rodent models of prenatal alcohol exposure (PAE). Here we show that a mouse model with daily maternal drinking, resulting in a BAC of 85 mg/dl throughout gestation, results in maladaptive perseveration, or repetitive incorrect errors, on a visual touch-screen reversal paradigm. Reversal of visual touch screen learning has been shown to be mediated by the orbital frontal cortex (OFC), while associative learning is mediated by the dorsal striatum (DS). However, no studies have addressed the *in vivo* changes in neural signaling that occur after PAE that result in maladaptive perseveration. Pairing PAE with *in vivo* electrophysiology we have shown that spike firing changes in the OFC and DS may explain prolonged perseveration, however the magnitude of change suggests they are not the sole underlying mechanism of impaired reversal learning. Our data suggest that during early reversal, decreases in functional connectivity and over coordination of spikes with low frequency oscillations may be driving perseveration in PAE treated mice. Therefore, future treatments should be targeted to increase coordinated activity during executive function tasks to help correct negative repetitive behaviors in FASD individuals.

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INTRODUCTION

1a. Fetal Alcohol Spectrum Disorder

Fetal alcohol spectrum disorders (FASD) caused by alcohol consumption during pregnancy, are currently the leading source of preventable developmental disabilities despite the surgeon general's warning about the teratogenic effects of alcohol instituted in 1988. In 2009, 30% of pregnant women reported drinking alcohol, and 8.3% of those reported binge drinking (Ethen et al., 2009). While, Fetal alcohol syndrome (FAS), the most severe form of FASD, is easily identifiable by characteristic facial abnormalities including wide set narrow eyes and a smooth philtrum, the majority of FASD cases are not associated with physical malformations leading to difficulty diagnosing patients. In addition, maternal drinking information is often incomplete or unavailable, suggesting that the estimated FASD prevalence rate of 1% of live births is likely a vast underestimation (Sampson et al., 1997).

Despite the lack of outward physical characteristics, individuals with FASDs have a range of cognitive deficits including poor and inappropriate social interactions (Thomas et al., 1998, Kodituwakku, 2007, Greenbaum et al., 2009), antisocial behaviors (Nash et al., 2006), poor attention (Nanson and Hiscock, 1990, Coles et al., 2002), and poor executive functions, including decision-making, working memory, behavioral inhibition and cognitive flexibility (Mattson et al., 1999, Kodituwakku et al., 2001a, Kodituwakku et al., 2001b, Green et al., 2009). There is also evidence that brain malformations may occur even with lack of outward physical changes. Poor visual processing in some children with FASD has been associated with microstructure abnormalities in their

posterior corpus callosum, measurable through diffusion tensor imaging (Wozniak et al., 2006, Lebel et al., 2008, Wozniak et al., 2009).

Regardless of the wide variety of observable behavioral and sometimes structural changes of the brain after prenatal alcohol exposure (PAE), individuals with FASD are often overlooked for developmental disability because of an intelligence quotient (IQ) score that is above 70 points (2 standard deviations below a mean score set by the government as disability cutoff). Unfortunately, without special attention awarded by developmental disability these children do not fare well. In a study done in 2004, only 11-17% of the individuals diagnosed with an FASD actually had an IQ below 70 (Streissguth et al., 2004). However, by the age of 21, 29% of these individuals had drug or alcohol problems and 61.1% had been arrested, with 46% actually being incarcerated (Streissguth et al., 2004). For comparison, the highest national reported average arrest rate for teens by the age of 18 is 35.8% (Brame et al., 2012) and national estimates of illicit drug use is below 10% for teens (Kann, 2014). In addition, these kids have monumental difficulties in classroom settings, as school suspensions, dropouts or expulsion increases from 14% in middle school aged children to 62% in high school children. Clearly, even with the majority of FASD individuals having an IQ above pre-defined disability levels, these children struggle not only with school but also in leading a fulfilling life. While many of the behavioral problems in individuals with FASD contribute to these issues, it has been proposed that deficits in executive functioning are a more reliable predictor of behavioral difficulties in school than IQ (Kodituwakku et al., 2001a, Kodituwakku et al., 2001b). This makes executive functions a useful target for

therapies to widely improve behavior. However, an understanding of the underlying brain activity changes that cause deficits in executive functions after prenatal alcohol exposure (PAE) is required to develop future treatments. Pre-clinical rodent models allow for exact dissection of exposure outcomes and in-depth analysis of neuronal involvement.

1b. Considerations in Use of Rodent Models for FASD

The outcomes from various maternal drinking patterns are difficult to establish in human populations due to unreliable reports or incomplete data, making it unclear if any amount of drinking during pregnancy is safe (Ethen et al., 2009). Therefore, the use of pre-clinical rodent models has been helpful in establishing the outcomes of PAE. A study comparing intoxication levels showed analogous levels of intoxication in both rodents and humans based on blood alcohol level (BAC), allowing for direct comparison of maternal BAC on offspring behaviors between humans and rodents (Driscoll et al., 1990b). However, across the decades of research, difficulty has arisen in determining general outcomes of PAE as amount, timing, and route of administration of ethanol in dams can effect outcomes in pups (Marquardt and Brigman, 2016).

To control for the exact intake of alcohol, intraperitoneal injection (i.p.), intragastric gavage, or vaporized ethanol inhalation are utilized as alcohol administration techniques (for a comprehensive review of administration, see (Patten et al., 2014)). However, these methods can be stressful, due to extremely high levels of alcohol given (BAC 200-300 mg/dl), daily handling, or restraint. Drinking paradigms, like limited access or liquid-diet, are used to minimize handing and replicate human motivations for alcohol consumption.

Liquid diets, in which only an alcohol solution is constantly present, do not result in extremely high BAC (~150 mg/dl) as the methods above, however they are a good model of constant level drinking across the period tested. Limited access models, allow drinking of an alcohol solution for typically 4 hours a day, modeling a more binge like exposure in which alcohol is consumed in a limited period to rapidly increase BAC. However, unless bred for high alcohol consumption (Li et al., 1993, Crabbe et al., 2011), rodents do not consume large bouts of alcohol, which allows for more moderate PAE exposures to model FASD-like conditions. What is considered heavy versus moderate exposure within humans and rodents is still disputed between pre-clinical and clinical divisions. In rodent literature a resulting BAC at the end of a drinking period of less than 100 mg/dl, is considered moderate to mild, as 80 mg/dl is the legal intoxication limit. Exposure paradigms often occur across multiple days, sometimes across the entirety of gestation, and drinking *everyday* to intoxication is not considered mild in the clinical field.

The National Institute on Alcohol Abuse and Alcoholism defines binge drinking as a single bout of consumption that results in a BAC of 80 mg/dL within two hours (NIAAA, 2016), which is roughly 4 drinks for women and 5 drinks for men. Many direct administration methods (ex: i.p. injection, intra-gastric gavage) reach and greatly exceed this criterion. One study showed that even one binge-like injection given on gestational day seven, corresponding to human gestational week three, was sufficient to induce the most severe outcome of in utero alcohol exposure, FAS (Sulik, 1984). This time point may even be before the mother is aware she is pregnant, demonstrating that both the amount and timing of alcohol exposure must be considered carefully.

Preclinical models often aim to replicate common timing patterns of human drinking (Ethen et al., 2009). The first and second human trimester growth equivalents in rodents are gestation days 1-10 and 11-21, respectively, allowing for direct comparison of maternal drinking effect during these points in development. However, the human third trimester equivalent in developmental growth corresponds to postnatal days 0-12 in the rodent (Schneider et al., 2011). Therefore, models aiming to study exposure during the third trimester require direct administration to pups. Depending on the method chosen, this may also add daily stress of pup handing and maternal separation, which must be considered when interpreting results of these studies.

In summary, rodent studies aim to elucidate direct prenatal alcohol effects on offspring behavior. However, considerations of timing, dose and route of administration to mimic human consumption patterns must be weighed against caveats such as daily handling and exceedingly high doses of prenatal alcohol that may not be clinically relevant.

1c. Rodent Models of FASD and Executive Function Deficits

There is significant evidence that rodent models of PAE can recapitulate behavioral deficits seen in FASD individuals including social behaviors, memory and attention deficits (for review see (Marquardt and Brigman, 2016)). However, significantly less pre-clinical work has focused specifically on executive functions.

Reversal learning, a direct measure of cognitive flexibility, is the most widely used paradigm for accessing executive functions in PAE models. These tasks require a subject

to first learn which one of two stimuli leads to a positive outcome, and once the association is well learned, the subject must reverse, and now respond to the other stimulus. Difficulty or an inability to shift behavior results in perseveration, or repeated responses to the previously correct stimuli without reward. Many rodent reversal learning studies utilize a maze-based task (Y-Maze, T-Maze, Morris Water Maze), in which a subject learns location-outcome associations in the initial discrimination, and is required to switch location responding upon reversal.

In the most moderate PAE paradigms, utilizing limited access models throughout gestation, with resulting daily dam BACs of ~80 mg/dl, PAE treated rats and mice were significantly less flexible and performed worse than control mice when altering their behavior upon reversal (Allan et al., 2014, Hamilton et al., 2014). Liquid diet paradigms that also occurred throughout gestation, but had a slightly higher maternal BAC of ~180 mg/dl also resulted in less cognitive flexibility, in which PAE rodents required more trials or committed more perseverative errors before re-obtaining criterion (Riley et al., 1979, Wainwright et al., 1990, Lee and Rabe, 1999). These studies suggest that alcohol exposure throughout gestation might result in a flexibility deficit irrespective of dose. An extremely high level of alcohol (BAC ~372 mg/dl), given during the third trimester equivalent of postnatal days 4-9 also resulted in behaviorally inflexible PAE animals that committed more perseverative errors during reversal than controls (Thomas et al., 2004b). Together, these studies demonstrate that deficits in reversal learning occur after multiple different exposure windows and alcohol concentrations, however, it is not yet

clear if reversal deficits are a universal feature of PAE that occur irrespective of timing and dose.

In further support of PAE altering cognitive flexibility, a set of studies modeling PAE in guinea pigs found similar difficulties in controlling behavior utilizing a different set of cognitive tasks. Exposure to high levels of ethanol (~250 mg/dl) chronically across gestation increased impulsivity on a go/ no-go task by only increasing responses on no-go trials, where behavior is supposed to be omitted (Olmstead et al., 2009). In addition, these animals showed increased repetitive error responses on a cued alternation task, where treated guinea pigs required more trials to alter their behavior after being cued to do so (Olmstead et al., 2009). Although impulsivity and alternation are different measures of cognitive function than reversal learning, these studies suggest that these animals are unable to inhibit responding, particularly after learning a behavior, similarly to increased perseveration seen in reversal deficits.

As ubiquitous as cognitive flexibility deficits may seem after PAE, impairments are not universal across all stimulus modalities. In conditioned eye blink and auditory-based paradigms reversal is not impaired by moderate or high levels of PAE (Mihalick et al., 2001, Brown et al., 2007). The lack of effect in these models could be due to differences in modality processing, or in the case of conditioned eye blink, masked by overall learning deficits. Importantly, the maze-based reversal studies discussed above show specific reversal deficits without significant impairments in initial spatial discrimination learning. While many studies have shown that more moderate PAE does not impair

spatial learning until the task increases in complexity (Popovic et al., 2006, Hamilton et al., 2010a, Cullen et al., 2014), it must be noted that some studies have shown impaired spatial learning and retention after high and moderate doses of PAE (Thomas et al., 2004a, Dursun et al., 2006, Savage et al., 2010, An and Zhang, 2013). Since reversal deficits are well established in maze-based paradigms, the next step is to determine the underlying neural processing that may contribute to inflexible behavior. However the exact timing of behaviors can be difficult in maze-based paradigms, making it difficult to associate neural activity and behavior. Therefore, the use of operant reversal tasks, that allow for precise timing of behaviors and minimal experimenter involvement, should be studied in models of PAE.

In humans, reversal learning is measured through visual computer tasks, such as the Cambridge Neuropsychological Test Automated Battery (Green et al., 2009). Based on the human version, a rodent visual touch-screen discrimination reversal task has been utilized to determine the effects of chronic intermittent alcohol in adult mice (DePoy et al., 2013), and elucidate contributions of neuronal receptor types to reversal learning (Brigman et al., 2008, Brigman et al., 2013). However, no study has tested visual reversal learning in a rodent model of PAE. Operant visual paradigms have the distinct advantage of recording exact timing of behaviors, such as stimulus touch response or reward retrieval, which combined with *in vivo* electrophysiology allows for powerful combined analysis of a behavior and the neural activity. It will be essential to the field of PAE going forward to replicate reversal deficits in a translatable visual paradigm to confirm

reversal specific deficits and to study neuronal signaling changes after PAE that may promote cognitive inflexibility.

1d. Cortico-Striatal Circuitry in Reversal Learning

The rodent prefrontal cortex (PFC) is indisputably less complex compared to humans' and non-human primates'; however, detailed studies of connectivity and function of the frontal cortex show that rodents have frontal regions that serve analogous functions (Uylings et al., 2003, Wise, 2008). The rodent PFC is commonly separated into three distinct regions, delineated by connectivity and function: medial prefrontal cortex (mPFC), medial orbital frontal cortex (mOFC) and the lateral orbital frontal cortex (lOFC).

The mPFC, thought to be analogous to the human dorsolateral PFC, is implicated in attention, conflict processing and error detection (Bissonette et al., 2013). The mPFC is further subdivided into the anterior cingulate (ACC), prelimbic (pl) and infralimic (il) regions. Lesion of the plPFC or ilPFC, across species, results in impairments of set-shifting but not reversal (Dias et al., 1996, Birrell and Brown, 2000, Ng et al., 2007). Set-shifting is a hierarchically more complex cognitive flexibility task, which requires subjects to shift behavior based on abstract rules instead of switching between dichotomous behaviors as in reversal learning. Each sub-division of the mPFC has been shown to mediate a unique part of set-shifting behavior. ACC is important on focusing attention and in generalizing reward rules, for example, when utilizing the same reward rule on a novel set of stimuli (intra-dimensional shift) (Pardo et al., 1990, Seamans et al.,

1995, Bussey et al., 1997, Ng et al., 2007, Bissonette et al., 2013). Lesion of either the plPFC or ilPFC results in deficits in switching behavior once rule contingencies have changed. However, studies suggest that the plPFC is important in representing the current rule, while the ilPFC is required for initiation of a novel behavioral pattern (Bussey et al., 1997, Rich and Shapiro, 2009, Oualian and Gisquet-Verrier, 2010). Lesion of any sub-region of the mPFC does not impair reversal learning (Bissonette et al., 2008), therefore, while it is an important region in flexibly altering rule states and error signaling, there must be another prefrontal region that subserves reversal learning.

The medial and lateral OFC regions are reciprocally connected, but receive inputs from different regions (Hoover and Vertes, 2011, Zingg et al., 2014) indicating they serve distinct functions. Like the lOFC, the mOFC is thought to encode stimulus value associations (Rushworth et al., 2011). Particularly, the mOFC is postulated to be involved in encoding of value when information about outcome is partially uncertain, like during probabilistic tasks, and communicates this information to the mPFC (Rushworth et al., 2011, Bradfield et al., 2015, Gourley et al., 2016). Despite seemingly similar functions, selective lesion or inactivation of the lOFC impairs reversal learning while mOFC inactivation does not (Bissonette et al., 2008, Mar et al., 2011, Brigman et al., 2013, Rudebeck and Murray, 2014).

Many studies have focused on the neuronal action of the OFC *in vivo* during paradigms that require stimulus value outcome associations, and flexible shifting of these associations. lOFC neurons increase firing rate after a stimulus is chosen and the reward

is expected (Thorpe et al., 1983, Simmons et al., 2006, Riceberg and Shapiro, 2012, Moorman and Aston-Jones, 2014, Amodeo et al., 2016, Rich and Wallis, 2016). Specifically, lOFC expected value firing is greatest for the stimulus that has the largest subjective value associated with it (Cai and Padoa-Schioppa, 2012). The lOFC requires repetition to form stimulus-outcome associations as rats with lOFC lesions are not impaired on reversal when contingencies are changed rapidly (Riceberg and Shapiro, 2012). However, it is noteworthy that a small percentage of neurons encode the lesser or non- valuable option (Rich and Wallis, 2016) and that the lOFC also increases firing rate to infrequent incorrect trials (Nobre et al., 1999). When reward contingencies are reversed, lOFC neurons loose responsiveness to the initially reward stimulus, and switch response to the newly rewarded stimulus (Thorpe et al., 1983, Schoenbaum et al., 1999, Schoenbaum et al., 2003, Riceberg and Shapiro, 2012). This holds true in a probabilistic reversal task as well, when stimuli were associated with a probability of reward instead of a fixed value (Amodeo et al., 2016). In summary, current literature suggests that the lOFC functions as a current stimulus-value outcome cognitive map that is updated when expected contingencies change.

The lOFC is a unique PFC region in that it is heavily innervated by primary visual and auditory sensory cortices and secondary posterior parietal association cortices (Zingg et al., 2014). In addition, the lOFC projects to multiple regions of the striatum, a structure that is important for both action and reward processing (**Figure 1**) (Voorn et al., 2004, Schilman et al., 2008, Pan et al., 2010, Hoover and Vertes, 2011, Liljeholm and O'Doherty, 2012). These connectivity patterns mean the lOFC is uniquely situated to

process individual stimulus-value associations and inform actions based on that value. However, selective lesion or inactivation of the lOFC does not impair rule or stimulus-outcome learning, which means another region must be involved in association learning (Bissonette et al., 2013). In humans, cortico-striatal functional connectivity has been associated with flexible control over behavior and that individual differences in connectivity may affect intrinsic variability between subject task performance (Forstmann et al., 2010). Furthermore, in a visual touch-screen discrimination reversal learning paradigm, the lOFC and dorsolateral striatum (DLS) were differentially active during reversal and learning, respectively, indicated by early gene *c-fos* activation (Brigman et al., 2013). Evidence suggests that reversal learning is therefore mediated through a lOFC-striatal circuit.

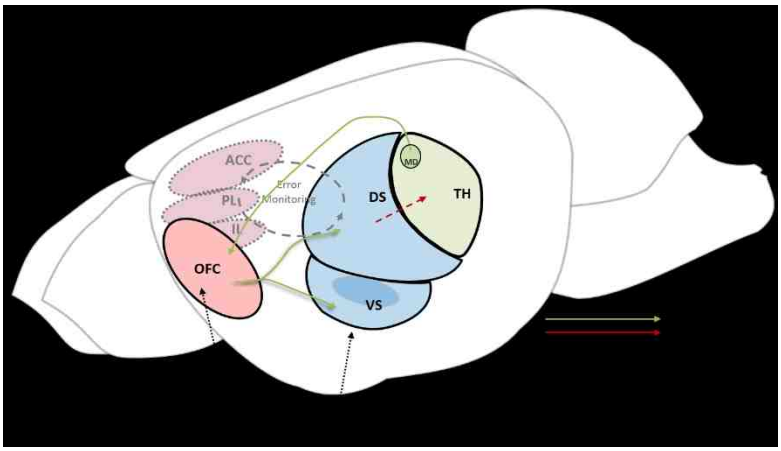

Figure 1. Reversal Cortico-Striatal Circuitry. The orbital frontal cortex (OFC) projects directly to both ventral (VS) and dorsal striatum (DS) in a medial-lateral and anterior-posterior fashion, meaning the most dorsolateral OFC projects to the dorsolateral striatum. Connectivity back to the OFC is through the medial dorsal nucleus (MD) of the thalamus (TH). While not expanded here the medial prefrontal cortex (anterior cingulate cortex (ACC), prelimbic (pl) and infralimbic (il)) also project to the striatum as an error monitoring system.

The striatum is a well-studied region that has been shown across species to be involved in associative and instrumental learning and is optimally positioned to encode action

outcomes and receive value-informative input from the OFC (Liljeholm and O'Doherty, 2012, Calabresi et al., 2014). The striatum is roughly divided into three regions: ventral striatum (VS), dorsomedial striatum (DMS) and the dorsolateral striatum (DLS) (Voorn et al., 2004). Unlike the prefrontal cortex, the delineations between the striatal regions are not well demarcated by connectivity or function. However, it is largely accepted that the VS is heavily involved in motivational performance and Pavlovian learning, while the DS is involved in instrumental learning (Liljeholm and O'Doherty, 2012). The DS is innervated not only by the prefrontal cortex, but also the dopamine system; therefore, with its outputs to the thalamus and motor pathways, the DS is a key component in integrating cortical and motivational information for action selection and initiation during learning (Voorn et al., 2004, Balleine et al., 2007). Two types of action selection: goal directed and habitual, are mediated by separate areas in the DS. Goal directed behavior is distinct from habitual responding due to its sensitivity to devaluation, when a subject will stop responding when the reward is no longer valued due to satiation. The DMS mediates goal-directed behavior as lesion or inactivation results in inflexibility, while lesion of DLS promotes goal directed behavior showing it is involved in habit formation (Yin et al., 2004, 2005, 2006, Clarke et al., 2008, Gremel and Costa, 2013). During a motor learning paradigm *in vivo* electrophysiology shows DMS neurons are more active during early, goal-oriented learning, and DLS neurons respond only once the behavior is well learned (Yin et al., 2009). While this suggests that DS sub-region activation is sequential across learning from goal-directed to habit formation, dual region recordings during a lever press operant task suggest that DMS and DLS activity is actually parallel. Neurons in both regions increase firing rate to both goal and habitual reward contexts at lever

press, however, neurons respond in a gradient to each condition (Gremel and Costa, 2013). Sequential recordings across learning stages show that DLS activity begins low and grows more stable as contingencies are learned, but DMS activity waxes and wanes as novel contingencies are learned. This suggests that while both regions function in parallel the DMS may modulate the DLS's ability to influence action when a contingency is not well established, thus promoting goal directed behavior (Thorn et al., 2010).

Alcohol consumption is thought to progress to a habitual, often detrimental, behavior due to a shift from DMS and VS motivated to DLS habitual mediated behavior. In humans, heavy drinkers show more blood-oxygen-level dependent (BOLD) activity in the DS when shown an alcohol related cue, which correlated with craving, while social drinkers showed higher activation in the VS and less cravings (Vollstädt-Klein et al., 2010). This suggests that alcohol consumption has become a habit in heavy drinkers, thus activating the DS preferentially over the VS. To further support alcohol use transitioning to habit, during alcohol self-administration in rodents, inactivation of the DMS attenuates alcohol administration, but only during early sessions. Once self-administration is well learned, inactivation of the DMS is ineffective while DLS inactivation returns alcohol self-administration to a goal oriented behavior and sensitive to devaluation (Corbit et al., 2012). Similarly, DMS region activation by agonism of the adenosine A_2A receptor was able to promote goal-directed drinking patterns over habitual (Nam et al., 2013).

During acute alcohol self-administration on either fixed interval (goal) or variable interval (habit) reinforcement schedules, both DMS and DLS neurons were active

(Fanelli et al., 2013) supporting that regions perform in parallel. However, DMS activity was time-locked to reinforcement, while DLS activity was at time of lever press, and magnitude of response in both regions was modulated by the reinforcement schedule, showing that within parallel activity each sub-region encoded different information based on regional function (Fanelli et al., 2013). In a chronic intermittent ethanol paradigm, which has been shown to produce alcohol dependence (Griffin et al., 2009), the DLS in mice was primed for habit learning, with increased rates of learning and faster cognitive shifts on a visual touch-screen discrimination reversal paradigm (DePoy et al., 2013). The DLS had greater dendritic complexity and larger neuronal response after a correct choice, which may indicate that the DLS exerted greater control over learning than the goal-directed DMS, facilitating the development of habits after alcohol dependence was formed (DePoy et al., 2013). While not yet tested, it may be that prolonged alcohol exposure, either in adults or prenatally, leads to an increase in drive of the DLS, promoting repetitive behaviors and habits.

In relation to PAE, considerably less work has investigated the DS, OFC or the striatal-prefrontal connectivity. Positron emission topography in non-human primates, shows that exposure to PAE can affect the dopamine, D2, receptor concentration in the striatum (Schneider et al., 2005). This suggests that PAE may affect striatal function and behavior due to the high concentration of D2 receptors in the striatum (Calabresi et al., 2014). Like PAE, both acute and prenatal cocaine exposures impair reversal learning (Goto and Grace, 2005, Calu et al., 2007). In a rat model of cocaine dependence, anesthetized evoked local field potential synchrony between the OFC and VS decreased during

intravenous cocaine exposure, which implicates OFC to striatal communication disruptions may be a factor underlying disrupted reversal after extended cocaine exposure (McCracken and Grace, 2013). However, no studies have investigated the neural coordination or activity in the OFC or DS after PAE during a behavioral flexibility task leaving a large gap in the literature about underlying functional changes that could be addressed in behavioral therapies.

1e. Contributions of Local Field Potentials to Neural Processing

While most of the accompanying literature to the function of the lOFC and DS in reversal learning in rodents is spike-firing data, which looks at individual or small populations of neurons, most clinical work focuses on less invasive methods by measuring neuronal electrical activity at the scalp by electroencephalography (EEG). An EEG is the summation of all the oscillatory electrical currents summed at the scalp at each recording electrode, with respect to a reference potential (Buzsaki et al., 2012). Oscillatory activity captured at-depth, within a region, typically completed in rodents, due the technical difficulties of scalp or skull level EEG on a diminutive subject, is called the local field potential (LFP).

The amplitude and timing (phase) of the oscillatory waves are used to determine the power and coordination of the changes in ion current from contributing areas. Action potentials are the largest contributing factor to oscillations, due to the large flow of ion currents across the membrane. However the cytoarchitecture of the brain creates cancelation and summation of ion changes making the determination of source and

intensity of neuronal activity that create power changes extremely challenging, even within LFP data collected in rodents (Buzsaki et al., 2012, Herreras, 2016). However, increases in theta (4-8 Hz) power changes have been linked to cognitive control functions in the mPFC of humans and rats (Narayanan et al., 2013, Cavanagh and Shackman, 2015). Interestingly, a study in rodents failed to show significant power changes in the OFC across learning in a T-maze paradigm (Young and Shapiro, 2011), suggesting power contributions are an intrinsic factor to the behavior and do not change significantly across a learning paradigm. Furthermore, one study has linked human resting state EEG stability overtime to the gene locus for the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) receptor A, further suggesting power is an intrinsic stable pattern across time (Porjesz et al., 2002). Together these studies suggest that while power may give an overall pattern of ion current of a region during a task, it may be a set pattern that is not dynamic across learning.

The phase of the oscillation, or timing of the wave is highly dynamic. If the phase of the oscillatory signal is consistent across occurrences of a behavior, meaning the oscillation is reset every time a behavior occurs, it suggests the underlying current changes contribute to that behavior. In addition to increased power, theta phase becomes coherent after an error in the mPFC (Cavanagh et al., 2009, Narayanan et al., 2013), reinforcing a role of theta oscillations in behavioral control. The timing of the oscillation can also align to timing of spike firing, which maximizes or minimizes neural excitability dependent upon timing compared to peak or trough of the oscillation (Anastassiou et al., 2011, Cohen, 2014b). In the primary visual cortex, spike-field coupling to the gamma (>30 Hz)

frequency amplifies signals from targets and decreases coherence to distractors, resulting in increased attention to target stimuli (Fries et al., 2001, Haider et al., 2016). Interestingly, loss of spike-field coordination to auditory stimuli is associated with greater psychotic symptoms in schizophrenic patients (Calderone et al., 2014), which may be interpreted as disordered brain function. Similarly the theory behind trans-cranial direct current stimulation (tDCS) is to synchronize neuron response with the oscillations by manually setting the electrical currents, which would allow for optimal response and learning (Calderone et al., 2014). In support of this theory, tDCS activation over the prefrontal cortex increases performance in healthy controls in the Stroop and Iowa Gambling executive function tasks that require cognitive control (Ouellet et al., 2015). Furthermore, regional coherence can be compared across electrodes or region as a measure of functional connectivity. If two regions' oscillations are synchronous, information from one region will have a larger impact on the downstream region. Together spike-field coupling and inter-region synchrony are thought to be mechanisms of information transfer between regions (Womelsdorf et al., 2007). Spike-field coupling between regions has been shown to be a modulator of visual attention and a mechanism for transfer of information to downstream regions including the motor cortex for stimulus-motor integration (Womelsdorf and Fries, 2006, Gregoriou et al., 2009b). Together oscillatory phase based measures may help bridge the gap between individual neuronal firing and brain-wide coordinated activity patterns that are required to learn and change behaviors.

During cognitive tasks multiple frequencies have been associated with distinctive task behaviors, allowing for coordination of multiple neural functions and complex behavior during learning and reversal in the OFC. Increases in gamma (>30 Hz) are seen in response to odor stimulus sampling during a go/ no-go task, regardless of associated reward, while theta power increased only during reward anticipation after correct stimulus sampling (van Wingerden et al., 2010a, van Wingerden et al., 2010b). Interestingly, gamma frequency oscillations became entrained with a population of neurons in the OFC that became suppressed during reward approach after odor sampling (Pennartz et al., 2011). However, neuron firing was positively coupled with theta oscillations during reward approach, and similarly to spike-firing, spike-field coupling in the theta frequency range followed stimulus contingency changes during reversal, collapsing upon initial reversal but re-establishing during re-learning (van Wingerden et al., 2010a, Pennartz et al., 2011). Interestingly, blockade of N-methyl-D-aspartate receptors (NMDAR) in the OFC creates hyper-synchrony between spikes and the theta, beta (10-30 Hz) and high frequency bands (>30 Hz), which destroys the differential signaling between stimuli values and impairs ability to shift behavior during reversal (van Wingerden et al., 2012). Dual encoding of two types of signals that separately carry information about the stimulus and value allows for complex encoding of all stimuli and outcome associations that may be used for action selection in downstream regions. Impairment of one or multiple streams of information to downstream regions may be an underlying cause of reversal impairments that should be further investigated.

LFP phase coherence and coordination with neuron firing suggest that oscillations time neural response to behaviors and coordinate inter-region activity to facilitate learning. In the striatum increases in theta phase coherence are associated with voluntary movement in a T-maze paradigm, which may indicate a necessary signal to initiate an action (DeCoteau et al., 2007b). In a similar T-maze paradigm DMS neurons were entrained to a higher ~10 Hz frequency, compared to ~5 Hz neuron entrainment in the DLS (Thorn and Graybiel, 2014). Importantly, the differential spike-field coupling only became apparent after a behavioral response pattern was learned. Separation of coding information between striatal regions may be important for simultaneously coordinating information between the regions, while also allowing for independent function. This separation in striatal region coordination may become important when considering inter-region communication throughout learning. During learning of a radial arm maze task VS neurons became entrained to a hippocampal theta frequency, while DLS neurons became entrained to alpha (8-13 Hz) oscillations (Berke et al., 2004). Further separation between neuron type and function was distinguished by relative timing to the phase, as fast spiking interneurons (FSI) in the striatum have been shown to be entrained to an earlier hippocampal phase than medium spiny neurons (MSN) (Berke et al., 2004). It is hypothesized that the earlier FSI coordination allows for amplified MSN activity. Synchrony between the striatum and dorsal CA1 hippocampus increased in the theta frequency range during the choice period in a T-maze task, suggesting that the striatum and hippocampus increased communication as a spatial based memory was formed (DeCoteau et al., 2007a). Notably, there is evidence that disease states can alter cortico-striatal coordination and disruptions in communication between regions can result in

impaired behavior. Evoked synchrony between the OFC and downstream VS is disrupted in anesthetized cocaine dependent animals after four weeks of abstinence, a time period correlating with impairments in reversal learning (McCracken and Grace, 2013). Together, these studies suggest that coordination between prefrontal cortical areas and the striatum in unique frequency bands may be a useful tool for distinguishing which striatal circuits are utilized in downstream control of action selection during tasks utilizing cognitive control.

1f. Effects of Alcohol on Local Field Potentials

Acute alcohol intoxication in humans at the legal intoxication limit (80 mg/dL) has been shown to increase alpha oscillations in resting state EEG, which is thought to represent an "idling" brain (Schwarz et al., 1981, Ehlers et al., 1989, Cohen et al., 1993, Nikulin et al., 2005). During cognitive tasks, alcohol ingestion decreases theta frequency power response during tasks requiring inhibitory control, resulting in decreased ability to perform executive functions (Kovacevic et al., 2012, Rosen et al., 2016). Interestingly, alcohol may also interfere with cortical functions by interfering with visual stimuli processing, as peak gamma frequency response to stimuli while intoxicated is lower than in non-alcohol conditions (Campbell et al., 2014). Little work has been done after chronic alcohol consumption; however, decreased theta power during cognitive tasks is a persistent feature in 30 day abstinence individuals (Kamarajan et al., 2004).

Magnetoencephalography (MEG) studies, which have decreased temporal resolution compared to EEG but increased spatial resolution, suggest that adolescents with FASD

have decreased gamma power responses in frontal regions during prosaccade visual tasks, and further suggest they have differences in cortical network function (Stephen et al., 2013). EEG studies in individuals with FAS also show alterations in oscillatory power, however it is unclear if these changes affect cognition. In FAS neonates of alcoholic mothers, EEG power is significantly increased across multiple low frequency oscillations (.1-25 Hz) during the sleep cycle (Havlicek et al., 1977, Chernick, 1982). In two cases of FAS in adolescents, awake resting state EEG recordings again showed excessive power, but only in beta oscillations; however other frequencies were sparse or missing across regions (Mattson et al., 1992). While it is unclear if EEG power differences are maintained across the lifespan of individuals with less severe FASD, studies in individuals with at least one parent with an alcohol abuse disorder have shown altered EEG response during cognitive tasks, suggesting that alterations in EEG may be a permanent effect of in utero alcohol exposure and impair cognitive processing. In both go/ no-go and odd ball tasks, which require attention and inhibition, adolescents with at least one alcohol abusing parent show decreased low frequency delta and theta power after response in the frontal cortex (Kamarajan et al., 2006, Rangaswamy et al., 2007). However, decreased power responses did not necessarily predict impaired behavior. Deficient EEG theta response becomes more pronounced during more difficult versions of the task, in which cognitive control was impaired, suggesting that although there are EEG differences at all difficulty levels, the more cognitive demand required, the less adolescents' with alcohol abusive parents can compensate (Kamarajan et al., 2006). This is reminiscent of animal behavior in which deficiencies are more readily seen in more

difficult tasks (Savage et al., 2002, Brady et al., 2012) and suggests the underlying neuronal changes are present even during non-cognitive taxing paradigms.

Part of these cognitive difficulties may stem from impaired visual attentional processing in these individuals, evidenced by decreased P300 amplitude in the event related potential (ERP), an averaged EEG signal, during visual attentional tasks (Kamarajan et al., 2006, Rangaswamy et al., 2007). The P300 is a positive increase in voltage that occurs 300 to 700 ms after the onset of a noteworthy visual stimulus that is thought to represent late cognitive processing, and therefore attentional load allocated to the task (Rangaswamy et al., 2007, Burden et al., 2009). Decreases in amplitude of P300 are thought to indicate a decrease in the ability of the individual to filter out irrelevant information (Klimesch et al., 2000). Other studies have shown that the ERP in FAS individuals indicates difficulties in early visual processing and perceptual discrimination during response inhibition (Kaneko et al., 1996, Burden et al., 2009, Burden et al., 2011), but these differences in ERP latency and amplitude may not be detectable after more moderate PAE in FASD (Burden et al., 2010). However, deficits in auditory and visual responses are supported by MEG studies in FASD children and adolescents who showed delayed stimulus processing (Stephen et al., 2012, Coffman et al., 2013). It is clear, from data with adolescents with alcoholic abusive parents and MEG studies of FASD individuals that underlying oscillatory activity is changed and needs to be correlated with PAE exposure to more fully understand the outcomes.

A solitary study was completed in PAE mice injected with a single postnatal day 7 alcohol bolus that tested anesthetized network function of the olfactory bulb, piriform cortex and dorsal hippocampal circuit at three months of age. There was a significant amount of brain wide neurodegeneration after PAE, and although object place memory was impaired, odor investigation was unimpaired. Not only were there increases in theta power responses to previously trained odors in all three regions, but there was also an increase in synchrony between each step in the circuit (Wilson et al., 2011). While overarching conclusions cannot be drawn from a single study, in conjunction with human EEG data from adolescents of alcohol abusive parents, it does suggest that prenatal exposure to alcohol affects brain wide oscillations within and between regions, which may be a contributing factor to cognitive impairments. However, further research is needed to identify within task differences caused by PAE that may ultimately affect behavioral outcomes.

1g. Hypothesis and Further Rationale for Current Study

Several decades of research has been put into uncovering the behavioral and cognitive outcomes of PAE, however, many questions still remain, particularly in uncovering consistent behavioral and neural activity changes after more moderate alcohol exposures. As individuals with FASD struggle greatly with maintaining behavioral norms and have great difficulty altering behaviors, which often lands them in trouble with the law or results in school expulsion, it is essential to understand how PAE changes brain activity to cause these deficits for future development of interventional therapies. Pre-clinical rodent models of PAE have shown a consistent deficit in reversal learning in maze-tasks

across both high and low dose models. However, research has fallen short in integrating behavioral paradigms and *in vivo* electrophysiology techniques to elucidate how brain activity and coordination changes during reversal and after PAE. The current project addresses these gaps in the PAE literature and proposes to further elucidate how both single unit and oscillatory activity plays a role in reversal learning.

The following study utilizes an established model of moderate PAE in which pregnant dams have limited voluntary access to an alcohol solution for four hours a day,

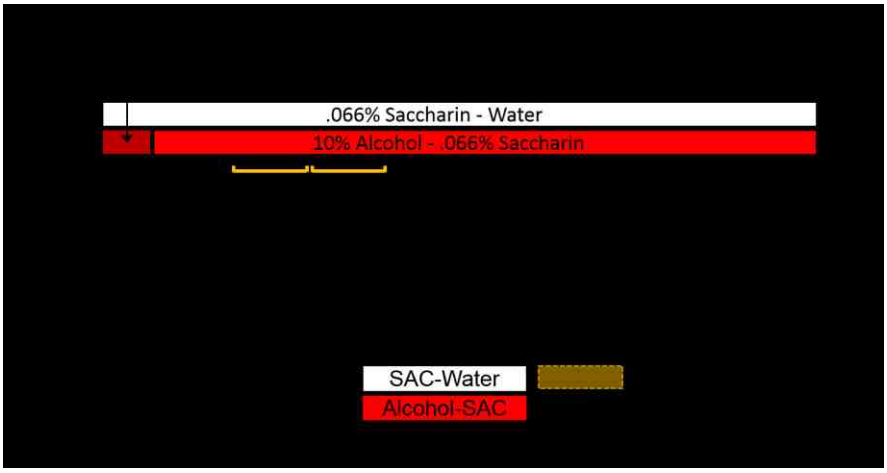

Figure 2. Limited Access Prenatal Alcohol Exposure Model. Dams had daily limited access to either saccharin (SAC)–water or alcohol–SAC solution throughout gestation. Alcohol drinking is established with four days of 5% alcohol-.066% saccharin solution, and at least one week of drinking a 10% alcohol- .066% saccharin solution prior to breeding rounds. Alcohol consumption continued through five-day breeding rounds, with two hour breeding periods after a drinking session. Upon pup birth, dams were weaned off of solutions, by halving the concentration every other day, over six days to prevent withdrawal effects that may affect maternal care. This model reliably produces blood alcohol concentrations of 80-90 mg/dl (Brady et al., 2012).

throughout gestation (**Figure 2**) (Brady et al., 2012, Brady et al., 2013). This model is equivalent to exposure during the first and second trimester in humans, which is one of the top ten drinking patterns reported by pregnant women (Ethen et al., 2009). The voluntary limited access paradigm results in bouts of drinking across the four hour period that end in ~80 mg/dL (Brady et al., 2012). While this may not be considered moderate in

the clinical literature because it occurs daily, it is minimal exposure compared to other pre-clinical paradigms that utilize liquid diets or direct alcohol administration as discussed above, and thus makes an ideal model for determining exposure of more clinically relevant PAE doses on behavioral outcomes.

Combining moderate PAE with *in vivo* electrophysiology techniques I hypothesize that **moderate prenatal alcohol exposure is sufficient to impair cognitive flexibility in adulthood by altering cortical-striatal function and connectivity.** The following three proposed studies will elucidate the effects of moderate PAE on visual touch screen reversal learning, define optimal lOFC and DS neuron and LFP function and coordination during visual reversal learning and finally, analyze changes that occur in the OFC-DLS circuit in adult mice after moderate PAE.

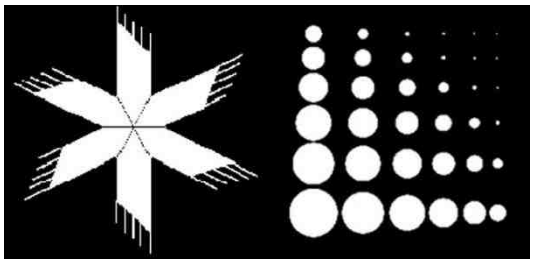

Figure 3. Discrimination Reversal Visual Stimuli. FAN (*left*) and MARB (*right*) are equiluminescent visual touch-screen stimuli that are used for all subjects. Initial discrimination S+ is counterbalanced across subjects in all studies. Stimuli have been previously used in a visual touch-screen discrimination reversal paradigm (Brigman et al., 2008).

Study 1. To test the hypothesis that moderate PAE is sufficient to selectively impair reversal learning in an operant visual touch-screen discrimination reversal task.

Our PAE model has been shown to impair both spatial and hippocampal based fear learning (Brady et al., 2012, Caldwell et al., 2013). To minimize the impact of potential spatial learning deficits in our model of PAE, we utilized a previously established visual paradigm in which spatial location of stimulus was never informative of reward (Brigman

et al., 2008, Brigman et al., 2010b, Brigman et al., 2013, Brigman et al., 2015). Furthermore, the use of this operant touch-screen discrimination reversal task will allow for discrete timing of behavioral events, like touch-response, for use in future neural activity studies. In this paradigm, two visual stimuli (**Figure 3**) are presented side-by-side on a touch-screen, wherein nose-poke to the screen will result in either reward or time-out period based on stimulus reward contingencies. Furthermore, this paradigm is similar to those used in clinical settings in human patients (Green et al., 2009) and extends PAE research beyond more rodent-based tasks to determine if reversal deficits are universal across modalities. I hypothesize that our moderate PAE model is sufficient to impair reversal on a visual touch-screen task that mirrors paradigms used in the clinical setting.

Study 2: To test the hypothesis that during visual reversal learning in the mouse lOFC neurons encode expected value of choice responses and neuron coordination with the LFP may be a method of value signal propagation to downstream regions to promote or discontinue choice behaviors.

Combining our visual touch-screen operant task with *in vivo* electrophysiology allows for linking millisecond timing of behaviors and operant cues to neural activity. Previous research shows lOFC neurons increasing firing rate in response to expected positive reward outcomes, however work was completed in either non-human primates in visual tasks or rodents in maze-based or lever press tasks. I hypothesize that the lOFC in the rodent also processes visual stimuli reward expectations due to the innervation from the visual cortex (Zingg et al., 2014). However, how these value signals are coordinated with simultaneous action selection in the striatum is unclear. Network synchronization by

oscillations and coordination of neuron spike timing to these oscillations are a proposed mechanism of directed communication between regions to direct attention and initiate behaviors (Pennartz et al., 2011, van Wingerden et al., 2012, Thorn and Graybiel, 2014). I hypothesize that lOFC neuron response to rewarded and unrewarded stimuli will be differentially coordinated with the oscillations, and change across reversal sessions in a manner that correlates with behavior promotion or cessation.

Study 3: To test the hypothesis that PAE impairs lOFC activity and decreases functional connectivity to the DLS during reversal, resulting in over activation of the DLS, driving maladaptive perseveration.

Cortico-striatal loops containing the lOFC and DLS are required for optimal performance in discrimination reversal learning. However dynamics across learning and reversal within each region and the synchronous activity between regions during the switch from automatized to goal directed behavior has not been directly studied in a PAE model. I propose using *in vivo* electrophysiology recording with single region bilateral, and dual ipsilateral region electrodes to elucidate the local field phase-based dynamics within and between regions in our task during early reversal to examine how PAE alters coordinated brain function resulting in impaired cognitive flexibility. Chronic intermittent alcohol exposure increases dendritic complexity in the DLS and improves reversal performance by increasing the DLS drive of habit learning (DePoy et al., 2013). Since alcohol exposure has been shown to increase DLS drive, but PAE impairs reversal, I hypothesize that PAE increases the influence of the DLS, but at a detriment to reversal due to concomitant impairments in lOFC neuron activity resulting in maladaptive perseveration.

Cocaine exposure, which also impairs reversal learning, may do so through the disruption of synchrony between the OFC and downstream VS and BLA regions (McCracken and Grace, 2013). Therefore, I hypothesize that functional connectivity between the lOFC and DLS will be disrupted by PAE, particularly during early reversal when deficits are seen in behavior. Collectively, this study aims to add to the knowledge of cortico-striatal loop function, as well as identify important neuronal and oscillatory activity changes after PAE.

From the proposed studies I aim to determine effects of moderate PAE on a visual reversal paradigm to confirm that impairments in cognitive flexibility are a common outcome across exposure paradigms. Furthermore, I hope to advance the field by clearly defining how lOFC and DLS intra- and inter-region activity and coordination are affected by PAE during reversal learning. Defining disruptions in organized neural activity will provide critical information for the development of interventional therapies for individuals with FASD to help ameliorate executive function deficits and improve long-term outcomes.

CHAPTER 2

Prenatal Ethanol Exposure Impairs Executive Function in Mice into Adulthood

Marquardt, K., Sigdel, R., Caldwell, K., and Brigman, J.L. (2014). Prenatal ethanol exposure impairs executive function in mice into adulthood. ACER 38(12), 2962-2968.

2a. INTRODUCTION

It has been over 30 years since the Surgeon General of the United States advised that women who were, or were considering becoming, pregnant abstain from alcohol. Despite a wealth of evidence that consumption of alcohol during pregnancy can have profound and wide-ranging consequences on offspring, rates of drinking during pregnancy remain high. Recent reports suggest that as many as one third of all women drink at some time during pregnancy and between 5-10% report binge drinking behavior (Ethen et al., 2009). This disconnect may be partially explained by conflicting reports regarding the safety of consuming alcohol at low levels, both from the research literature and primary care providers.

There is strong evidence that high levels of prenatal alcohol exposure (PAE) during pregnancy have negative consequences on the physical and cognitive development of offspring (Streissguth et al., 1991). Studies in clinical populations have found that high dose PAE is associated with a wide-range of symptoms that include impaired growth, deficits in cognitive function and executive control (Mattson et al., 1999, Day et al., 2002, Olesen et al., 2004, Willford et al., 2006, Green et al., 2009) and increased behavioral and emotional problems (Richardson et al., 2002). Close comparison of end-points between human patients and rodent ethanol exposure models suggest a congruent

effect of blood alcohol content (BAC) on behavioral outcomes across species (Driscoll et al., 1990). Controlled-dose studies using rodent models have underlined the detrimental effects of PAE, demonstrating that high levels of exposure (BAC 300 - 400mg/dl) can alter motor behavior, impair learning, and decrease cognitive flexibility (Riley et al., 1979, Thomas et al., 2004a, Thomas et al., 2004b, Morton et al., 2014).

While these and other studies have helped to establish a link between high dose exposure and later cognitive and behavioral deficits, the effects of more moderate dose prenatal alcohol exposure remain controversial (Henderson et al., 2007, Todorow et al., 2010). Clinical studies have shown that lower dose PAE can lead to increased risk of behavioral issues in adolescence including aggression and emotional problems (Sood et al., 2001, Sayal et al., 2007, O'Leary et al., 2010). More moderate PAE has also been associated with cognitive deficits in both children and adolescents (Coles et al., 1991, Burden et al., 2005a, Burden et al., 2005b). In particular, adolescents with fetal alcohol spectrum disorders (FASD) who do not show the hallmark morphological abnormalities associated with Fetal Alcohol Syndrome exhibit impairments in executive control, including, behavioral inflexibility, which can interfere with everyday classroom functioning (Green, 2007). However, other studies failed to find an association between more moderate PAE and impairment in either behavioral or emotional development (Kelly et al., 2009, Bay and Kesmodel, 2011, Kelly et al., 2012). Several preclinical studies have shown that PAE can lead to alterations in brain function and behavior. PAE resulting from maternal daily ethanol consumption resulting in a BAC of 80-90mg/dl, has been shown to impair hippocampal mediated working memory and N-methyl-D-aspartate receptor- (NMDAR)

mediated synaptic plasticity (Brady et al., 2012, Brady et al., 2013). Additionally, PAE has been shown to alter cerebellar-motor coordination, cortical organization and social behavior (Valenzuela et al., 2012). However, others have failed to find any impact of moderate PAE on cognition (O'Leary-Moore et al., 2006). Although measures of cortically-mediated cognition have been shown to be sensitive to high dose ethanol (EtOH) exposure during development in rodents (Riley et al., 1979, Thomas et al., 2004a, Thomas et al., 2004b) studies using spatial tasks to measure flexible behavior have had mixed results (Riley et al., 1979, O'Leary-Moore et al., 2006).

Here we show that clinically-relevant levels of PAE can impair cortically mediated behavioral flexibility using a hallmark task of executive control across species: reversal learning. Mice tested on a touch-screen based paradigm during adulthood showed a significant and specific increase in maladaptive perseverative responding on the task. To our knowledge, these are the first data to demonstrate that even moderate PAE can have long-lasting negative impact on executive control.

2b. MATERIALS AND METHODS

Prenatal Alcohol Exposure Model. Male and female C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were used in a limited access PAE paradigm as previously described (Brady et al., 2012, Brady et al., 2013). Briefly, two hours into the dark cycle, female mice were given access to either 0.066% (w/v) saccharin or an ethanol solution (5% w/v for two days, then 10% w/v) sweetened with 0.066% (w/v) saccharin for four hours (from 1000 to 1400 hr). After one week of drinking 10% ethanol or the saccharin

control solution, individual females were placed into the cage of a singly-housed male for two hours immediately following the drinking period. Females continued to consume ethanol and saccharin solutions throughout the five-day mating period. Pregnancy was positively determined by monitoring weight gain every 3-4 days. Access to alcohol was withdrawn beginning on post-natal day 0 using a step-down procedure over a 6-day period and offspring were weaned at approximately 23 days of age. We have shown this protocol reliably produces blood ethanol concentrations of 80-90 mg/dL at the end of the 4-hour drinking period (Brady et al., 2012, Brady et al., 2013). Offspring were housed in groupings of 2-4 per cage in a temperature- and humidity- controlled vivarium under a reverse 12 h light/dark cycle (lights off 0800 h) and tested during the dark phase. All behavior was conducted on adult male and female offspring (n=7-9 per sex/treatment; ~9 weeks at onset of testing, **Figure 4**). All experimental procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee.

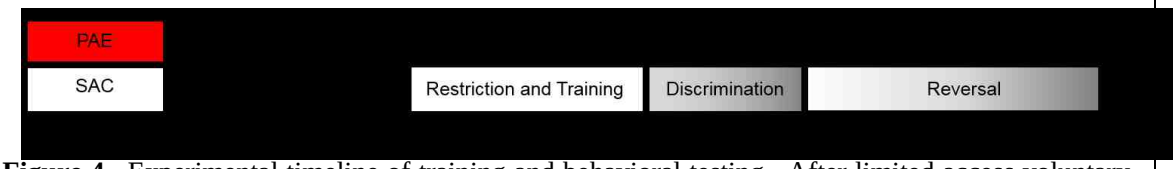

Figure 4. Experimental timeline of training and behavioral testing. After limited access voluntary PAE or SAC treatment mice were weaned and allowed to age to 6 weeks. The functional observational battery (FOB) was conducted immediately prior to food restriction and the three stage pre-training paradigm that acclimated mice to initiate and respond to visual stimuli. Next, discrimination learning was tested followed by reversal on the session immediately following criterion performance.

Functional observation battery. PAE and saccharin control mice (SAC) were assessed for physical, neurological or gross behavioral abnormalities as previously described

(Brigman et al., 2010a). Briefly, mice were individually placed in a bare empty home cage and observed over 60 sec for the presence of freezing, trembling, wild running, grooming, sniffing, licking, rearing, jumping, spontaneous seizure, defecation, urination, head bobbing, circling, abnormal gait, and retropulsion. Basic physical health was evaluated by examining for missing whiskers, bald patches, exophthalmus, straub tail, kinked tail, kyphosis, lordosis, body weight, and core body temperature. Simple sensory reflexes were measured via orienting responses to an approaching probe and to physical touch, and via palpebral closure on touch of the eye, twitch of the pinna on touch and an orienting response to tail pinch. Basic motor and neurological functions were assessed by observing the instance of splayed limbs, forepaw clutch and hind limb clutch when mice were tail suspended. Grip strength was measured by placing the mouse on a grid surface made of 2-mm diameter metal rods running lengthwise at 10-mm intervals. This was then slowly rotated and the latency of the mouse to fall was manually recorded (60-sec maximum) (Boyce-Rustay and Holmes, 2006). Observers were blinded to treatment conditions throughout the assessment.

Operant apparatus. Touch-screen discrimination and reversal learning were assessed as previously described (Brigman et al., 2010a). Briefly, operant behavior was conducted in a chamber measuring 21.6 x 17.8 x 12.7 cm (model # ENV-307W, Med Associates, St. Albans, VT) housed within a sound- and light-attenuating box (Med Associates, St. Albans, VT). The standard grid floor of the chamber was covered with a solid acrylic plate to facilitate ambulation. A pellet dispenser delivering 14 mg dustless pellets (#F05684, BioServ, Frenchtown, NJ) into a magazine, a house-light, tone generator and

an ultra-sensitive lever was located at one end of the chamber. At the opposite end of the chamber there was a touch-sensitive screen (Conclusive Solutions, Sawbridgeworth, U.K.) covered by a black acrylic aperture plate allowing two 7.5 x 7.5 cm touch areas separated by 1 cm and located at a height of 0.8 cm from the floor of the chamber. Stimulus presentation in the response windows and touches were controlled and recorded by the KLimbic Software Package v1.20.2 (Conclusive Solutions, Sawbridgeworth, U.K.).

Pretraining. Mice were first slowly reduced and then maintained at 85% free-feeding body weight. Prior to testing, mice were acclimated to the 14 mg pellet food reward by provision of ~10 pellets/mouse in the home cage for 3-5 days. Mice were then habituated to the operant chamber and to eating out of the pellet magazine by being placed in the chamber for 30 min with pellets available in the magazine. Mice retrieving 10 pellets within 30 min were moved to a three-stage pre-training regimen. First, mice were able to obtain reward by pressing a lever within the chamber. Mice pressing and collecting 30 rewards in under 30 minutes were moved to touch training. Here, a lever press led to the presentation of a white (variously-shaped) stimulus in 1 of the 2 response windows (spatially pseudorandomized). The stimulus remained on the screen until a response was made. Touches in the blank response window had no response. Mice initiating, touching and retrieving 30 pellets within 30 min were moved to the final stage of pre-training. This stage was identical to touch-training except that responses at a blank window during stimulus presentation now produced a 15 sec timeout (signaled by illumination of the house light) to discourage indiscriminate screen responding. Errors on this stage were

followed by correction trials in which the same stimulus and left/right position was presented until a correct response was made. Mice making ≥75% (excluding correction trials) of their responses at a stimulus-containing window over a 30-trial session were moved onto discrimination.

Discrimination and Reversal Learning. Following pre-training all mice were tested on a pairwise discrimination-reversal paradigm during daily sessions of a maximum of 60 minutes. For discrimination learning, 2 novel approximately equiluminescent stimuli were presented in a spatially pseudorandomized manner over 30-trial sessions (5 sec inter-trial interval). Responses at 1 stimulus resulted in reward; responses at the other stimulus resulted in a 15 sec timeout (signaled by illumination of the house light) and were followed by a correction trial. Designation of initially rewarded stimulus was randomized across treatment. Stimuli remained on screen until a response was made. As during pre-training, errors on first presentation trials were followed by correction trials which continued until a correct response was made or the session ended. Mice were trained to a criterion of ≥85% correct responding (excluding correction trials) over 2 consecutive sessions. Reversal training began on the session after discrimination criterion was attained. Here, the designation of stimuli as correct versus incorrect was reversed for each mouse. Mice were trained on 30-trial daily sessions (as for discrimination) to a criterion of ≥85% correct responding (excluding correction trials) over 2 consecutive sessions.

Statistical Analysis. The following dependent measures were taken during discrimination and reversal: correct responses made, errors (=incorrect responses made), correction errors (=correction trials made) which are a putative measure of perseveration during reversal (Brigman et al., 2013b), stimulus response (=time from trial initiation to touchscreen response) and reward response (=time from touchscreen response to reward retrieval). As correct and incorrect response measures were consistent on all analysis, incorrect responses are shown throughout. Discrimination performance was analyzed across all sessions required to reach criterion (**Figure 5A**). In order to examine distinct phases of reversal (early perseverative and late learning) mediated by cortical and striatal subregions respectively, we separately analyzed errors and correction errors for sessions where performance was below 50% and performance from 50% to criterion, as previously described (Brigman et al., 2010a, Brigman et al., 2013) (**Figure 6A**). Main effects of sex, treatment (PAE vs. SAC) and interaction were compared for all measures using analysis of variance (ANOVA).

2c. RESULTS

We found that the limited access paradigm yielded ethanol consumption levels in dams similar to those producing BACs of approximately 80-90 mg/dL (Brady et al., 2012, Brady et al., 2013). Offspring tested were taken from litters born to dams with an average consumption of 6.31±0.34 g of EtOH/kg of body weight/d. Our analysis of the functional observation battery revealed no obvious physical, neurological or gross behavioral abnormalities in PAE as compared to SAC control animals (**Appendix A**). In correspondence with previously reported findings PAE mice also showed no significant

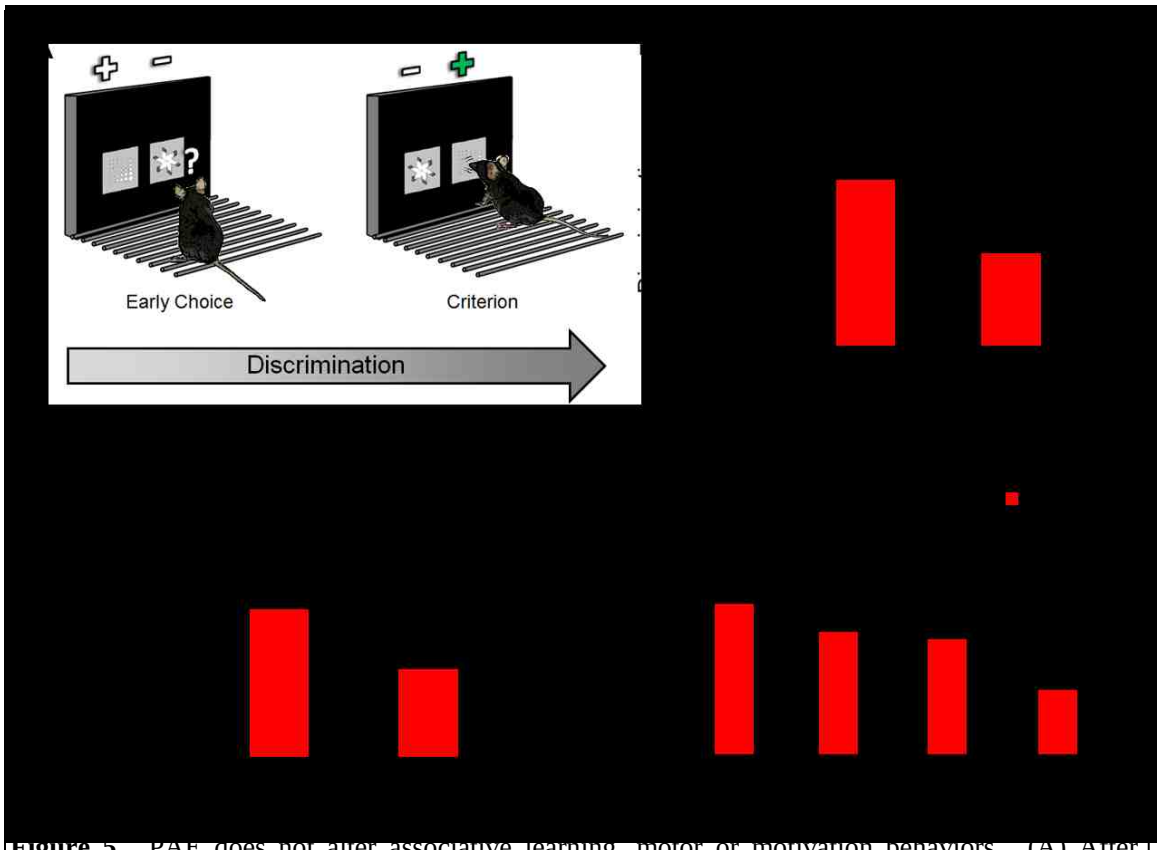

Figure 5. PAE does not alter associative learning, motor or motivation behaviors. (A) After initially responding at approximately chance, mice learn to respond to a rewarded stimuli (+) and ignore the unrewarded (-) to high level of criterion (85% over 2 consecutive sessions). Female mice required significantly more errors (B) and correction errors (C) than males to reach discrimination criterion but did not significantly differ by treatment. (D) There were no significant differences between sex or treatment on stimulus response or reward response after correct choice across the problem. *=*P*<.01 main effect of sex.

differences in ad lib weight at time of testing versus saccharin control mice (Male SAC=24.2±1.0, Female SAC=21.6±0.8; Male PAE=25.1±1.2, Female PAE=22.2 ±0.7).

All mice were able to successfully complete the three-stage pre-training and no significant differences were seen by sex or treatment (**Appendix B**). We next examined PAE and SAC performance on the pairwise visual discrimination task (**Figure 5A**). We found that all mice tested attained performance criterion with no main effect of sex, treatment or interaction on any stage of pre-training as measured by sessions to completion (**Appendix A**). There was a significant main effect of sex on errors (F1,25=16.79, *P*<.001; **Figure 5B**) and correction errors (F1,25=25.14, *P*<.001; **Figure**

5C) to discrimination criterion with females making significantly more of both error types. No main effect of treatment or sex and no significant interaction was seen on either errors or correction errors to attain criterion performance on the discrimination problem (ANOVA: *ns).* No main effects of sex, treatment or interactions were seen on motivation to respond to visual stimuli or work for food reward as measured by stimulus or reward response time respectively (**Figure 5D**).

Analysis of performance across both stages of reversal (early perseverative and later learning phases; **Figure 6A**) revealed no significant main effect of treatment on total errors, correction errors or response times (ANOVA: *ns*). However, analysis of reversal performance divided by phase revealed a profound perseverative impairment in PAE mice. PAE mice made significantly more correction errors, or repeated incorrect responses, after an initial error ($F_{1,27}=8.40$, $P<.001$; **Figure 6C**), but not initial errors (ANOVA: *ns;* **Figure 6B**), during the early perseverative phase (sessions <50% correct) while choice re-learning (sessions ≥50% correct) was intact. Importantly, the significant increase in correction errors, a measure of maladaptive perseveration, during early reversal was not due to motivation to respond or retrieve reward as measured by stimulus and reward response times on either phase of the reversal stages (ANOVA: *ns;* **Figure 6D**).

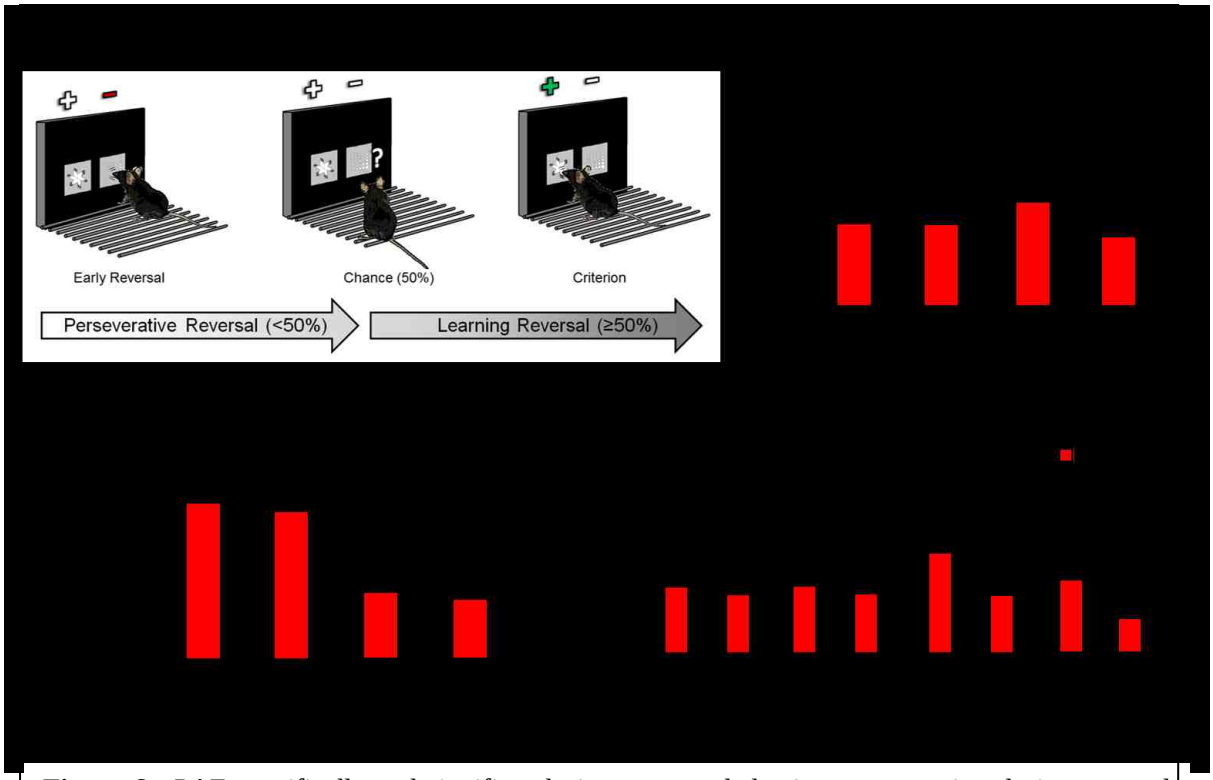

Figure 6. PAE specifically and significantly increases maladaptive perseveration during reversal learning. (A) Mice show high levels of perseverative responding to the previously rewarded and now unrewarded stimulus (-) before re-attaining chance over several sessions (perseverative phase= sessions <50% correct). Over several sessions mice then learn to respond to the newly rewarded (+) stimuli to high level of criterion (learning phase= sessions ≥50% correct). (B) Female and male PAE and SAC controls did not make significantly different rates of errors during early perseverative or late learning phases of reversal. (C) Both female and male PAE mice made significantly more correction errors during early perseverative sessions, but not late learning reversal performance. (D) There were no significant differences between sex or treatment on stimulus or reward response times after correct choice during either phase of the reversal problem. *=*P*<.01 main effect of treatment.

2d. INTERIM DISCUSSION

Although there is increasing awareness of the detrimental effects of heavy alcohol intake during pregnancy, the effects of more moderate drinking are still controversial. The current results demonstrate that lower, but still clinically relevant, doses can lead to impairments in executive control that persist into adulthood. These higher-order mental processes, which include attention, working memory, future planning and behavioral flexibility are essential to succeed in a complex, constantly changing environment

(Mattson et al., 1999, Green et al., 2009). Not surprisingly, impairments in executive control are associated with reduced quality of life due to negative impact on employment, managing finances and personal relationships (Royall et al., 2002). The specific impairment seen in the current study is particularly intriguing given evidence from the clinical literature that executive control is impaired in individuals with Fetal Alcohol Syndrome (McGee et al., 2008). Further, executive functioning has been shown to predict level of social skill, suggesting that alterations in these domains may underlie a wide range of deficits after PAE (Schonfeld et al., 2006).

Using a previously established PAE model, we found that dams drank to levels previously shown to induce BACs that correspond to a physiological relevant moderate dose (Driscoll et al., 1990, Brady et al., 2012). In agreement with previously published results in this model, PAE did not lead to gross morphological, motor or sensory alterations in offspring. Importantly, this moderate exposure paradigm has previously been shown not to alter dam-pup interactions, as measured by time-on-nest and pup retrieval time by dams (Brady et al., 2012). Additionally, adult PAE mice did not show alterations in ad lib weight or feeding behavior prior to, or after the cessation of behavioral testing that might significantly alter performance in an appetitive operant paradigm. Our analysis of pairwise visual discrimination learning showed that female mice required more errors and correction errors to learn the problem than males, although all animals of both sexes were able to learn the problem to criterion at levels comparable to non-treated control strain performance (Izquierdo et al., 2006). The effect did not persist into either phase of the reversal, suggesting that sex differences in weight prior to

training may have initially driven lower motivation to perform for food reward that increased as food reduction was adjusted for normal growth through the problem sequence. Despite the lack of interaction with PAE treatment, this sex difference is potentially important given the common practice of using combined groups of male and female mice in behavioral studies of cognition.

Previous studies using the mouse PAE model have demonstrated robust impairments in hippocampal plasticity and hippocampal dependent spatial learning behaviors (Brady et al., 2012, Brady et al., 2013). Here we found that PAE did not impact visual associative learning, either during discrimination or during the learning phase of reversal. While the hippocampus likely plays a role in the working memory necessary for visual discrimination performance, associative learning has been shown to be mediated by the dorsal striatum both during initial learning and later re-learning of the new association in late reversal (Featherstone and McDonald, 2004, Yin et al., 2004, Brigman et al., 2013). Although high-dose exposures in adult mice have been shown to prime associative processes, our PAE model did not alter discrimination learning, suggesting non-spatial tasks may be uniquely suited for examining flexible behavior after intact learning in these models (Depoy et al., 2013). In contrast, early perseverative reversal learning in rodents is mediated by cortical subregions, particularly the lateral orbitofrontal cortex (lOFC) (Schoenbaum et al., 2002, Chudasama and Robbins, 2003, Izquierdo et al., 2013). The lOFC has been hypothesized to be critically important in monitoring expected reward value and signaling when expectations are altered or violated (Rudebeck et al., 2013). We have recently shown that optimal early-reversal in the touch-screen paradigm

specifically recruits lOFC in the mouse and that this region is functionally necessary for optimal behavioral flexibility on the task (Graybeal et al., 2011, Brigman et al., 2013). Together, the profile of intact discrimination learning and increased maladaptive perseverative shown here suggest that PAE may be primarily altering cortical development, leading to a loss of top-down control of striatal-subregions. This hypofrontality in turn leads to continued responding to a previously learned cue even when it ceases to be beneficial, due to a failure to monitor expected outcomes and update reward contingencies as needed. This is consistent with previous findings in PAE models which have been shown to alter neocortical development, alter immediate early gene expression and decrease social behavior in the rat (Cuzon et al., 2008, Hamilton et al., 2010a, Hamilton et al., 2010b). Recent evidence in both rat and mouse PAE showing both alterations in neurochemistry and spatial flexibility further support the current findings and suggest that even moderate exposure can alter cortical function long-term (Allan et al., 2014, Hamilton et al., 2014).

In conclusion, our study provides the first evidence using a highly-translatable touchscreen learning paradigm that prenatal alcohol exposure can impair behavioral flexibility, a common measure of cortically-mediated executive control. These data provide strong support for both the voluntary prenatal exposure model and operant behavioral measures for investigating cortical alterations after developmental insult. More importantly, given reports of executive control impairments in adolescents with FASD, the present data provide strong evidence that even a low amount of alcohol

consumption during pregnancy may have detrimental effects on cognition that last well into adulthood.

Acknowledgements

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CHAPTER 3

Touch-Screen Visual Reversal Learning is Mediated by Value Encoding and Signal Propagation in the Orbitofrontal Cortex

Marquardt, K., Sigdel, R., and Brigman, J.L. (2017). Touch-screen visual reversal learning is mediated by value encoding and signal propagation in the orbitofrontal cortex. Neurobiology of Learning & Memory 139, 179-188.

3a. INTRODUCTION

Behavioral inflexibility is a common cognitive symptom of numerous neuropsychiatric and neurodevelopmental disorders, including but not limited to schizophrenia obsessive compulsive disorder, addiction, fetal alcohol- and autism spectrum- disorders. Inflexibility can have a profoundly negative impact on quality of life as a failure to adapt to changes in environmental conditions leads to intransigent patterns of behavior that affect relationships, financial management and the ability to maintain employment. The orbitofrontal cortex (OFC) has been implicated in mediating decision making and behavioral flexibility across species (Stalnaker et al., 2015). Targeted lesion and inactivation studies have demonstrated that OFC function is necessary for optimal reversal learning behavior, a hallmark task of behavioral flexibility (Hamilton and Brigman, 2015, Izquierdo et al., 2016). Across modalities, reversal tasks require subjects to form expectations of outcome based on associative cues, perform based on those expectations and control changes in response to altered reward contingencies (Wilson et al., 2014, Costa et al., 2015, Jang et al., 2015, Saez et al., 2015, Stalnaker et al., 2015).

Studies using single and multi-unit recording during lever-press and spatial paradigms in the rat (Schoenbaum et al., 2000, Moorman and Aston-Jones, 2014) and olfactory/tactile

paradigms in the mouse (Bissonette et al., 2008) suggest that the OFC responds with increased firing to an expected outcome, and tracks the efficient switching of reward value across reversal sessions. This supports the notion that OFC forms representations of expected outcomes based on previous trial outcomes, and that these representations are required to successfully switch choice behaviors when contingencies change (Padoa-Schioppa, 2007, Kennerley et al., 2011, Cai and Padoa-Schioppa, 2014). However, the OFC is also required to successfully monitor when learned actions fail to lead to the expected outcome. Responses to unexpected outcomes, or prediction errors, first described in midbrain dopamine neurons have been characterized in a subpopulation of OFC neurons which fire to an unexpected outcome (Thorpe et al., 1983, Mirenowicz and Schultz, 1994, Morris et al., 2006, Roesch et al., 2007). Inactivation of these neurons in the OFC can impair new learning once contingencies are changed, specifically when previous contingencies were well defined (Takahashi et al., 2009, Sul et al., 2010, Riceberg and Shapiro, 2012). Touch-screen reversal has similarly been shown to be sensitive to OFC lesion and targeted antagonism in mice, but to date, it has not been established that outcome value is similarly tracked in these more complex visual learning tasks in the rodent (Graybeal et al., 2011, Brigman et al., 2013).

In order to exert control over choice behaviors, changes in reward expectancies tracked by OFC must be communicated to downstream regions (Schoenbaum and Esber, 2010) involved in reward, habitual and goal directed behaviors (Schilman et al., 2008, van der Meer et al., 2010, Hoover and Vertes, 2011). It has been previously been shown that the dorsal striatum (dS) tracks behavioral responses during touch-screen learning, but how

OFC value-signal propagation occurs has not been studied in this paradigm (Brigman et al., 2013). Given their hypothesized role in temporally coordinating neuronal firing within and between regions, oscillatory local field potentials (LFP) have been posited as the putative mechanism for propagating changes in neuronal firing across regions (Buzsaki and Draguhn, 2004, Womelsdorf et al., 2007, Cohen, 2014b). In addition, they have been proposed to behaviorally select single unit signals by frequency tuning (Fries et al. 2001). Recent evidence from rodent studies suggest that oscillations in the OFC can lock with spike-firing to distinct behaviors such as odor sampling and waiting for reward delivery (van Wingerden et al., 2010a, van Wingerden et al., 2010b) or spatial choice in a T-maze (Young and Shapiro, 2009). Understanding how local oscillations encode information and coordinate with single unit spikes during reward and error cues to signal changing reward contingencies could greatly improve our understanding of how OFC value encoding exerts influence over future choice behaviors across paradigms.

Touch-screen automated paradigms have become increasingly utilized to screen rodent models of numerous neuropsychiatric disorders (Marquardt et al., 2014, Yang et al., 2015, Copping et al., 2016, Leach et al., 2016) as these paradigms closely model tools used in the clinical assessment and may increase translational potential of preclinical studies (Mar et al., 2013, Talpos and Steckler, 2013, Hvoslef-Eide et al., 2016). While previous studies have demonstrated that lesion and/or inactivation of the region is sufficient to disrupt visual touch-screen reversal, it has not yet been demonstrated that the rodent OFC mediates reversal of complex visual stimuli in an analogous manner to those seen in primates using visual stimuli (Clarke et al., 2008a), or to more species-specific

stimuli such as spatial, olfactory or tactile stimuli in rodents (Hamilton and Brigman, 2015).

Here we examined whether the OFC tracked reward expectancies during discrimination and reversal of visual stimuli in a touch-screen operant paradigm. We hypothesized that in agreement with lever, odor and spatial approaches, during distinct stages of touch-screen visual reversal, single units would signal changes in value and expectancy after choice behaviors differentially based on the previous trial response. We further hypothesized that these signals would be differentially coordinated with the local field potential to either increase or decrease likelihood that signals would be propagated to downstream regions to guide flexible behavior. To test this hypothesis, we utilized a touch-screen paradigm that provided immediate feedback on reward and error choices via concomitant tone and light cues and allows for recording of behavior and neuronal activity at well-established phases of reversal learning. Using this framework, we analyzed spike-firing and oscillatory activity during rewarded trials following a previously correct trial (*win*-stay) or following a previous error (*lose-shift)* and error trials that followed a rewarded trial (*regressive*) or followed another error in a series (*perseverative*) across discrimination and reversal to determine more precisely what behaviors the OFC encodes and potentially propagates at specific points of learning.

3b. MATERIALS AND METHODS

Subjects. Male C57BL/6J mice obtained from The Jackson Laboratory (Bar Harbor, ME) were housed in groupings of 2-4 per cage in a temperature- and humidity- controlled

vivarium under a reverse 12 h light/dark cycle (lights off 0800 h). A total of 12 male mice were used for all experiments and tested during the dark phase. Beginning at 7 weeks of age, mice were food-restricted to 85% of their free-feeding body weight. Operant training began once mice reached food-restricted weight. All experimental procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee.

Touch-screen Apparatus. All operant behavior was conducted in a chamber measuring 21.6 x 17.8 x 12.7 cm (model # ENV-307W, Med Associates, St. Albans, VT), housed within a sound- and light- attenuating box (Med Associates, St. Albans, VT) as previously described (Marquardt et al., 2014). The standard grid floor of the chamber was covered with a solid acrylic plate to facilitate ambulation. A pellet dispenser delivering reward (14 mg dustless pellets; #F05684, BioServ, Frenchtown, NJ) into a magazine, a house-light, tone generator and an ultra-sensitive lever was located at one end of the chamber. At the opposite end of the chamber there was a touch-sensitive screen (Conclusive Solutions, U.K.) covered by a black acrylic aperture plate allowing two 2 x 5 cm touch areas separated by 0.5 cm and located at a height of 6.5 cm from the floor of the chamber. Stimulus presentation in the response windows and touches were controlled and recorded by the KLimbic Software Package (Conclusive Solutions, U.K.).

Pre-training. Mice were habituated to the operant chamber and to eating out of the pellet magazine by being placed in the chamber for up to 30 min with pellets available in the

magazine. Mice retrieving 10 pellets within 30 min were moved onto pre-training. Mice began a three-stage pre-training regimen by first being trained to obtain reward by pressing a lever within the chamber on an FR1 schedule. Mice pressing and collecting 30 rewards in under 30 minutes were moved to touch training. During this stage, a lever press led to the initiation of a trial in which a white (variously-shaped) stimulus was presented in 1 of the 2 response windows. Lever press-initiation was included to clearly distinguish between initiation and reward seeking behaviors in later recording sessions. Throughout the paradigm images were spatially pseudorandomized preventing side bias and ensuring location was not an informative variable. The stimulus remained on the screen until a response was made. Touches in the blank response window had no effect, while a touch to the white stimulus resulted in reward delivery, immediately cued by a tone and illumination of the magazine light on the opposite side of the operant chamber from the touch screen. Mice initiating, touching and retrieving 30 pellets within 30 min were moved to the final stage of pre-training. This stage was identical to touch-training except that responses at the blank window during stimulus presentation produced an immediate 10 sec timeout, signaled by illumination of the house light, to discourage indiscriminate screen responding. Errors on this, and all subsequent stages, were followed by correction trials in which the same stimuli and left/right position was presented until a correct response was made. Mice making $\geq$75% (excluding correction trials) of their responses at a stimulus-containing window over a 30-trial session were moved onto discrimination.

Stereotaxic Array Implantation. After completing pre-training and at least two consecutive days of free-feeding, mice were anesthetized with isoflurane and placed in a stereotaxic alignment system (Kopf Instruments, Tujunga, CA) for implantation of a microelectrode array. The array (Innovative Neurophysiology, Durham, NC) comprised 16 individual 35 μm-diameter tungsten microelectrodes arranged into 2 bundles of 2x4 electrodes (150 μm row/column spacing, 2.75 mm spacing between bundles) targeting bilateral orbitofrontal cortex (center of array: AP +2.60, ML ±1.38, DV -2.60). After 7 days of recovery, body weight reduction resumed and mice were given a post-surgery reminder consisting of the last pre-training session to ensure retention of pre-training criterion.

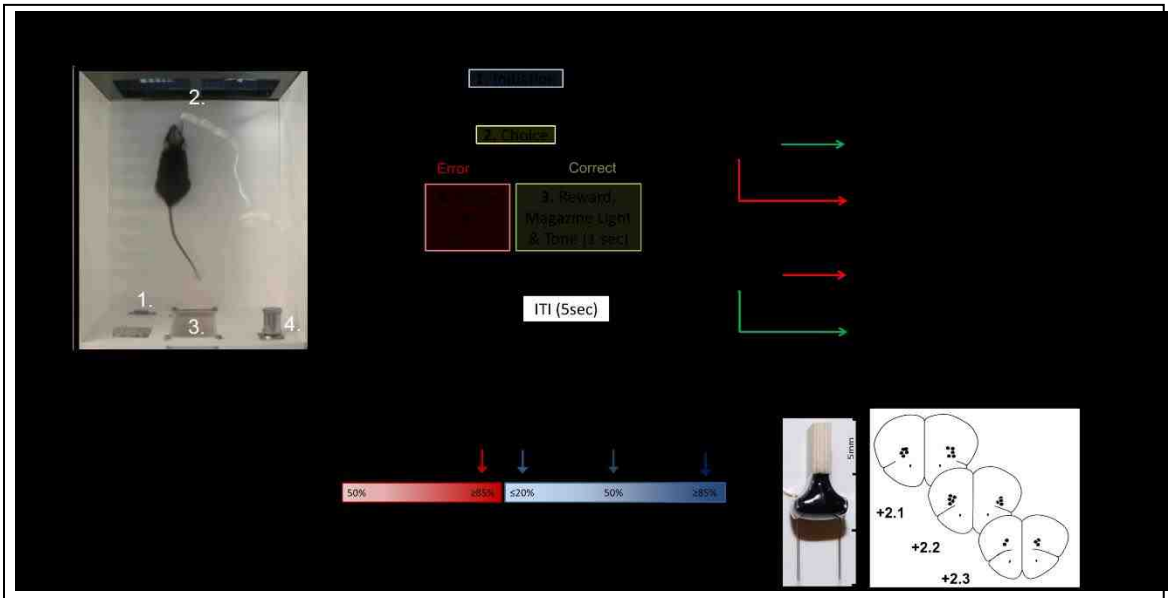

Figure 7: Behavioral Paradigm and Recording Sessions Timeline. (A-B) Trials were initiated through a lever press (1) which led to onset of pairwise visual stimuli on a touch-sensitive screen (2). Touch of the rewarded stimulus resulted in delivery of reward in the magazine (3) concomitant with 1 sec tone (3b) and illumination of the magazine light. Touches at the unrewarded led to illumination of the house light (4) for 10 sec. for an incorrect response. Error choices were followed by correction trials in which a subsequent initiation led to the stimuli presented in the same spatial orientation until a correct response was made to prevent side-bias and measure perseveration. (C) Trials were sorted into four distinct categories, based on the outcome of the previous trial: rewarded trial following rewarded trial= *win-stay*, rewarded trial following error= *lose-shift*, error trial following error trial= *perseverative*, and error trial following rewarded trial= *regressive*. Trial types can also be generally categorized by outcome (rewarded vs. unrewarded) and strategy (stay vs. switch). (D) After training and electrode implantation mice were recorded at 4 distinct learning stage. Discrimination and Reversal criterion= ≥85% correct, Early reversal= First session of reversal with performance <20% correct, Chance reversal= ~50% correct. Recording sessions are marked by arrows. (E) Array image and verification of electrode placement with each point representing the midpoint location of the electrode bundle. Adapted from (Paxinos and Franklin, 2001).

Discrimination and Reversal Learning. Following array implantation, all mice were tested on a pairwise discrimination reversal paradigm as previously described (Brigman et al., 2010b). Mice were first trained to discriminate two novel, approximately equiluminescent stimuli (Fan/Marble (Brigman et al., 2006, Brigman et al., 2010a, Brigman et al., 2013, Marquardt et al., 2014)), presented spatially pseudorandomized across 30-first presentation trials (not including correction trials) per daily session (5 sec ITI) (**Figure 7A**). As in pre-training, responses at the correct stimulus resulted in reward, immediately cued by the onset of a 1 sec tone; responses at the incorrect stimulus resulted in timeout, immediately cued by a 10 sec house-light followed by correction trials until a correct response was made (**Figure 7B**). Correct stimuli were balanced across mice. Discrimination criterion was of ≥85% correct responding (excluding correction trials) over two consecutive sessions. Reversal training began on the session after discrimination criterion was attained. Here, the designation of correct verses incorrect stimuli was reversed for each mouse. Mice were trained on 30-trial daily sessions (same as for discrimination) to a criterion of ≥85% correct responding (excluding correction trials) over two consecutive sessions. In order to measure performance differences across distinct sessions, percent correct responses, total errors, reaction time (time from lever press initiation to screen touch) and magazine latency (time from screen touch to reward retrieval) were analyzed. In order to analyze use of feedback for learning, correct and incorrect responses were further categorized based on previous trial outcome: correct responses were characterized as *win-stay* (following correct response) or *lose-shift* (following an error trial), while error trials were characterized as *perseverative* (following an error trial) or *regressive* (following a correct response; **Figure 7C**).

Neurophysiological Recording. Neuronal activity was continuously recorded using a multichannel acquisition processor (OmniPlex, Plexon, Dallas, TX) as previously described (Brigman et al., 2013, DePoy et al., 2013). Single and oscillatory activity was captured during the following 4 stages: **Discrimination Criterion** = session of discrimination criterion attainment (% correct >85%), **Early Reversal** = first session of reversal were perseverative responding is highest (% correct <20%), **Chance Reversal** (Chance) = session of reversal where chance performance was re-attained (% correct =50%, and **Reversal Criterion**= session of reversal where criterion is re-attained (% correct >85%; **Figure 7D**). Continuous spike signal was sampled at 40 kHz and waveforms were manually sorted during recording, based on manually set voltage threshold. Local field potential was sampled from the same electrodes at 1 kHz and automatically low band pass filtered at 200 Hz. Neuronal recording data was timestamped by responses from k-limbic software by TTL pulse to reward tone and punishment house light. At the completion of testing, array placement was verified via electrolytic lesions made by passing 100 μA through the electrodes for 20 sec (S48 Square Pulse Stimulator, Grass Technologies, West Warwick, RI). Brains were removed post perfusion with 4% paraformaldehyde, 50 μm coronal sections cut with a vibratome (Classic 1000 model, Vibratome, Bannockburn, IL), stained with cresyl violet and placement verified with reference to a mouse brain atlas (Paxinos and Franklin, 2001)(**Figure 7E)**.

Waveforms Analysis. Waveforms were re-sorted offline using principal component analysis of spike clusters and visual inspection of waveform and inter-spike interval <1%

shorter than 2ms using Offline Sorter (Plexon Inc, Dallas, Texas). Recording across multiple sessions increases the likelihood of repeatedly sampling individual units. However, tracking of individual units across sessions could not be verified, sorted putative neurons were therefore treated as independent units between sessions. TTL pulse timestamps recorded concurrently with neuronal data were used to create epochs of firing rate spanning 1 sec pre-choice to 3 sec post-choice in averaged bins of .05 sec with NeuroExplorer software (NeuroExplorer; NEX Technologies, Littleton, MA). Firing rate was analyzed during epochs of choice behaviors which were defined by the previous trial completed: correct responses following a previously correct response (*win-stay*) or a previous error (*lose-shift*), and errors following a previous error (*perseverative*) or correct trial (*regressive*). The 3 sec post-choice analysis window overlapped with immediate tone, during correct choice, and first 3 sec of house-light during incorrect trials allowing for analysis of immediate response to secondary associative cues. If reward retrieval occurred prior to the end of the 3 sec analysis window on correct trials, the epoch for that trial was truncated as to prevent overlap with reward signaling. Less than 5% of neurons showed baseline firing rate of >15Hz, were categorized as fast-spiking interneurons and excluded from analysis as previously described (Brigman et al., 2013). Normalized firing rates were calculated by Z-score of 3 sec post-choice to 1 sec pre-choice baseline. Pattern of firing rate changes were examined for the period during immediate cue delivery (choice → 1 sec post) and magazine approach (2 → 3 sec.) using repeated-measures ANOVA followed by the Newman–Keuls post-hoc test. The threshold for statistical significance was $P < 0.05$.

In addition to spike-firing activity changes, the proportion of the population of neurons that significantly increased their firing rate in the post-choice period (compared to individual pre-choice baseline using Student's t-test) were analyzed across type and discrimination reversal session via chi square. All choice- responsive neurons were analyzed, independent of significant response to other timestamped events as single unit signaling in the OFC is extremely heterogeneous and selection of single event-responsive neurons would unfairly represent overall response (Thorpe et al., 1983, Moorman and Aston-Jones, 2014, McMurray et al., 2016).

Local field potential power. Time-frequency analyses, as previously described (Cavanagh et al., 2009) were adapted to depth recordings and computed using custom Matlab (TheMathWorks) scripts. Each of the defined choice types was convolved separately with a set of complex Morlet wavelets, power was computed as the magnitude of the convolved data and decibel normalized to an average baseline (see Appendix A. Supplementary Material). Normalized time-frequency power was compared across frequency bands, trial types and sessions. Difference power graphs were calculated by subtracting each reversal session time-frequency spectrum from discrimination reversal, individually. Using a non-parametric approach, significance of reversal difference from discrimination was determined by two standard deviations difference from randomly distributed data within trial type and session.

Spike Field Coupling. In order to compare timing of spikes relative to shifts in phase within the LFP analysis of spike field coupling was conducted (Cohen, 2014b). For each

of the four defined trial types, single instances of spike firing across waveforms were marked and 500ms of oscillatory LFP data around each spike was set into an individual epoch with the spike occurrence at time point 0. The spike-LFP phase angle was computed as previously described (Cohen, 2014a) and used to calculate the paired phase consistency (PPC0) value (see Appendix A. Supplementary Material; (Vinck et al., 2010)). To control for unequal trial numbers across all behavioral bins and trial types 10 spike- locked LFPs were randomly selected to perform the PPC calculation over 1000 permutations to give an average, unbiased by spike number. Bins were combined for a final average of the 3 sec post-choice time epoch and PPC within each trial type, session and time epoch was compared to scrambled data within the same type. Significance was determined by a difference greater than two times the standard deviation from scrambled data.

3c. RESULTS

Behavior Profile of Touchscreen Reversal Learning. Mice with multichannel electrode arrays readily re-attained pre-training criterion (1.45 ± 0.4 sessions) and demonstrated a clear pattern of flexible shifting from a well-learned behavior to a new response. Mice progressed through stages of discrimination and reversal learning requiring similar numbers of trials as seen previously (**Figure 8A**; ANOVA effect of stage on correct trials: $F_{3,39}=72.43$, $P<.01$, followed by *post hoc* tests). Four distinct target trial types resulted from sorting based on the N-1 previous trial response, which allowed for the analysis of the microstructure of rewarded behavior. *Win-stay* trials (**Figure 8B**), were significantly more prominent on discrimination criterion, indicating a well-learned and

beneficial response strategy. *Win-stay* response significantly decreased during early reversal before increasing in a step-wise pattern across chance reversal and returned to high levels upon re-attainment of criterion on reversal ($F_{3,10}=263.618$, $P<.001$ followed by *post-hoc* test). In contrast, *lose-shift* trials represent a positive change in strategy to obtain a reward due to the previous error response (**Figure 8C**). Low levels of this exploratory behavior are seen during criterion stages and early reversal. This behavior increases during chance reversal during which the new association is being learned, before again decreasing at reversal criterion ($F_{3,36}=18.117$, $P<.001$ followed by *post hoc* tests).

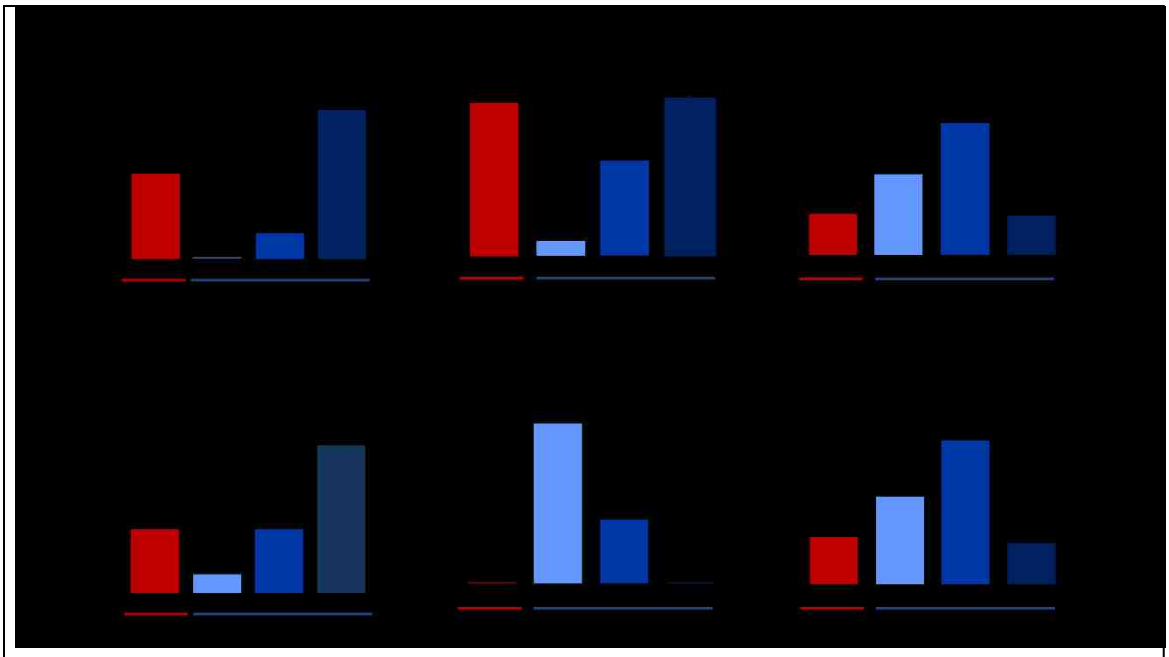

Figure 8: Microstructure of Behavior Matches Learning Stage during Recorded Sessions. (A) Cumulative trials required to attain each stage of discrimination and reversal learning. (B) Total error trials significantly increased during the first session of reversal and subsequently decreased significantly across each subsequent stage of reversal. (C) *Win-stay* closely reflected total percent correct, significantly decreasing during early reversal and significantly increasing across reversal sessions. (D) Cumulative total errors to attain each stage of discrimination and reversal learning. (E) *lose-shift* trials did not significantly increase until chance reversal, before significantly decreasing again at criterion. (F) *Regressive* error trials, another switch trial type, closely mirrored *lose-shift*, increasing at chance before decreasing again at criterion. Data are means ±SEM. N=12 mice per recording session. * = $P<.05$, compared to Discrimination criterion; #= $P<.05$, compared to early reversal; and †=$P<.05$, compared to chance reversal.

Analysis of total error choices as mice progressed through discrimination and reversal were similar to those seen in previous *in vivo* recording experiments (**Figure 8D;** ANOVA stage effect: $F_{3,39}=42.94$, $P<.01$, followed by *post hoc* tests). Analysis of the microstructure of error responses confirmed previous findings that *perseverative* (error-error; **Figure 8E**) responses significantly increase during early reversal, dominating all other responses before tapering off by chance and becoming virtually non-existent by criterion reversal ($F_{3,36}=26.359$, $P<.001$ followed by *post hoc* tests;). *Perseverative* errors continued during chance reversal, even with the presence of the more beneficial *win-stay* trials within the same session, indicating the difficulty in initiating a change in response strategy. Similar to *lose-shift* responses, *regressive* errors, a non-beneficial change in behavior, which is not prompted by changing contingencies (reward-error; **Figure 8F**), occur at very low levels during well-learned criterion discrimination and increase during early reversal, but not significantly. *Regressive* errors significantly increase in conjunction with *lose-shift* trials as new associations are learned during chance reversal, before decreasing again when criterion reversal is obtained ($F_{3,36}=20.088$, $P<.001$ followed by *post hoc* tests;).

Analysis of secondary behavioral measures showed a small but significant increase in latency to make a screen response on early reversal (**Appendix D. Figure S1**; $F_{3,11}=3.466$, $P<.05$). This is unlikely to be a change in general motivation as the latency to retrieve a reward showed no significant differences (**Appendix D. Figure S1**), but an alteration in behavior caused by the shift in paradigm rules during early reversal.

Choice-Responsive Neurons are Learning Session Specific. We recorded single unit activity from 166 putative neurons. There was no significant difference in 1 sec pre-choice baseline between any trial types within a session, or between sessions (**Appendix D. Figure S2**). During distinct phases of discrimination and reversal populations of OFC neurons increased firing significantly to specific reward and error types. *Win-stay trials* were the dominant signaling type during well learned behavior, showing significantly more choice-responsive neuronal recruitment (χ^2=6.44, *P*<.05; **Figure 9A right**). Analysis of all neurons found a significant increase in firing to both *win-stay* and *lose-shift* rewarded responses 2 seconds after correct choice response during reward approach (**Figure 9A left**; MAIN EFFECT OF TIME: F3,291=4.39, P<.001). There was also a significant interaction between rewarded trial types, revealing that *win-stay* had a significantly increased firing rate compared to *lose-shift* trials (INTERACTION: F3,291=2.70, P<.05). In contrast, there was a significant sustained increase in firing after both *regressive* and *perseverative* errors immediately after negative reinforcement cue onset during discrimination criterion (**Figure 9A center**; MAIN EFFECT OF TIME: F3,210=4.79, P<.001). However, there was no significant difference in the strength or pattern of firing between error trial types.

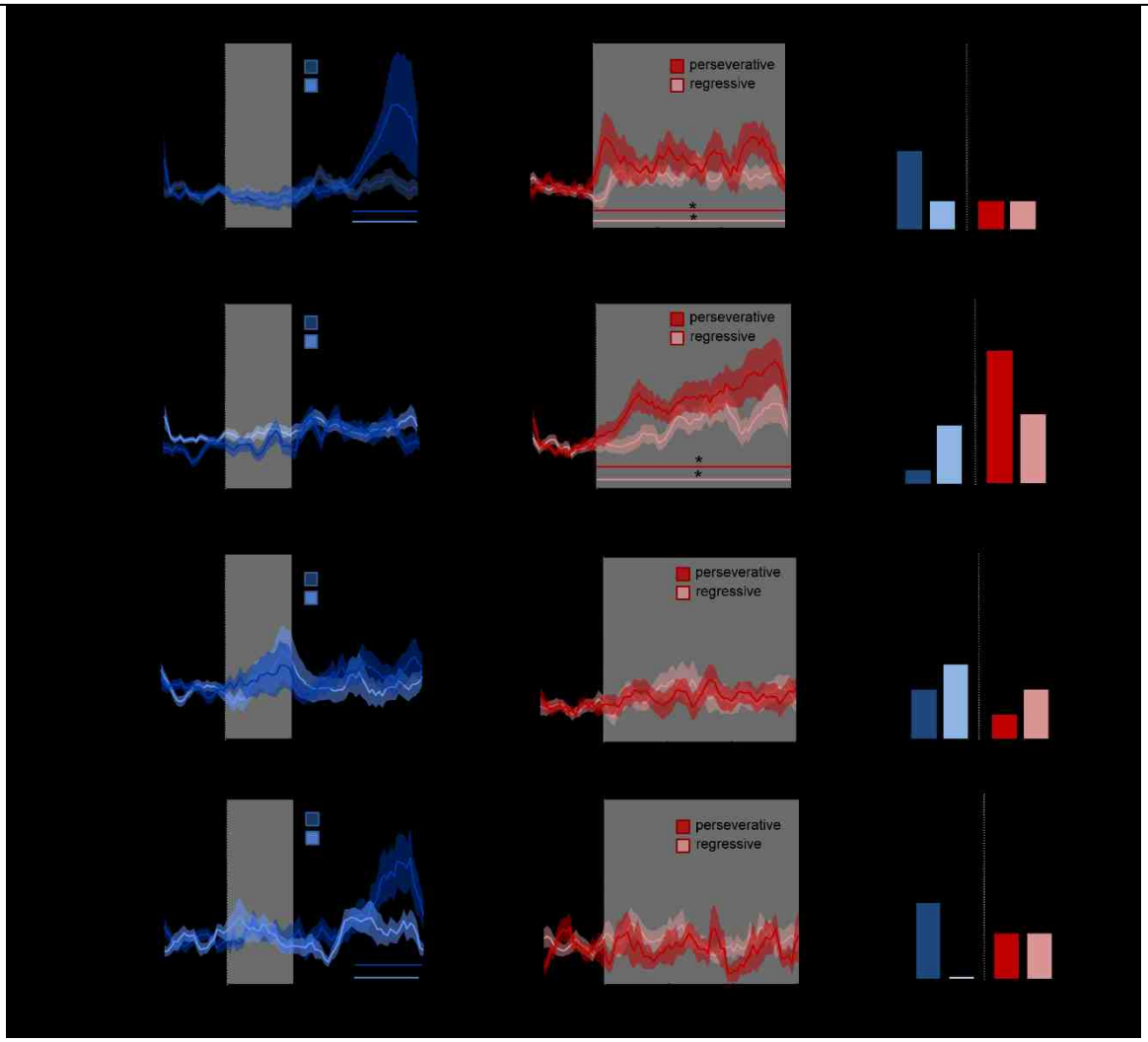

Figure 9. Average Spike- Firing Rate and Phasic Neuron Recruitment for each Trial Type is Specific to Learning Session. (A) Discrimination Criterion: Average firing significantly increased during the 2- 3 sec epoch for both reward trial responses, with *win-stay* firing increasing significantly more than *lose-Shift* (left). Average neuronal firing significantly increased across all post-choice epochs for both error trial types (center). Significantly more neurons were responsive to *win-stay* versus *lose-shift* rewarded trials, while there was no difference between number or error responsive neurons (right). (B) Early Reversal: There was no significant increase in average firing for either rewarded trial type for any epoch (right). Both error trial types significantly increased across all post-choice epochs (center). Significantly more *lose-shift* responsive neurons were seen versus *win-stay* and significantly more *perseverative* than *regressive* error responsive neurons (right). (C) Chance Reversal: Both reward trial types had increased firing to cue onset but neither was significantly above baseline (left). Neither error trial type showed significant increases in average firing (center). No significant difference was seen between event-responsive neurons to reward trials while *regressive* responsive neurons were significantly greater than *perseverative* errors (right). (D) Reversal Criterion: Average firing significantly increased during the 2-3 sec epoch for both rewarded trial responses with firing after *win-stay* increasing significantly more than *lose-shift* (left). No significant increase was seen in average firing rate for error trials (center**).** Significantly more *win-stay* responsive neurons were seen with no lose-shift responsive neurons recorded and no difference in percentage of error responsive neurons (right). X-axis indicates if the responsive neuron was found for a correct response (rewarded cue) or incorrect response (error cue) Data are means ±SEM. Gray shading indicates the presence of the associative cue: tone (correct) or house-light (error-cue). Bar indicates significant difference from baseline by type (color). * = P<.05

During early reversal the percent of *win-stay* trial responsive neurons significantly decreased while *lose-shift* responsive neurons became the dominant rewarded trial type (**Figure 9B right**; χ^2=11.83, *P*<.01). Neither *win-stay* nor *lose-shift* rewarded trials responded with a significant increase in firing rate (**Figure 9B left**). Both error trial types had more responsive neurons than discrimination criterion, with *perseverative* trial responsive neurons dominating (χ^2=22.78, *P*<.01). *Perseverative* and *regressive* error trials led to an immediate and sustained significant increase in firing rate during early reversal (**Figure 9B center**; F3,297=4.79, P<.001), with no significant difference between firing intensity.

During chance reversal, there continued to be more choice-responsive neurons to *lose-shift* rewarded trials than *win-stay* trials, but not significantly. However, the proportion of choice-responsive neurons to error trials overall decreased, and the proportion of *regressive* choice-responsive neurons are significantly higher than *perseverative* (**Figure 9C right**; χ^2=3.97, *P*<.05). Neurons responsive to correct responses did increase firing rate to the end of the associative signal (tone), but did not reach significance. Similarly, neither error trial response led to an increase in firing.

When reversal criterion was attained, the pattern of choice-responsive neurons mirrored discrimination criterion. *Win-stay* responsive neurons were the dominant type of reward responsive neurons with no neurons responsive to *lose-shift* (χ^2=103.15, *P*<.01). While slightly elevated over discrimination, there was no significant difference between proportions of neurons responsive to *regressive* or *perseverative* errors (**Figure 9D**

right). As in discrimination criterion, there was a significant increase in firing to both rewarded responses two seconds after choice (**Figure 9D left**; MAIN EFFECT OF TIME: $F_{3,120}=5.73$, $P<.001$). Similarly, *win-stay* responses had a significantly increased firing rate compared to lose-shift trials (INTERACTION: $F_{3,120}=3.18$, $P<.05$). In contrast to discrimination, during reversal criterion, *perseverative* nor *regressive* error trials were followed by significant increases in firing during any epoch.

Spike-Field Coupling Tracks Reinforced Behavior. Comparisons of time-frequency power spectra did not reveal any consistent changes that were greater than two standard deviations from chance, during any session of reversal in any trial type (**Appendix D. Figure S3**) indicting the time-frequency power spectra of each trial type was independent of learning session. However, analysis of synchrony between spike firing and oscillatory activity revealed distinctive patterns of coupling across learning sessions. Trials in which a switch in behavior occurred (*lose-shift* and *regressive*) had significantly greater than chance spike- theta field coupling (4-10 Hz) during discrimination criterion (**Figure 10A**). In contrast, both consistent behavioral trial types (*win-stay* and *perseverative* responses) had extensive cross- frequency decreases in synchrony that were two standard deviations *lower* than chance indicating a decrease in signal coordination. However, *win-stay* decreases were limited to beta (10-30 Hz) and portions of gamma (30-40 Hz), while neurons responsive to *perseverative* trials were de-coupled across every measured frequency (5-40 Hz).

Lose-shift, *regressive*, and *perseverative* responses returned to chance level of spike-field coupling during early reversal (**Figure 10B**). In contrast, *win-stay* trials were strongly de-synchronized (greater than two standard deviations less than chance) across every

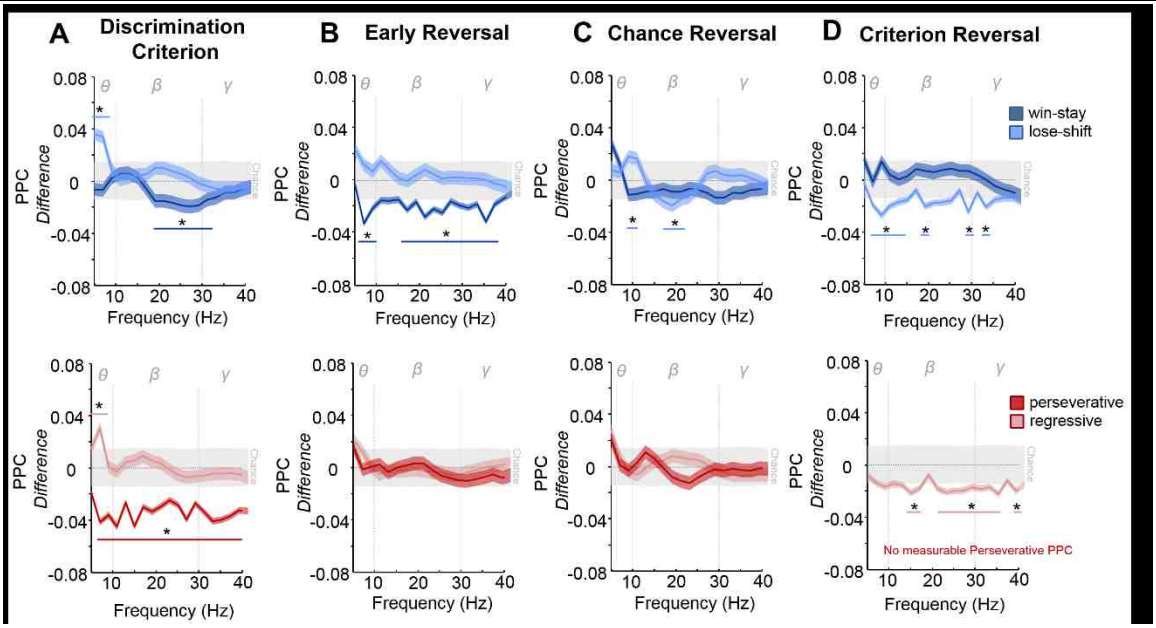

Figure 10. Spike- Oscillatory Synchrony Pattern is Distinct During the Post-Choice Phase of Each Learning Session. (A) Discrimination Criterion. *Lose-shift* trials showed significant increases in spike-field coupling in the theta frequency while *win-stay* trials showed significant decoupling in the beta frequency (upper). *Regressive* error trials had significant increases in the theta range while *perseverative* responses showed significant decoupling across all frequencies (lower). (B) Early Reversal: *Lose-shift* trials showed no significant coupling or decoupling across frequencies while *win-stay* trials now showed decoupling across Theta, the majority of Beta and all Gama frequencies (upper). Neither error trial type showed significant coupling or decoupling during this stage (lower). (C) Chance Reversal: *Win-stay* trials showed no significant coupling or decoupling while *lose-shift* now had strong coupling in the Theta frequency and significant decoupling in the mid Beta range (upper). While more variable, neither error trial type showed significant coupling or decoupling (lower). (D) Criterion Reversal: While increasing from chance, *win-stay* trials showed no significant coupling or decoupling while *lose-shift* trials now showed significant decoupling in all frequencies (upper). *Regressive* errors now had significant decoupling across Beta and Gama frequencies, while non-measurable coupling response was seen to *perseverative* trials (lower). Dotted Lines mark boundaries of frequency ranges of theta (θ, 4-10 Hz), beta (β, 10-30 Hz) and gamma (γ, 30-40 Hz). Data are means ±SEM. Colored lines indicate the frequency ranges of significance for the corresponding color trial types. *= significant difference (P<.05) from chance as calculated by two standard deviations away from random shuffled distribution of the data (grey bar).

frequency tested, except for a small band between 11 and 15 Hz, mirroring decreases in signal coordination seen during perseverative trials on the previous learning stage. *Win-stay* trials return to chance levels of synchrony by chance reversal (**Figure 10C top**)

while both error responses continue to have non-significant changes in spike field coupling (**Figure 10C bottom**). Only *lose-shift* trials had significant changes exceeding two standard deviations from chance in spike-field coupling during chance reversal, a small increase from 9-11 Hz and decrease from 15 to 25 Hz.

Patterns of spike field synchrony do not return to discrimination criterion patterns during reversal criterion. *Win-stay* trials at this stage were consistent with chance reversal with no significant changes in spike field coupling. This is in stark contrast to both *lose-shift* and *regressive* errors that became strongly decoupled across multiple frequency ranges (greater than two standard deviations lower than chance). *Lose-shift* responses were decoupled across all three-frequency bands of interest, while *regressive* is limited to changes in beta and gamma frequencies. *Perseverative* PPC was immeasurable due to extremely low spike responsiveness, indicating it is strongly decoupled during criterion reversal.

3d. INTERIM DISUSSION

Consistent with previous studies in primate visual and rat spatial paradigms, our data suggest that the OFC distinctly encodes values of specific choices during different stages of learning in touch-screen visual reversal (Moorman and Aston-Jones, 2014, Rich and Wallis, 2016). We also saw dynamic alterations in coupling between neuronal spike-firing and oscillatory activity that suggests selective propagation of choice-responsive signals to influence future behaviors. Taken together, our data support the role of choice-responsive OFC neurons in encoding value in a rodent touch-screen task, and further

suggests spike coupling with local oscillations as a putative mechanism by which signals are propagated to downstream regions to increase or decrease the likelihood of a behavioral action.

Spike-firing encodes value-expectancy across discrete behavioral choices. During touch-screen visual performance, OFC neurons showed distinct choice-responsive firing rates based on the likelihood a given response would lead to reward at that stage, suggesting that OFC choice-responsive neurons encode a pre-reward value expectancy (Young and Shapiro, 2009, Sul et al., 2010, Cai and Padoa-Schioppa, 2014, Moorman and Aston-Jones, 2014). During criterion performance, *win-stay* choice firing dominated all other signaling. Thus, this signal, which was increased when the expected value of the choice matched the outcome, is highly congruent with reward expectancy signaling in the OFC seen across rat spatial tasks and primate visual studies (Young and Shapiro, 2009, Sul et al., 2010, Cai and Padoa-Schioppa, 2014, Moorman and Aston-Jones, 2014). In contrast, during early reversal where outcome *least* matched the learned expectancy, firing shifted to robustly track error choices, suggesting these signals may reflect a negative expectancy violation. In non-human primates, spike-firing responses switch target responding within several trials (Thorpe et al., 1983). In the current study, where reversal takes several sessions, we see a clear response to the unexpected negative outcome on *perseverative* choice trials due to the un-signaled shift in stimuli contingencies. This suggests that during early reversal in our paradigm, OFC neuronal signaling that was previously tied to an expected reward shifts, to now signal the failure of that reward to occur. While we are unable to differentiate whether our recorded neuronal populations are consistent across

sessions, our data suggest the OFC tracks choice values differentially when expectancies are consistently met (criterion) versus violated (early reversal). As we and others have shown loss of OFC function significantly increases these *perseverative* responses and extends the timeframe to exit the preservative period (Clarke et al., 2008, Graybeal et al., 2011), we hypothesize this signaling is critically important to alter the choice-value has changed and facilitate flexible behavior. Similarly, *regressive* choice errors increased both in total number of responsive neurons and firing strength during this period. While both error types do not result in reward when it is expected, differences in signaling pattern, and number of responsive neurons in *regressive* compared to *perseverative* errors, indicate that there may be differences in downstream influences. It has been recently reported that the OFC does not differentially signal responses between stay and exploratory trials during lever press probabilistic reversal in an extended post-choice analysis window (Amodeo et al., 2016). Our results suggest that differences in signaling between consistent versus exploratory trials may only occur immediately after the choice event and may not be captured when data is analyzed over extended periods. While firing rates did not change, we also detected a significant increase in number of choice-responsive neurons to *lose-shift* during early reversal, suggesting the OFC also tracks these infrequent unexpected rewards, as has been previously shown in a Pavlovian odor task in the rat (Takahashi et al., 2009)

The recruitment of choice-responsive neurons when expectations of value become ambiguous also provides evidence of online value encoding in the OFC. During chance reversal, when the animal is no longer highly perseverative, but has not learned the new

association, *lose-shift* and *regressive* trials recruit more choice-responsive neurons than other responses. This switch in OFC recruitment may represent a shift to a more exploratory behavior pattern, as mice sample the outcome of different choices. While at criterion stages we found OFC firing strongly increased during reward approach, at chance reversal, firing increased to the immediate reward cue as described in non-human primates (Padoa-Schioppa and Assad, 2006). While our previous studies show that the OFC is not functionally necessary to establish the new choice values during touch-screen reversal (Brigman et al., 2013), the shift in OFC firing to immediate reward cues suggest the OFC tracks cue value to provide immediate feedback when the optimal choice is ambiguous. In contrast, when choice-values are well-learned, the cue may be too far removed in time from reward retrieval to continue this association, and therefore the approach and other un-intended secondary cues, like the pellet delivery, become informative for value.

Spike-field coupling correlates with behavioral strategy continuation or cessation. As reported previously in spatial reversal tasks, we did not detect large pattern changes in power across discrimination-reversal sessions (Young and Shapiro, 2011). In line with human EEG studies of consistent resting state power over time, analysis of power by trial types found distinct patterns that were consistent across sessions, suggesting OFC power patterns are set for particular behavioral responses (Porjesz et al., 2002). Local field potential power is a measure of the strength in the local oscillations, and changes in power can represent changes in activity or attention directed to the task suggesting that attentional capacity did not significantly vary across task stages (Buzsaki and Draguhn,

2004, Buzsaki et al., 2012, Calderone et al., 2014). The lack of changes in power across reversal suggests that meaningful value signals likely depend on dynamic coupling of varying event-dependent spike-firing events to the more consistent oscillatory signals.

Synchronization of spikes by local oscillations leads to coordinated large scale networks by amplifying or attenuating spike signals in multiple regions across the brain during spatial reversal and visual attention tasks (Womelsdorf et al., 2007, Gregoriou et al., 2009a, Cohen, 2014b, Womelsdorf et al., 2014). Here we show that during reversal, the dynamics of the spike-field coupling and decoupling correlated with the stereotypical reversal behavioral pattern, suggesting amplification and attenuation of specific trials. When discrimination was well learned, theta spike-field coupling was increased during more exploratory choice-trials independent upon rewarded outcome. This is in contrast to an odor discrimination task where theta spike-field coupling was linked to reward anticipation (van Wingerden et al., 2010a). During criterion performance, these exploratory trials occur infrequently, suggesting feedback generated by preferentially aligning signaling during these trial types to the oscillations may serve as a general monitoring function, for matching expectancy and outcome. Previous reports suggest spike de-synchronization in low frequency ranges reduces spike co-occurrences focusing visual attention (Fries et al., 2001). Multi-frequency spike-field de-synchronization in the current visual reversal task may strongly impact behavior by decreasing the signaling influence of particular trial types. During well-learned behavior the OFC strongly decreases the value- signaling impact of any *perseverative* trials, thus promoting *win-stay* behavioral response. Early reversal, dominated by negative- value signaling in the OFC,

responses to infrequent strings of *correct* choices are not propagated resulting in strings of errors.

Spike-field coupling differences between criterion stages likely reflect differences in the two stages from a value encoding perspective. During discrimination, stimulus choices have a fixed never varied value, while after reversal stimuli have multiple values, which vary based on *when* they are advantageous. Therefore, differences in spike-field coupling may be the result of reversal experience. These alterations may facilitate future reversals by holding both new and previous expectancies online, which would prime for future changes in reward associations (Boulougouris et al., 2007, Klanker et al., 2013). Overall, both spike-field coupling and decoupling patterns appear to correlate with potentiation of behavioral outcomes that results in maintenance of choice value signaling or marks unexpected changes in contingencies.

Conclusion. Consistent with previous studies utilizing lever and spatial rodent approaches, we found that neuronal firing responds to value expectancy in a complex visual reversal task. Spike-firing responses during visual reversal are sensitive to prior reward outcomes, supporting that the OFC is continually monitoring value. Additionally, patterns of behavior were associated with significant alterations in spike- coupling with the local oscillations, which may be a mechanism whereby neuronal responses caused by actions that lead to a desired outcome are propagated to downstream areas required for efficient association learning. Together, this data helps bridge the gap between previous operant and spatial tasks with touch-screen visual learning approaches and provides

evidence for spike-field coupling as a putative mechanism of how value expectancy signals may be propagated to influence behavior.

Acknowledgements

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CHAPTER 4

Coordination by Local Field Potentials is Disrupted During Reversal Learning in Moderate Prenatal Alcohol Exposed Mice

Marquardt, K., Cavanagh, J., Caldwell, K. and Brigman, J.L. (2017). Coordination by local field potential is disrupted during reversal learning in moderate prenatal alcohol epxosed mice. *In Preparation*

4a. INTRODUCTION

Fetal alcohol spectrum disorders (FASD) are the leading cause of preventable developmental disability, with estimated rates of up to 5% of live births (Ethen et al., 2009). In a study of over 400 individuals with either the most severe form, fetal alcohol syndrome (FAS) or an FASD, less than 20% had an IQ below 70, qualifying them as mentally challenged (Streissguth et al., 2004). However, in the same study, 61% of school aged children between 12 and 20 years were reported to have a disrupted school experience including suspension and expulsion (Streissguth et al., 2004, Green et al., 2009). Studies suggest that in children with FASD, difficulties in executive functioning, including planning, cognitive flexibility, and inhibition are better predictors of behavioral problems than intelligence (Mattson et al., 1999, Kodituwakku et al., 2001a). However, there are limited studies published on the neural activity changes that may be the cause of disruptions in executive functions and no studies have addressed frontal cortex activity during a task of executive function after prenatal alcohol exposure (PAE).

Discrimination reversal learning is a widely used paradigm for assessing behavioral flexibility across species. Regardless of stimulus modality, these tasks require a subject to learn a stimulus-outcome association, and then switch the reward association once it is well-learned. It is well established in rodent models of PAE utilizing spatial paradigms

that reversal learning is impaired (Wainwright et al., 1990, Thomas et al., 2004b, Allan et al., 2014). This appears to be a common feature across PAE models, as deficits have been reported across many exposure levels and exposure times (Marquardt and Brigman, 2016). Furthermore, we have shown that visual touch screen reversal learning is impaired after moderate PAE in adult mice (Marquardt et al., 2014).

Formation and reversal of stimulus-outcome associations are separately mediated by the dorsal striatum (DS) and orbital frontal cortex (OFC), respectively (Brigman et al., 2010a, Graybeal et al., 2011). Single unit studies have shown the OFC encodes value outcome and tracks this switch across reversal (Schoenbaum et al., 2003, Bissonette et al., 2008, Moorman and Aston-Jones, 2014). In addition, local field potentials (LFP), a regional measure of electrocorticographic data, synchronize in the theta frequency during expectancy outcome in the OFC (Pennartz et al., 2011). The DS is well known to signal action selection and mediate goal and habit, and signals well-learned reward outcome associations during visual touch-screen discrimination reversal learning (Yin et al., 2009, Corbit et al., 2012, Brigman et al., 2013). In addition, LFP studies show that during spatial decision making the DS synchronizes with the theta frequency in the hippocampus (DeCoteau et al., 2007b, Tort et al., 2008). However, within and between region activation and coordination has not been investigated utilizing either single-unit or LFP techniques in PAE models.

Here we examined the activity and coordination between the OFC and DS utilizing both single unit and LFP activity during a touch-screen visual discrimination reversal

paradigm to determine the underlying cause of cognitive inflexibility after PAE. We hypothesized that PAE would impair reversal by disrupting single unit and coordinated activity within and between the OFC and DS. This would lead to over activation of the DS, driving maladaptive perseveration during reversal. To test this hypothesis we utilized *in vivo* electrophysiology, paired with our visual touch-screen reversal, with single region bilateral electrodes to establish single unit responses separately in both regions across the entire discrimination reversal paradigm, recording at established learning criterions. Secondly, we utilized dual, OFC-DS region electrodes to analyze LFP dynamics and focused *in vivo* recordings at the beginning of reversal to examine coordinated activity within each region and establish functional connectivity between regions. Together, we aimed to establish the coordination within and between the OFC and DS during reversal learning to examine the neural activity changes that result in maladaptive repetitive behaviors after PAE.

4b. MATERIALS AND METHODS

Prenatal Alcohol Exposure Model. Male and Female C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were housed singly in a temperature- and humidity-controlled vivarium under a reverse 12 h light/dark cycle (lights off 0800 h). Female mice underwent a limited access voluntary prenatal alcohol exposure paradigm previously established (**Figure 2**) (Brady et al., 2012, Brady et al., 2013, Marquardt et al., 2014). Two hours into the dark cycle, female mice were given access to either 0.066% (w/v) saccharin or an ethanol solution (5% w/v for two days, then 10% w/v) sweetened with 0.066% (w/v) saccharin. Access was given daily for four hours (from 1000 to 1400 hr)

during the dark cycle. Voluntary consumption was recorded by weight of solution consumed per kg of female weight. After one week of drinking 10% ethanol + saccharin or the water-saccharin control solution, individual females were placed into the cage of a singly housed male overnight, immediately following the drinking period. Females continued to consume ethanol and saccharin solutions throughout the consecutive five-day mating period. Pregnancy was positively determined by monitoring weight gain every 3-4 days. Dams were weaned off alcohol solution beginning on post-natal day 0 using a step-down procedure over 6-days, halving ethanol concentration every other day. Offspring were weaned at approximately 4 weeks of age and housed in groupings of 2-4 per cage in a temperature- and humidity- controlled vivarium under a reverse 12 h light/dark cycle (lights off 0800 h) and tested during the dark phase. All behavior was conducted on adult male offspring (n=15 per treatment). Beginning at 6-7 weeks of age, offspring were food-restricted to 85% of their free-feeding body weight. Operant training began once mice reached food-restricted weight. All experimental procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee.

Touch-Screen Operant Chambers. All operant behavior was conducted in a custom made acrylic chamber measuring 21.6 x 17.8 x 12.7 cm covered in matte white contact paper, based on the Med Associates design (model # ENV-307W, Med Associates, St. Albans, VT). The chamber was housed within a sound- and light- attenuating box (Med Associates, St. Albans, VT). A pellet dispenser delivering reward (14 mg dustless pellets;

#F05684, BioServ, Frenchtown, NJ) into a magazine, a house-light, tone generator and an ultra-sensitive lever was located at one end of the chamber. At the opposite end of the chamber there was a touch-sensitive screen (Conclusive Solutions, U.K.) covered by a black acrylic aperture plate allowing two 2 x 5 cm touch areas separated by 0.5 cm and located at a height of 6.5 cm from the floor of the chamber. Stimulus presentation in the response windows and touches were controlled and recorded by the K-Limbic Software Package (Conclusive Solutions, U.K.).

Operant Training. Mice were habituated to the operant chamber and to eating out of the pellet magazine by being placed in the chamber for up to 30 min with pellets available in the magazine. Mice retrieving 10 pellets within 30 min were moved onto pre-training. Mice were given a three-stage pre-training regimen. First, mice were trained to obtain reward by pressing a lever within the chamber on an FR1 schedule. Mice pressing and collecting 30 rewards in under 30 minutes were moved to touch training. During this stage, a lever press led to the presentation of a white (variously-shaped) stimulus in 1 of the 2 response windows. Throughout the paradigm, images on the touch screen were spatially pseudorandomized preventing side bias and ensuring location was not an informative variable. The stimulus remained on the screen until a response was made. Touches in the blank response window had no effect, while a touch to the white stimulus resulted in reward delivery, immediately cued by a tone and illumination of the magazine light on the opposite side of the operant chamber from the touch screen. Mice initiating, touching and retrieving 30 pellets within 30 min were moved to the final stage of pre-training. This stage was identical to touch training except that responses at the blank

window during stimulus presentation produced an immediate 10 sec timeout, signaled by illumination of the house light, to discourage indiscriminate screen responding. Errors on this, and all subsequent stages, were followed by correction trials in which the same stimuli and left/right position was presented until a correct response was made. Mice making ≥75% (excluding correction trials) of their responses at a stimulus-containing window over a 30-trial session were implanted with a fixed electrode array.

Stereotaxic Electrode Array Implantation. After completing pre-training and at least two consecutive days of free-feeding, mice were anesthetized with isoflurane and placed in a stereotaxic alignment system (Kopf Instruments, Tujunga, CA) for implantation of a microelectrode array. Each array (Innovative Neurophysiology, Durham, NC) was comprised of 16 individual 35 μm-diameter tungsten microelectrodes arranged into 2 bundles of 2x4 electrodes (150 μm row/column spacing). Bilateral OFC targeting arrays had 2.75 mm lateral spacing between bundles with targeting coordinates: AP +2.60, ML +1.375, DV -2.60. Bilateral DS targeting arrays had 4.2 mm lateral spacing between bundles with targeting coordinates: AP +0.75, ML +2.04, DV -2.80. Dual OFC-DS arrays had a custom offset between bundles with targeting coordinates for OFC: AP +2.60, ML +1.375, DV -2.60; and targeting coordinates for DS: AP +0.75, ML +2.04, DV -2.80. After 7 days of recovery, body weight reduction resumed and mice were given a post-surgery reminder consisting of the last pre-training session to ensure retention of pre-training criterion.

Discrimination Reversal Paradigm. Following array implantation, all mice were tested on a pairwise discrimination reversal paradigm as previously described (Brigman et al., 2010a, Marquardt et al., 2014). Mice were first trained to discriminate two novel, approximately equiluminescent stimuli, presented spatially pseudorandomized across a possible 30-first presentation trials (not including correction trials) per daily session. As in pre-training, responses at the correct stimulus resulted in reward, immediately cued by the onset of a 1 sec tone; responses at the incorrect stimulus resulted in timeout, immediately cued by a 10 sec house-light followed by correction trials until a correct response was made. Due to the pseudorandomized location of the stimuli, correct performance during a session could not exceed 50% if a side bias was present. Correct stimuli were balanced across mice. Discrimination criterion was ≥85% correct responding (excluding correction trials) over two consecutive sessions. Reversal training began on the session immediately after discrimination criterion was attained, independently for each mouse. Here, the designation of correct verses incorrect stimuli was reversed for each mouse. Mice were trained on 30-trial daily sessions (same as for discrimination) to a criterion of ≥85% correct responding (excluding correction trials) over two consecutive sessions. In order to measure performance differences across distinct sessions, percent correct responses, total errors, reaction time (time from lever press initiation to screen touch) and magazine latency (time from screen touch to reward retrieval) were analyzed.

In vivo Electrophysiology. Neuronal activity was continuously recorded during specific performance sessions using a multichannel acquisition processor (OmniPlex, Plexon,

Dallas, TX) as previously described (Brigman et al., 2013, DePoy et al., 2013). Single unit and oscillatory activity was captured during the following stages in single region bilateral OFC and DS electrode implanted mice: **Discrimination Criterion** = session of discrimination criterion attainment (% correct >85%), **First Reversal Session** = first session of reversal when perseverative responding is highest (% correct <20%), **Chance Reversal** (Chance) = session of reversal where chance performance was re-attained (% correct =50%), and **Reversal Criterion**= session of reversal where criterion is re-attained (% correct >85%). Single unit and oscillatory activity was captured during the following concurrent states in dual OFC-DS electrode implanted mice: **Discrimination Criterion**, **First Reversal Session**, **Third Reversal Session** = third session of reversal when perseverative responding begins to decrease, **Fourth Reversal Session** = fourth sequential session of reversal, and **Chance Reversal**. If mice obtained chance criterion (50% correct) during the third or fourth sessions of reversal, recording session was labeled chance reversal and mice were not recorded further.

Continuous spike signal was sampled at 40 kHz and waveforms were manually sorted during recording, based on manually set voltage threshold. Local field potential was sampled from the same electrodes at 1 kHz and automatically low band pass filtered at 200 Hz. Neuronal recording data was timestamped by responses from K-Limbic software by TTL pulse to reward tone and punishment house light. At the completion of testing, array placement was verified by electrolytic lesions made by passing 100 µA through the electrodes for 20 sec using a current stimulator (S48 Square Pulse Stimulator, Grass Technologies, West Warwick, RI). Brains were removed post perfusion with 4%

paraformaldehyde, 50 μm coronal sections cut with a vibratome (Classic 1000 model, Vibratome, Bannockburn, IL), stained with cresyl violet and placement verified with reference to a mouse brain atlas (Paxinos and Franklin, 2001) (**Figure 12E**).

Waveform Analysis. Waveforms were re-sorted offline using principal component analysis of spike clusters and visual inspection of waveform and inter-spike interval <1% shorter than 2 ms using Offline Sorter (Plexon Inc, Dallas, Texas). TTL pulse timestamps recorded concurrently with neuronal data were used to create epochs of firing rate spanning 1 sec pre-choice to 3 sec post-choice in averaged bins of .05 sec with NeuroExplorer software (NeuroExplorer; NEX Technologies, Littleton, MA). The 3 sec post-choice analysis window overlapped with immediate tone, during correct choice, and the first 3 sec of house-light during incorrect trials allowing for analysis of immediate response to secondary associative cues. If reward retrieval occurred prior to the end of the 3 sec analysis window on correct trials, the epoch for that trial was truncated as to prevent overlap with reward signaling. Less than 5% of neurons showed baseline firing rate, defined at the 1 sec pre-event, of >15Hz and were categorized as fast-spiking interneurons and excluded from analysis as performed previously (Brigman et al., 2013). Neurons with average baseline-firing rate of 0 spikes/bin were also excluded from the analysis. Normalized firing rates were calculated by Z-scoring 3 sec post-choice activity to 1 sec pre-choice baseline. Pattern of firing rate changes were examined for the period during immediate cue delivery (choice → 1 sec post), movement time (1 → 2 sec post) and magazine approach (2 → 3 sec post) using repeated-measures ANOVA followed by the Fisher's post-hoc test.

In addition to spike-firing activity changes, the proportion of the population of neurons that significantly increased their firing rate in the post-event period (compared to individual pre-event baseline using Student's t-test; also known as behaviorally responsive neurons) were analyzed across type and discrimination reversal session using chi square. All event-responsive neurons were analyzed, independent of significant response to other timestamped events.

Local Field Potential Analysis. Time-frequency analyses were adapted to depth recordings from methods previously described (Cavanagh et al., 2009) and computed using custom Matlab (TheMathWorks) scripts. LFP data were averaged across electrodes within a region and aligned to time of choice, from 1 sec pre to 3 sec post. Each subject, during each recording session, was analyzed individually, and mice completing less than 18 of the choice type were excluded from that session to maximize the signal to noise ratio. The defined choice types: correct and error, were convolved separately with a set of complex Morlet wavelets, defined as a Gaussian-windowed complex sine wave: $e^{-i2\pi f}e^{-12/(2*\sigma 2)}$, where t is time, f is frequency (from 1 to 80 Hz in 80 logarithmic spaced steps to maximize lower frequency visualization), and σ is the width of each frequency band set at $4/(2\pi f)$. Convolved data was then used to separately calculate the instantaneous phase within each region, and the phase synchrony between regions. Instantaneous phase of the time-frequency oscillations was calculated at each frequency by finding the angle of the resulting convolved data: $ITPC_{R(t)} = \sum_{r=1}^{n}\left|e^{i(\theta_t)}\right|$, where *R(t)* was the convolved time-frequency data at each time point (*t*) and trial (*r*). Instantaneous phase synchrony between regions of the time-frequency oscillations was

calculated: $ISPC_{z(t)} = \sum_{t=1}^{n} \left| e^{i(\theta_{xt} - \theta_{yt})} \right|$, where *z(t)* is the convolved time-frequency data at each time point (*t*), and θ_{xt} θ_{yt} are phase angles from regions *x* and *y* at time point *t*. Two regions of interest (ROI) were defined by statistical significance above background levels, defined separately for ITPC and ISPC. ROIs were calculated from averaged subject, session and region ITPC or ISPC data to avoid subject, session or region bias. ROI boundaries were used to calculate average ITPC or ISPC values within each subject, session and region used for statistical comparison. A linear mixed model (R, lmr4), which is robust against missing data, was used to fit the repeated cross-session ROI averages with session and region as within factors, and treatment as a between factor, to determine the contribution and interaction of all dimensions of the data. Statistically significant contributions of factors and interactions were determined by ANOVA and post-hoc Tukey's tests.

Spike-Field Coupling Analysis.

Spikes during correct and incorrect choice types were analyzed separately, and mice completing less than 18 of the choice type were excluded from that session to maximize the signal to noise ratio. Spikes occurring during a trial were windowed into 4, 1000 ms bins, from -1000 ms pre-choice to +3000 ms post-choice (spike # average per epoch per subject: SAC DS 298; SAC OFC 602; PAE DS 200; PAE OFC 440). During each recording session, instantaneous phase angles were calculated at the time of spike occurrence: $A_j(f) = \arctan(\frac{imag(Z_j)}{real(Z_j)})$, where Z$j$ is the local field potential at every time point (*j*) after convolution with a complex Morlet wavelet family defined as a Gaussian-windowed complex sine wave: $e^{-i2\pi f} e^{-12/(2*\sigma 2)}$, where *t* is time, *f* is frequency set from

1 to 40 Hz in 40 linear spaced steps, and σ is the width of each frequency band set at $4/(2\pi f)$, for an average tradeoff between time and frequency precision (Cohen, 2014a). Within each bin, averaged over 500 bootstraps, 200 angles were used to compute the paired phase consistency (PPC1) value (Vinck et al., 2012) as the cosine of the angular difference between each angle within the vector: $PPC = \sum_{j=1}^{N}\sum_{k=j+1}^{N}\left|mean(e^{(i(\theta_j-\theta_k)})\right|$, where θ is the angle in radians and N is defined by length of LFP epoch and number of spikes. Furthermore, PPC1 removes angle comparisons from same trials, further reducing trial bias. Due to the trial count limitation criterion, PPC2, which further controls for within-trial bias, was not utilized, however preliminary tests indicate similar results as in PPC1 (data not shown). The 1000 ms bin beginning at tone offset was used for further analysis based on coherence data. Data were divided into three frequency bins, low (5-15 Hz), med (16-25 Hz), and high (26-40 Hz), and averaged for fit into a linear mixed model autogregression (R, lmr4), which is robust against missing data. Statistically significant contributions of factors and interactions were determined by ANOVA and post-hoc Tukey's tests.

4c. RESULTS

There was no significant effect of PAE treatment or electrode implant type on sessions to complete discrimination (SAC 11.4±1.2 sessions, PAE 10.6±2.0 sessions). In addition, there was no significant effect of treatment on total incorrect (TIC) or correction trials (CT) required to reach discrimination criterion (**Figure 11A, B**; TIC-SAC 104.7±19.0, CT-SAC 201.6±36.1; TIC-PAE 93.0±14.7, CT-PAE 195.3±23.7). There were no significant effects of PAE treatment on average number of sessions, incorrect or correct

trials (TC) to reach criterion after reversal (**Figure 11A, B**; SAC 17.5±1.7 sessions, PAE 15.9±6.7 sessions; TIC-SAC 287.8±30.9, CT-SAC 492±41.8, TIC-PAE 175.6±18.6, CT-PAE 466.9±38.2). However, SAC control mice implanted with bilateral OFC electrodes took significantly more sessions and total incorrect trials, but not correction trials to re-obtain reversal criterion (**Figure 11A**; Sessions Main Effect of Implant F2, 44=4.485, P<.05; TIC Main Effect of Implant F2,44=5.115, P<.05).

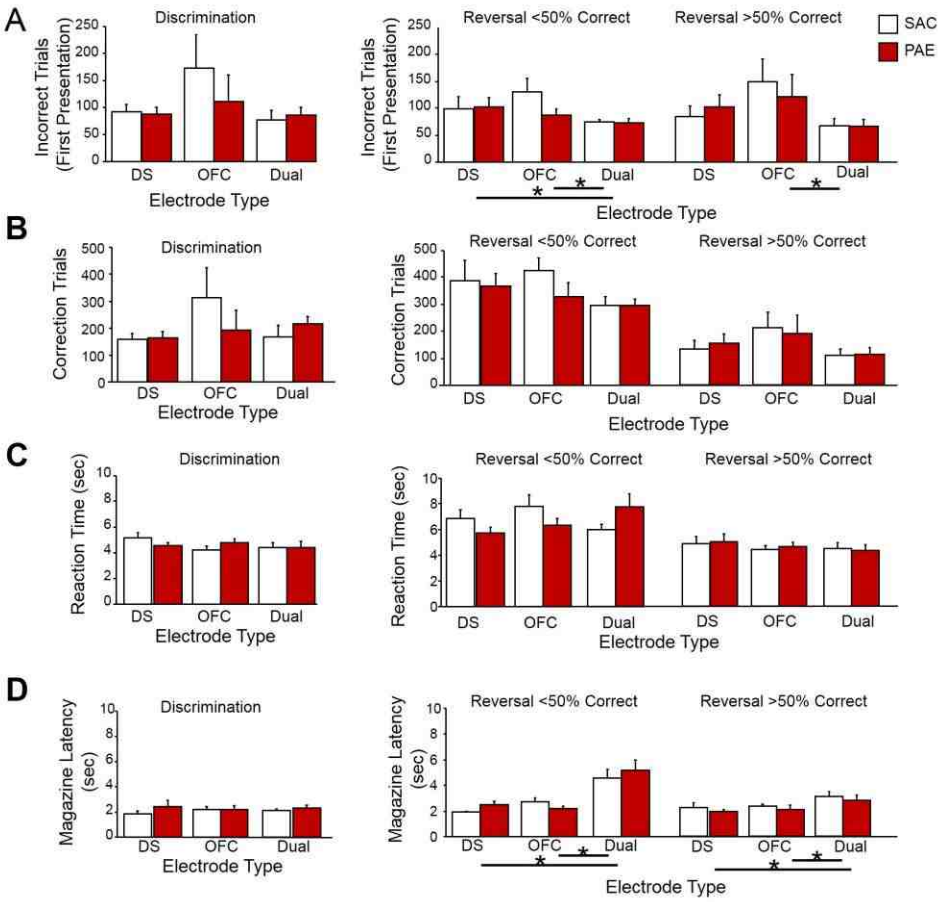

Figure 11. PAE Electrode Implanted Mice Are Not Impaired Across Discrimination or Reversal. (A) First presentation incorrect trials are not altered by PAE during either discrimination, perseverative (reversal <50% correct) reversal or re-learning during reversal (reversal >50% correct). However, Dual implanted mice completed significantly fewer incorrect trials during reversal than single region DS or OFC implanted mice. (B) Correction trials increase during perseverative phase of reversal in all implanted mice, regardless of treatment. There was no effect of treatment on either reaction time (C) or magazine latency (D). However, Dual implanted mice had significantly higher latency to retrieve reward (D) than either OFC or DS single region implanted mice. Data are means ±SEM. N=6 SAC OFC, 6 SAC DS, 12 SAC Dual, 7 PAE OFC, 7 PAE DS, 11 PAE Dual. *=*P*<.01, main effect of electrode type.

In single region bilateral electrode implanted mice first presentation incorrect trial number followed learning, with significantly higher levels during chance and first day reversal sessions compared to criterion sessions (**Figure 12A**, Main Effect of Session $F_{4,88}=40.510$, $P<.001$, followed by Fisher's Post-hoc test). First presentation errors were not significantly different between chance stages and the first session of reversal. There were no significant effects of PAE treatment on first presentation incorrect trials in single region implanted mice. In dual region implanted mice, first presentation incorrect trials

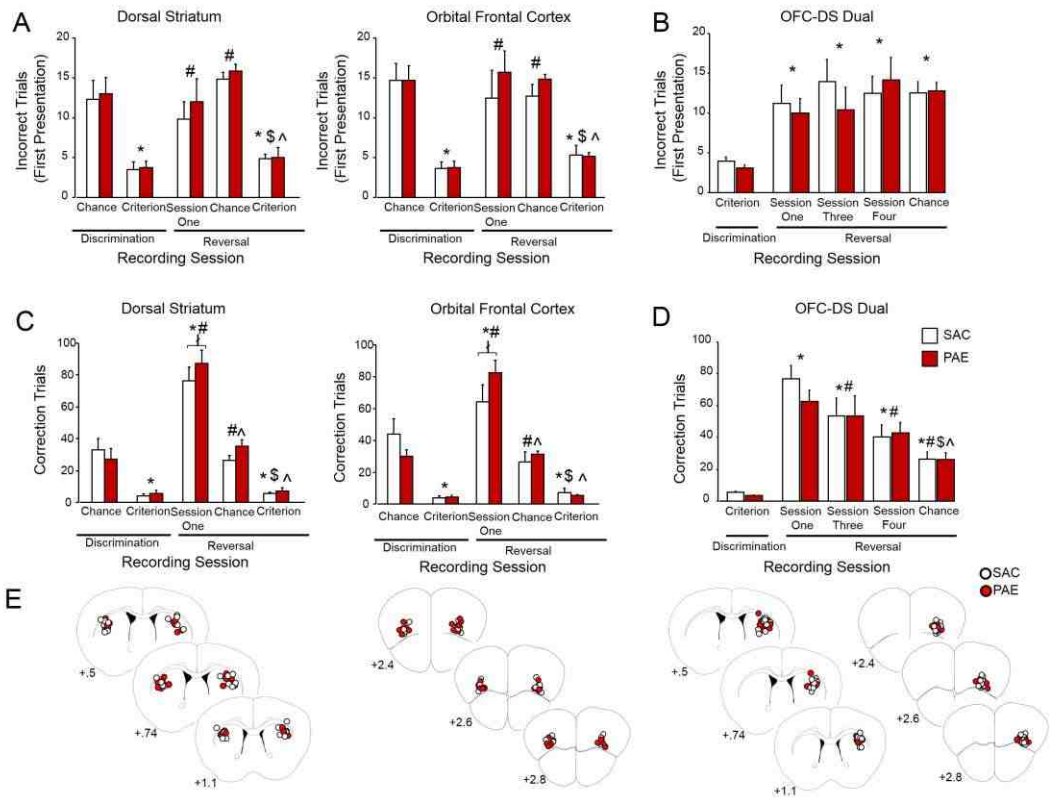

Figure 12. Selective Analysis of Recording Sessions Reveals PAE Reversal Impairment. PAE treatment did not alter the number of incorrect trials completed on each recording session for single region (A) or dual region implanted mice (B). (C) PAE treated mice completed significantly more correction trials on the first session of reversal in single region DS or OFC implanted mice. *=P<.05, difference from discrimination chance; #=P<.05, difference from discrimination criterion; ^=P<.05, difference from reversal session one; $=P<.05, difference from reversal chance; ǂ=P<.05, sig difference between treatments; *=P<.05, difference from discrimination criterion; #=P<.05, difference from reversal session one; ^=P<.05, difference from reversal session three; $=P<.05, difference from reversal session four (D) Correction trials decreased across early reversal sessions, however, PAE treated mice did not complete more correction trials than SAC controls on any recorded session. *=P<.05, difference from discrimination criterion; #=P<.05, difference from reversal session one; ^=P<.05, difference from reversal session three; $=P<.05, difference from reversal session four (E) Center placement of electrolytic lesions for implanted electrodes: bilateral DS (left), bilateral OFC (middle), Dual OFC-DS (right). Data are means ±SEM.

significantly increased from discrimination criterion to the first session of reversal (**Figure 12B**; Main Effect of Session $F_{4,60}=12.115$, $P<.001$, followed by Fisher's Post-hoc test), and remained elevated across early to chance reversal sessions.

Significantly more correction trials were completed during the first day of reversal than all other recorded sessions in single-region electrode implanted mice (**Figure 12C**; Main Effect of Session $F_{4,88}=127.685$, $P<.001$, followed by Fisher's Post-hoc test). PAE mice performed significantly more correction trials on the first reversal session than SAC controls, independent of single-region electrode implant type (**Figure 12C**; Session x Treatment Interaction $F_{4,88}=3.002$, $P<.05$). Dual electrode implant mice, with sequential early reversal day recordings showed significantly more correction trials completed on the first day of reversal than any other recorded session (**Figure 12D**; Main Effect of Session $F_{4,60}=28.658$, $P<.001$, followed by Fisher's Post-hoc test), with a decreased step-wise pattern across reversal. However, there was no significant effect of treatment on correction trials, during any day of reversal, in dual-region electrode mice.

There were no training differences in time to obtain the operant task between treatments or implant type. There were no significant differences in the average number of trials completed during a recording session between treatments or electrode implant type (data not shown). In addition, there were no differences in average reaction time for single region bilateral or dual electrodes implanted mice (**Figure 11C**). However, dual electrode mice took significantly longer to retrieve a food reward during reversal learning independent of treatment (**Figure 11D**; Main Effect of Implant $R<50$ $F_{2,43}=11.38$,$P<.05$,

R>50 F2,44=3.8, P<.05). There were no effects of maternal drinking level on correction trial number (**Appendix E. Supp. Figure 1**).

Experiment 1. Single region spike-firing analysis

We recorded 205 DS putative medium spiny neurons and 191 OFC putative pyramidal neurons in SAC controls and 233 DS and 236 OFC neurons in PAE mice.

DS neurons in SAC control animals significantly increased firing rate above baseline levels after a correct choice during reward approach, in every session (**Figure 13A-E middle**; Main Effect of Time D50: F3,312=16.492, P<.001; D85: F3,204=3.559, P<.05; RS1: F3,255=5.366, P<.01; R85: F3,228=6.659, P<.05). Firing rate response was not significantly modulated across sessions. In PAE animals, DS response to reward approach during all stages of reversal was increased within two second after choice, instead of three as in control mice (**Figure 13C-E center**; RS1: Time x Treatment Interaction F19,1615=1.610, P<.05; R50: Time x Treatment Interaction F19,1501=1.645, P<.05; R85: Main Effect of Treatment F19,1444=8.012, P<.01, Time x Treatment Interaction F19,1444=2.614, P<.01). Only during reversal criterion did DS response in PAE animals remain significantly greater than control levels three seconds after a correct choice (**Figure 13E**; Time x Treatment Interaction F19,1444=1.600, P<.05). Finally, the proportion of phasic neurons in response to a correct choice in the DS changed significantly across sessions in both SAC and PAE animals (SAC χ^2=17.74, PAE χ^2=19.79). However, in PAE treated animals, significantly more neurons responded to a

correct choice during both the first session (RS1) and chance criterion (R50) of reversal compared to SAC control mice (**Figure 13C, D right**; RS1 χ^2=7.27, R50 χ^2=63.62).

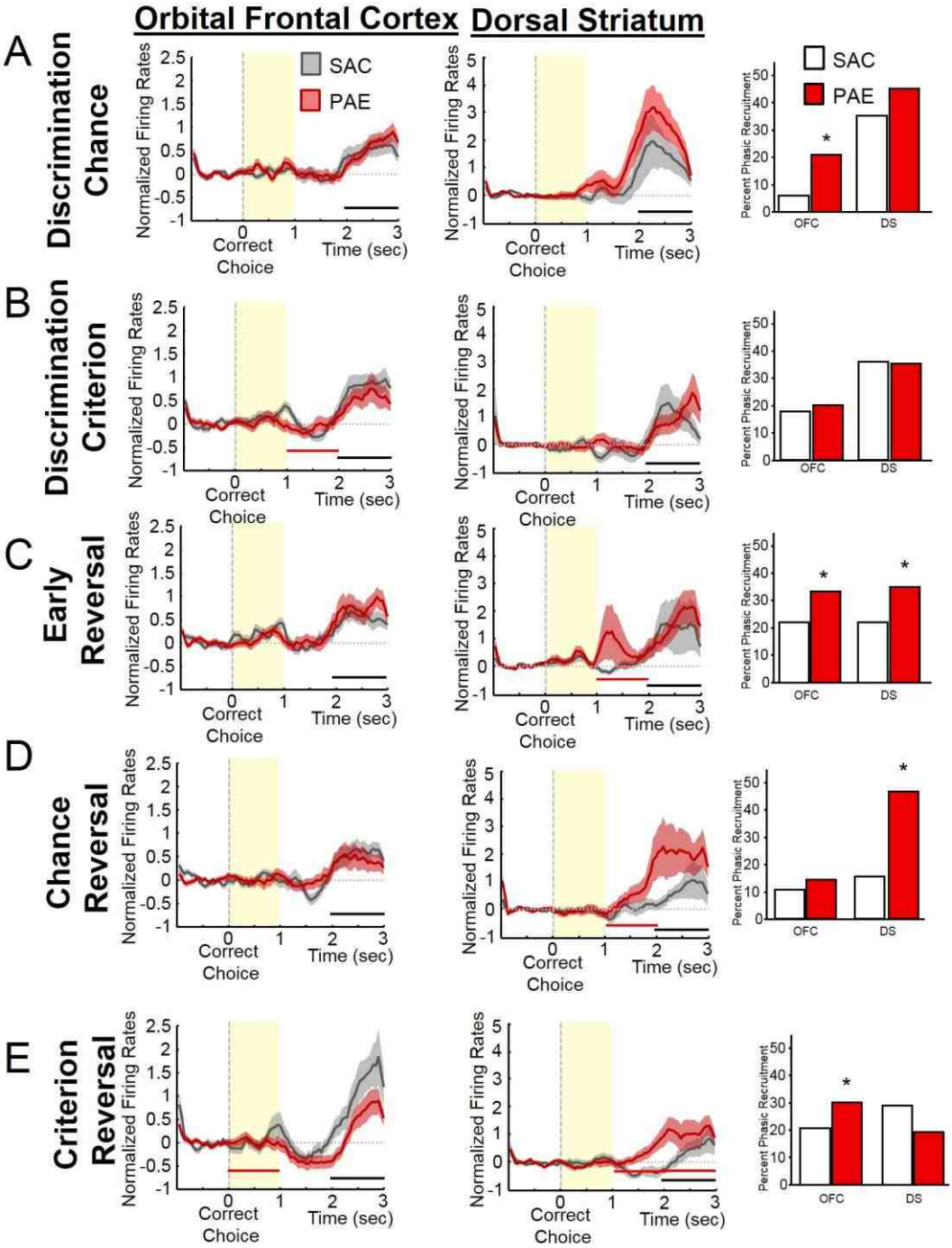

Figure 13. Single Unit Firing Changes and Recruitment in Response to Correct Choice. (A) Discrimination Chance: Average firing significantly increased during the 2- 3 sec epoch for both OFC (right) and DS (center). There were no significant effects of PAE on firing rate, but significantly more neurons were recruited in the OFC (right) while there were no differences in DS. (B) Discrimination Criterion: Average firing significantly increased during the 2- 3 sec epoch for both OFC (right) and DS (center). PAE significantly altered firing rate 1-2 sec after correct choice in the OFC, but did not alter either DS firing or neuronal recruitment.

Figure 13. continued from page 87.... (C) Early Reversal: Average firing significantly increased during the 2- 3 sec epoch for both OFC (right) and DS (center). PAE significantly increased firing rate from 1-2 sec after correct choice in the DS. In addition PAE significantly increased phasic neuron recruitment in both OFC and DS (right). (D) Chance Reversal: Average firing significantly increased during the 2- 3 sec epoch for both OFC (right) and DS (center). PAE significantly increased DS firing in the 1-2 sec post correct choice and increased the number of recruited neurons in the DS only (right). (E) Criterion Reversal: Average firing significantly increased during the 2- 3 sec epoch for both OFC (right) and DS (center). PAE altered the immediate response of the OFC after a correct choice and increased DS response from 1-3 sec post-correct choice. In addition, in the OFC significantly more neurons were recruited in PAE animals (right). Gray shading indicates the presence of the associative cue: tone (correct) or house-light (error-cue). Data are means ± SEM. Black bar=P<.05, difference from baseline; red bar=P<.05, difference from control. *=P<05

OFC response to correct choice is very similar to that of the DS, with increased in firing rate in response to reward approach three seconds after correct choice across learning sessions in SAC control animals (**Figure 13A-E left**; Main Effect of Stage D50: $F_{2,297}=18.472$, P<.001, D85: $F_{3,222}=16.448$, P<.001, RS1: $F_{3,231}=16.465$, P<.001, R50: $F_{3,252}=12.336$, P<.001, R85: $F_{3,228}=20.258$, P<.001). There were no significant differences in firing rates between sessions. Firing rate in the OFC after PAE treatment was decreased only during criterion stages, two seconds after a correct choice during discrimination criterion, and during the tone on reversal criterion (**Figure 13B,E**; D85: Time x Treatment Interaction $F_{19,1406}=2.302$,P<.05, R85: $F_{19,1444}=1.883$,P<.05). Phasic recruitment of neurons in the OFC at a correct choice significantly changed across sessions in both SAC and PAE animals (**Figure 13A-E right**; SAC $\chi^2=103.44$, PAE $\chi^2=12.87$). PAE treated animals had a greater proportion of choice responsive neurons during chance discrimination (D50), the first session (RS1) of reversal and reversal criterion (R85; **Figure 13A-E right**; D50 $\chi^2=37.38$, RS1 $\chi^2=5.55$, R85 $\chi^2=4.18$).

DS neurons in SAC control animals slowly ramped up an incorrect response to be significantly increased within two to three seconds post choice, during all stages except

discrimination and reversal criterion (**Figure 14A-E middle**; Main Effect of Time D50: F19,1900=1.769, P<.05, R50: Main Effect of Time F19,1387=2.458, P<.05).

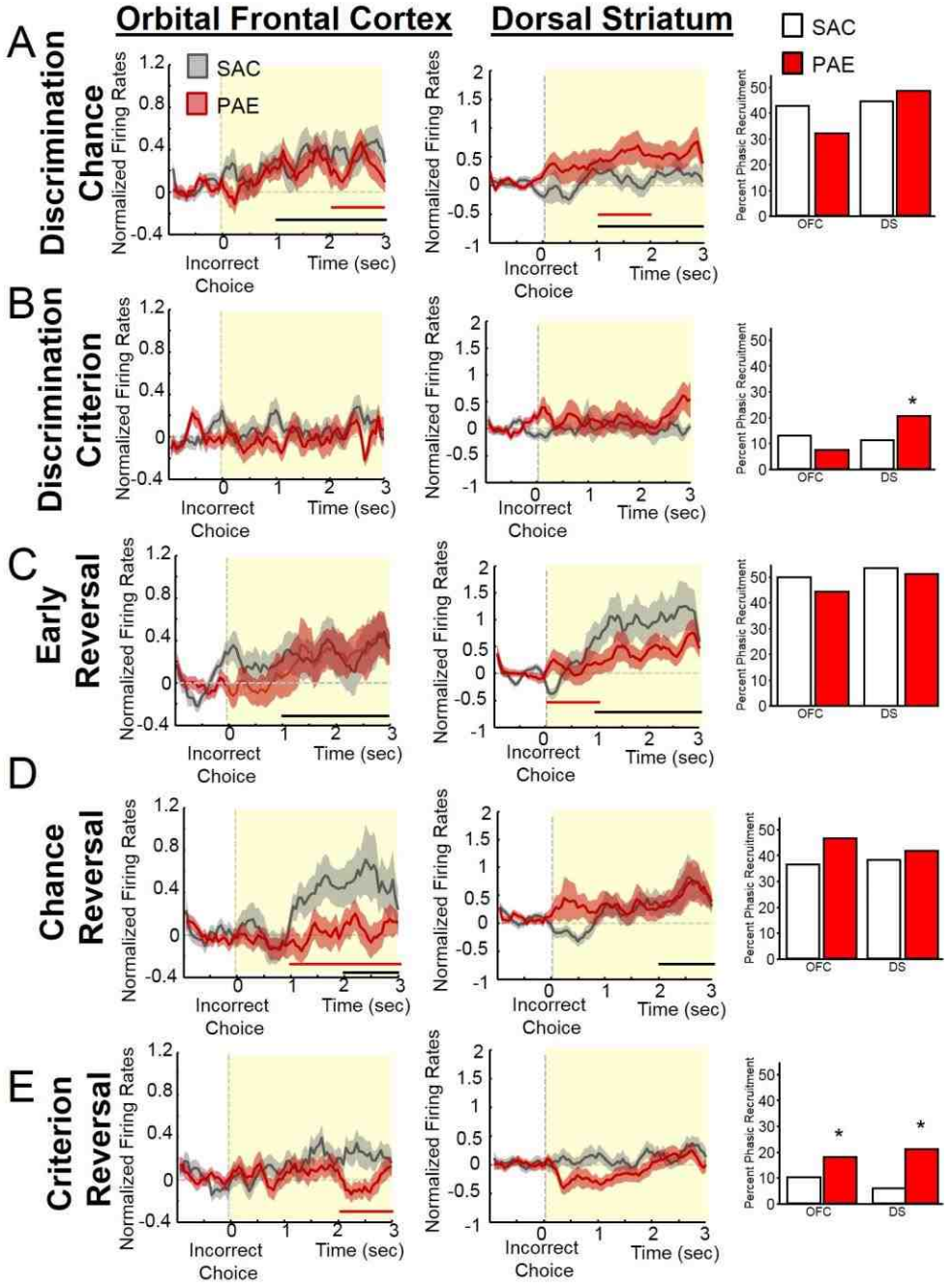

Figure 14. Single Unit Firing Changes and Recruitment in Response to Incorrect Choice. (A) Discrimination Chance: Firing rate significantly increased in SAC control and PAE animals in the time interval 1-3 sec post-incorrect choice in both the OFC (left) and DS (center). PAE treatment resulted in a significantly different firing rate pattern in the OFC during the last sec of the epoch (2-3 sec). In addition PAE treatment significantly increased DS firing from 1-2 seconds. There were no alterations in neuron recruitment (right). (B) Discrimination Criterion: There were no significant increases in average firing rate in either the OFC (left) or DS (center).

Figure 14. continued from page 89… No alterations were seen in firing rate response PAE treated mice. Significantly more DS neurons were recruited in PAE mice (right). (C) Early Reversal: Firing rate significantly increased in SAC control and PAE animals in the time interval 1-3 sec post-incorrect choice in both the OFC (left) and DS (center). PAE treatment altered immediate firing rate response in the DS (0-1 sec), but did not alter neuronal recruitment (right). (D) Chance Reversal: Firing rate significantly increased in SAC control and PAE animals in the time interval 2-3 sec post-incorrect choice in both the OFC (left) and DS (center). PAE treatment significantly decreased OFC firing rate response from control levels during the time interval 1-3 sec post-choice. No alterations in phasic recruitment were seen (right). (E) Criterion Reversal: There were no significant increases in average firing rate in either the OFC (left) or DS (center). OFC firing rate 2-3 sec post incorrect choice was decreased in PAE animals. In addition, neuron recruitment in both the OFC and DS was increased after PAE treatment (right). Gray shading indicates the presence of the associative cue: tone (correct) or house-light (error-cue). Data are means ± SEM. Black bar=P<.05, difference from baseline, red bar=P<.05, difference from control. *=P<05

Firing rate changes on the first session of reversal were significantly different compared to all other sessions (Main Effect of Session $F_{4,84}=4.184$, $P<.05$, followed by post-hoc test), with a continued increase across an epoch making the third second significantly greater than the first (**Figure 14C center**; Main Effect of Time $F_{3,264}=8.189$, $P<.01$, followed by post-hoc test). In PAE mice, firing rate response during chance discrimination, but not chance reversal, was increased two seconds post-response (**Figure 14A center**; Time x Treatment Interaction $F_{19,1900}=1.850$, $P<.05$). Additionally in PAE animals, immediate response to an incorrect choice during the first session of reversal was decreased compared to SAC controls (**Figure 14C center**; Time x Treatment Effect $F_{19,1672}=2.855$, $P<.01$). PAE treated and SAC control animals had equal levels of neurons responsive to an incorrect choice, except during criterion stages when PAE treated animals had more responsive neurons (**Figure 14A-E right**; D85 $\chi^2=8.13$, R85 $\chi^2=4.18$). Both PAE and SAC animals significantly changed phasic recruitment across learning sessions (SAC $\chi^2=53.67$, PAE $\chi^2=25.16$).

For incorrect choices in SAC control animals, the OFC increased firing rate within two seconds of an incorrect response during every session, except criterion sessions (D50:

F3,288=6.638, P<.05; RS1: F3,237=3.422, P<.05; R50: F3,252=5.029, P<.05). The magnitude of response to incorrect choices in SAC controls were not significantly different between sessions. However, firing rate changes to an incorrect response were significantly faster during chance discrimination than chance reversal, with an increased firing rate occurring two seconds instead of three post-incorrect response (**Figure 14A,D**; Main Effect of Time D50: F3,288=6.638, P<.05; R50:F3,252=5.029, P<.05, followed by post-hoc test). Response to an error on the first session of reversal in the OFC of control animals continued a steady increase in firing rate across a trial (**Figure 14C**; Main Effect of Time RS1: F3,237=.0180, P<.05, followed by post-hoc test). During discrimination chance, PAE treatment resulted in significantly different altered firing rate pattern, but not amplitude, three seconds after an incorrect choice (**Figure 14A**; Time x Treatment Interaction F19,1862=2.220, P<.01). There were no differences between PAE treated and SAC control firing rate responses to an incorrect choice during the first session of reversal in the OFC. On chance reversal, PAE treated mice had a significantly decreased firing rate two and three seconds after choice (**Figure 14D**; Main Effect of Treatment F1,1596=74.711, P<.06; Three: Time x Treatment Interaction F19,1596=2.075, P<.05). PAE response was significantly decreased during the third second of criterion reversal, even though control response was not significantly elevated above baseline levels (**Figure 14E**; Main Effect of Treatment F1,1425=5.691, P<.05). Phasic neuron proportions were significantly different across sessions in both SAC and PAE treated animals (SAC χ^2=41.69, PAE χ^2=39.63). In the OFC of PAE treated mice, a greater percentage of neurons remained choice-responsive during reversal criterion (**Figure 14A-E right**; χ^2=5.76).

Experiment 2. Local field potential analysis of early reversal sessions

Due to previous results suggesting behavioral impairment was limited to early reversal sessions, recording of dual regions was isolated to early reversal prior to re-obtainment of chance levels of performance, to further characterize the behavior and role of the OFC and DS in behavioral flexibility.

Phase. In both the DS and OFC, there was an increase in coherence at the cessation of the associative cue for correct trials that was significantly above background levels when averaged across treatments. The higher frequency ROI (hfROI) spanned across delta and theta frequencies from 1.75 to 7 Hz and the lower frequency ROI (lfROI) peak occurred approximately 500 ms later in the low frequency range of 1 to 1.75 Hz (**Figure 15A**).

Coherence in SAC control animals did not significantly change across learning sessions in either ROI frequency range after a correct choice, despite the increase in average coherence on reversal session one compared to discrimination criterion. Coherence in the DS was significantly different from OFC in SAC control animals, peaking at a higher level during reversal session four in the hfROI and reversal sessions three, four and chance in the lfROI (**Figure 15B-C**; $F_{1,260}=12.576$, $P<.01$). Average coherence in the lfROI was decreased in PAE treated animals during reversal sessions three, four and chance reversal (**Figure 15B**; $F_{4,240}=3.447$, $P<.05$, followed by post-hoc test). In PAE treated animals after correct choice, phase coherence in the hfROI was decreased on reversal session one, however this change was not significant in either region. Decreased coherence was significant on reversal sessions three and four, before returning to control

levels upon chance criterion in both regions (**Figure 15C**; F4,260=3.447, P<.05, followed by post-hoc test).

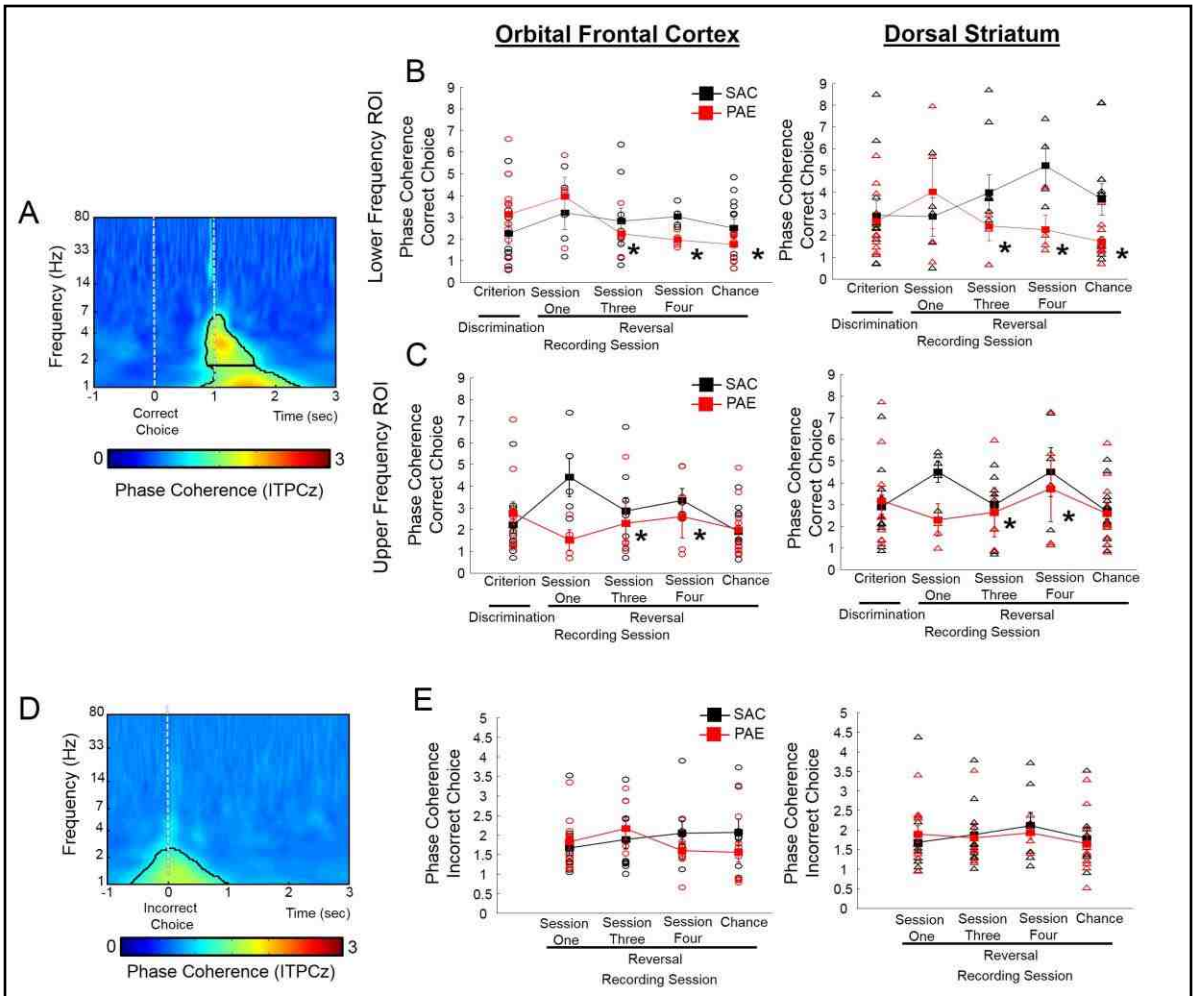

Figure 15. PAE Decreased Phase Coherence in the OFC and DS. (A) Region of interest for correct choice defined by significant difference from background. Upper frequency from 1.75 to 7 Hz. Lower frequency region from 1 to 1.75 Hz. (B) Average phase coherence for lower frequency region of interest after correct choice. PAE treatment significantly impairs coherence on reversal sessions three, four and chance in both the DS (right) and OFC (left). (C) PAE treatment decreases average phase coherence for upper frequency of interest after correct choice on reversal sessions three and four in both regions. First session of reversal is decreased, but not significant due to highly conservative statistical analysis and missing data due to few mice completing required trial numbers. (D) Region of interest for incorrect choice defined by significant difference from background. (E) PAE does not significantly alter average phase coherence in response to an incorrect choice across any session of reversal. Discrimination was unable to be measured due to the almost complete absence of incorrect trials during criterion sessions. Closed squares indicate treatment means with ±SEM. Open shapes are individual subject values, boarder color indicating treatment, with o = OFC and Δ = DS. * =P<.05, significant difference between treatments.

In both OFC and DS, during an incorrect response, and concurrent with error cue commencement, there was a significant increase in low frequency 1-2 Hz delta coherence (**Figure 15D**). Phase coherence was unable to be measured during discrimination criterion in either SAC or PAE animals due to low levels of incorrect responding, indicating no measurable corresponding brain related error response during this learning session. Increased coherence to an incorrect choice did not vary in intensity across reversal sessions, or differ between the OFC and DS in SAC control animals (**Figure 15E**). Additionally, while there is a visible decrease in phase coherence in PAE animals during reversal session 4 in the OFC, it did not reach statistical significance.

OFC – DS Synchrony. Average phase synchrony between regions occurred after a correct choice in response to the associative tone turning off, in accordance with phase coherence increases. Two ROIs were defined based on significant difference from background and an approximately 500 ms difference in time of peak synchrony (**Figure 16A**). Synchrony increased during reversal session one in the hfROI in SAC control animals, while remaining constant in the lfROI across all sessions, however no change reached statistical significance across recording sessions (**Figure 16B, C**). There were no significant differences in phase synchrony between SAC control and PAE treated animals utilizing a linear mixed model across sessions, in either ROI, even though synchrony was visually decreased in PAE animals in both frequency ranges. However, the *a priori* hypothesis that differences would be seen specifically during sessions of behavioral impairment led us to test reversal session one separately in a one-way ANOVA due to behavioral changes seen in single region mice. PAE treated animals had significantly less synchrony

during reversal session one in the hfROI (F1,7=7.528, P<.05; **Figure 16C**). There was no measurable synchrony in response to an incorrect choice in SAC control or PAE treated animals (data not show).

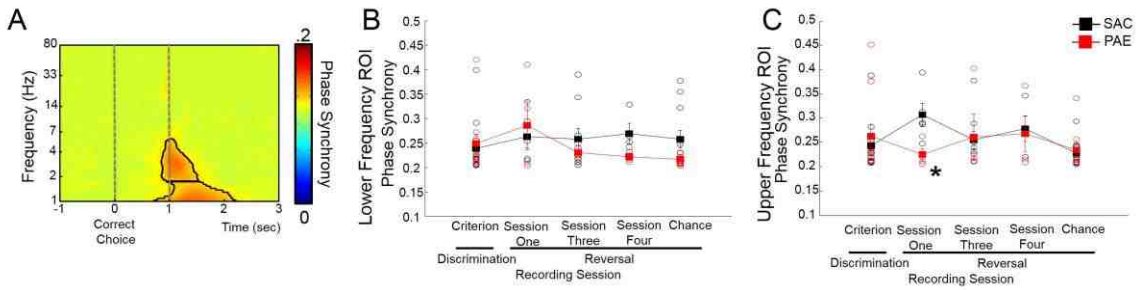

Figure 16. PAE Decreased Inter-region Phase Synchrony. (A) Region of interest for correct choice defined by significant difference from background. Upper frequency from 1.75 to 7 Hz. Lower frequency region from 1 to 1.75 Hz. (B) Lower frequency phase synchrony magnitude is not significantly altered after PAE. Nor is it modulated across reversal sessions. (C) Upper frequency synchrony increases in SAC control mice, on the first session of reversal, which is significantly decreased in PAE animals. * =P<.05, significant difference between treatments.

Spike-Field Coupling. Low frequency (5-15 Hz) spike field coupling was significantly different from both medium (15-25 Hz) and high (25-40 Hz) frequency coupling in both OFC and DS (Main Effect of Frequency F2,337=10.06, P<.01). Medium and high frequencies did not significantly differ from one another. In the OFC of SAC control animals there is high low-frequency spike-field coupling during discrimination criterion that decreases on the first session of reversal before returning to criterion levels by chance reversal (**Figure 17A-E top**). PAE mice spike-field coupling in the OFC was increased across reversal sessions 1 and 3 (**Figure 17B, C, F**), before falling below SAC control levels on reversal session 4 and chance (**Figure 17D-F**), although these differences did not reach significance (Treatment x Session Interaction F4,337=2.16, P=.073).

DS spike-field low-frequency coupling levels were high during discrimination criterion and low during early reversal sessions. Low- to mid-frequency coupling increased during reversal session four and mid- to high- frequency coupling increased during chance reversal (**Figure 17A-E lower**). DS spike-field coupling was significantly greater than OFC coupling during reversal session 4 (**Figure 17F**; Session x Frequency Interaction

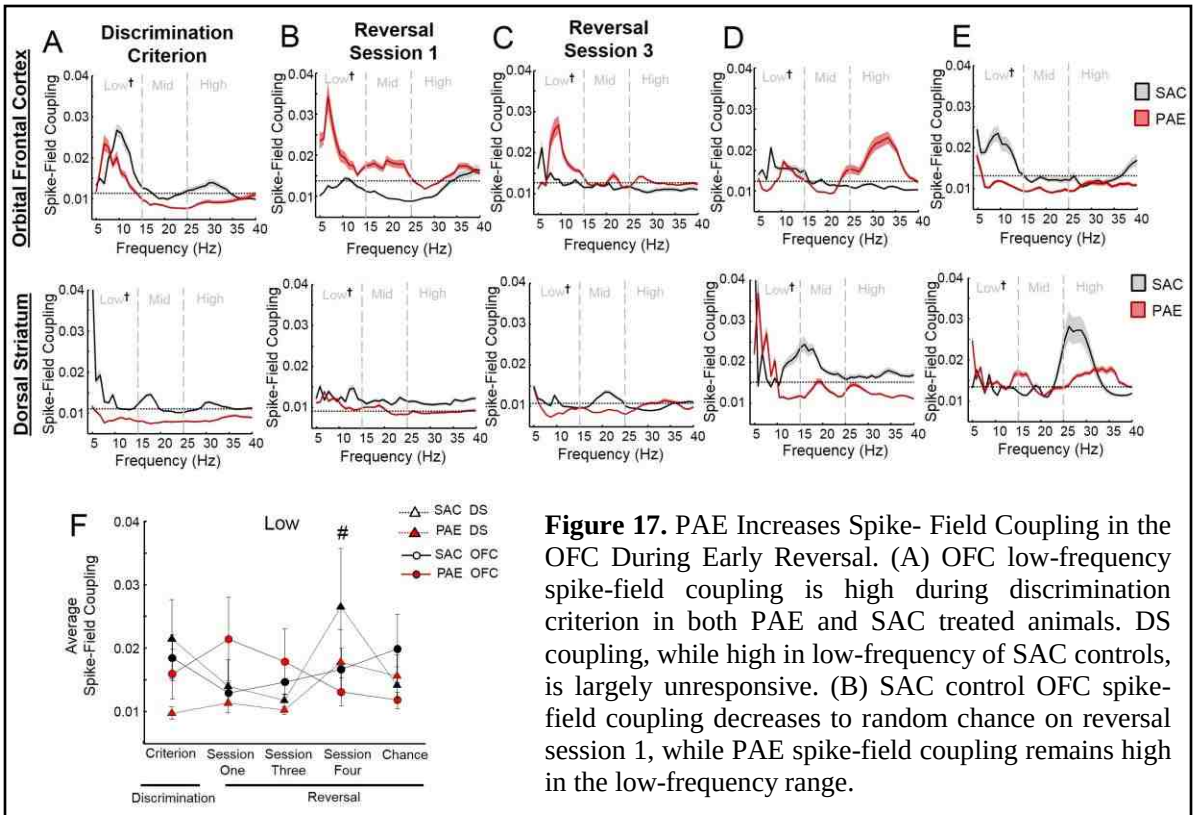

Figure 17. PAE Increases Spike- Field Coupling in the OFC During Early Reversal. (A) OFC low-frequency spike-field coupling is high during discrimination criterion in both PAE and SAC treated animals. DS coupling, while high in low-frequency of SAC controls, is largely unresponsive. (B) SAC control OFC spike-field coupling decreases to random chance on reversal session 1, while PAE spike-field coupling remains high in the low-frequency range. DS spike-field coupling remains low on reversal session 1. (C) During reversal session 3, PAE spike-field low-frequency coupling remains above SAC control levels in the OFC. DS spike-field coupling remains low in both SAC control and PAE animals across frequencies. (D) OFC low-frequency spike-field coupling in PAE returned to SAC control levels during reversal session 4, however PAE high-frequency coupling was increased. DS spike-field coupling increased in the low-medium range in SAC control animals. While DS spike-field coupling was also increase in PAE animals, the frequency range of this increase was lower. (E) SAC OFC spike-field coupling returned to low-frequency pattern seen during discrimination criterion, however, PAE spike-field coupling remained low across frequencies. In the DS, while PAE spike-field coupling was low across all frequencies, SAC control animals had increased high-frequency coupling. Vertical dashed lines indicate boundaries between low (5-15 Hz) medium (15-25 Hz) and high (25-40 Hz) frequency ranges. Horizontal lines indicate random chance derived from 5000 iterations of shuffled data within session. Spike-field x frequency graphs (A-E) are means ±SEM of bootstrapped spikes. Low frequency graph (F) is means ±SEM between subjects. #=P<.05, difference between regions; †=P<.05, significant difference between frequencies.

$F_{4,337}=3.15$, $P<.05$). PAE spike-field low-frequency coupling in the DS was decreased across reversal sessions, although not significantly (**Figure 17F**). There were no stable changes in spike-field coupling after an incorrect choice in either the DS or OFC in SAC control or PAE treated animals during any recording session (data not shown).

4d. INTERIM DISCUSSION

Utilizing *in vivo* electrophysiology paired with touch-screen discrimination reversal we found that while firing rates in neurons in moderate PAE treated animals were altered, the timing and coordination of the DS and OFC oscillations were more severely impaired by PAE treatment earlier in reversal, suggesting that LFP alterations may be the main mechanism underlying maladaptive perseveration. Changes in DS and OFC single unit firing rate and phasic recruitment may contribute to prolonged perseveration in PAE mice, but do not occur during early reversal and do not have a magnitude of change that suggests they are sufficient to explain an initial inability to flexibly alter behavior. Changes in regional oscillatory coordination, functional connectivity and spike-field coupling, begin earlier in reversal and are sustained, suggesting LFP changes impair the initiation and execution of flexible behavior in PAE mice.

Alterations in Perseverative Responding. The number of sessions and correct trials to criterion on discrimination and reversal were similar to those seen previously in non-implanted mice and were not affected by PAE treatment, reconfirming that PAE does not affect learning (Allan et al., 2014, Hamilton et al., 2014, Marquardt et al., 2014). Behavioral maladaptive perseveration in PAE treated mice was evidenced by increased

correction trials in single-region electrode implanted mice on the first session of reversal. While surgically naïve mice have been shown to be impaired across the early reversal perseverative phase, our data are in line with studies in rodents using maze-based paradigms, when only the first few trials or bins were impaired by PAE (Thomas et al., 2004b). However, compared to surgically naïve mice on a visual-touch screen reversal task, SAC control subjects with implants required double the total number of correction trials to exit the perseverative phase (Marquardt et al., 2014). This strongly suggests the implantation altered reversal behavior such that we observed a ceiling effect on perseveration, obscuring treatment deficits except on the first reversal session.

Interestingly in dual region implanted mice, the amount of time to retrieve reward was increased across reversal. This slowing of behavior, repeated over multiple sequential sessions, may have occluded behavioral deficits generally seen during these early reversal stages by allowing PAE treated animals increased processing time. However, it may be that the minor disruption caused by implantation of both regions simultaneously may have affected behavior differently than single region implants, resulting in an inability to differentiate treatments even on early sessions of reversal.

In summary, we have found evidence that we replicated a deficit in PAE treated mice in reversal learning. However, implantation of electrode increased perseveration in PAE and control mice, resulting in a ceiling effect and largely obscuring treatment impairments.

Single Unit Firing Changes Do Not Explain Increased Perseveration in PAE Animals. OFC firing rate increased in response to reward approach after correct choice in both SAC control and PAE treated subjects as seen in previous studies in rodents and non-human primates (Thorpe et al., 1983, Amodeo et al., 2016, McMurray et al., 2016). This was a stable response across all learning sessions, even during early reversal when value contingencies were changed, in contrast to the flexible shift of response seen in other studies where OFC did not strongly signal during early reversal (Schoenbaum et al., 1999, Schoenbaum et al., 2003, Marquardt et al., 2017). In the current paradigm, choice and reward retrieval were separated by several seconds, but reward outcome was signaled immediately by secondary associative cues, therefore it may be that the OFC is encoding the value of the associative tone across sessions, which never changes its meaning, even when presented with the opposite stimulus choice during reversal (Padoa-Schioppa and Assad, 2006). However, since more neurons become responsive to the correct choice during the first session of reversal, this indicates that although spike-firing responses are not altered, the cognitive load on the OFC may be increased, supporting its importance in reversal. While few studies have shown significant encoding of the un-rewarded stimulus, our data clearly support that when response outcomes are uncertain, during chance and early reversal sessions, the OFC encodes incorrect choices (Nobre et al., 1999, Rich and Wallis, 2016). Importantly, the firing pattern response is distinct from correct choices and signaling occurs immediately after the incorrect response, concurrently with the onset of the negative associative cue. During touch screen reversal learning, our data show the OFC encodes correct response value expectation and signals unexpected outcomes when contingences are not well learned.

The DS followed the same spike-firing patterns as the OFC for both correct and incorrect responses. The current study targeted specifically dorsolateral striatum, therefore previous literature suggests that activity would be greatest during criterion stages due to its role in promoting autonomic behavior (Clarke et al., 2008, Yin et al., 2009). While our data do not show specific activation of the DS during well-learned behaviors, our results are not in direct contrast to this role due to the parallel activation of striatal lateral and medial circuits (Gremel and Costa, 2013). Interestingly, for correct choices, neuron recruitment follows the traditional role of the dorsolateral striatum in learning and habit formation (Yin et al., 2009), with the greatest number of neurons responsive to correct choices during criterion stages. However, DS neurons were most recruited during learning stages and early reversal sessions during incorrect responses. It is likely that we are capturing the parallel nature of this circuit and its true involvement across all parts of learning and reversal, but that neuron recruitment is an indicator of intensity of response. We can conclude that the DS is both driving habits and participating in unexpected error learning in coordination with the OFC.

Both the OFC and DS neuronal firing responses are consistent with previously established roles in SAC control mice. In contradiction to our hypothesis, we did not find significant single unit firing alterations that would support DS driving of behavior or insufficient OFC involvement after PAE treatment that would fully support reversal deficits. We did see a decrease in firing rate to unexpected incorrect choices during chance reversal in the OFC, and a slight but only mildly significant increase in DS response during the same stage to correct choices, which together may partially explain

extended perseverative periods in these animals. Alterations in DS firing during any stage to correct choices are not the increased and prolonged firing rate responses seen in chronic intermittent alcohol models where the DS has been shown to drive behavior (DePoy et al., 2013). Intriguingly, we do see increased neuronal recruitment in both the OFC and DS during the first session of reversal to correct choices in PAE animals, suggesting that while there is little change in firing, the regions may be compensating for a discrepancy in function not revealed by single-unit firing. Together our data suggest that after moderate PAE, spike-firing changes may prolong perseveration, however, alone they may not drive initial maladaptive perseveration.

Decreased Neuronal Coordination after PAE Disrupts Reversal Learning. Low-frequency oscillation coordination at time of associative tone end, marking a correct choice, is required for encoding reward anticipation in both the OFC and DS, but has previously only been shown in the rat OFC (van Wingerden et al., 2010a). This occurs across discrimination and reversal indicating the associative tone was the reward informative factor, combined with the stimulus choice. Two distinct coherent signals focused on different frequencies and separated slightly in time were identified. Differences in dynamics across sessions between regions in these two regions of interest indicate that the unique timing of peak coherence in each region is likely important for optimal reversal. The OFC peaks on reversal session one, while the DS peaks again later on reversal session four when the perseverative phase is starting to decline. This may indicate that, while the OFC may signal the initiation of a reversal, DS activity is important in initiating the re-learning phase. Intriguingly, PAE treatment decreases phase

coherence to correct choice within both the OFC and DS significantly during these early reversal sessions, and although reversal session one was not significant, this was likely due to our conservative statistics. These data may indicate that PAE animals not only have deficient initial OFC activity and difficulties initiating a reversal, but also that they cannot exit the perseverative phase. We hypothesize that difficulties initiating a reversal and exiting the perseverative phase stem from deficient *information transfer*. Due to the disorganized state of oscillations in both regions in PAE mice, communication may be blocked both at the local level, by inappropriate spike-timing, and by insufficient functional commination between regions.

OFC spike-field coupling in control animals after a correct choice at the tone off was highest during sessions where reward contingencies are learned or being learned, as seen in previous studies (van Wingerden et al., 2010a, Pennartz et al., 2011). Increases in spike-field coupling have been linked with greater attention and information transfer in the visual system (Womelsdorf and Fries, 2006, Gregoriou et al., 2009b), suggesting that information about positive reward contingencies is favored when perseveration is low, similar to neuron signaling of positive reward contingencies (Schoenbaum et al., 2003, Bissonette et al., 2008, Moorman and Aston-Jones, 2014). This may function as an overseeing function and when stimuli do not elicit expected response, neurons are no longer timed to coherent oscillations. Intriguingly, despite both decreased synchrony and coherence, spike low-frequency coupling is maintained in PAE mice in the OFC across reversal sessions one to three, further supporting that, in PAE animals, decreased OFC function delays initiation of reversal. The increase in spike-field coupling concomitant

with coherence decreases may reflect the rigid nature of the spike-firing pattern of the OFC during reversal. The cause of this spike-firing intransience and behavioral delay may be synaptic inflexibility, as antagonizing N-methy-D-aspartate receptors (NMDAR) in the OFC impaired discrimination of olfactory cues by increasing theta locked spikes (van Wingerden et al., 2012) and NMDARs are well known for their role in synaptic plasticity (Sakimura et al., 1995, Liu et al., 2004, Nicholls et al., 2008). Global antagonism of NMDARs impairs both learning and reversal; however, targeted prefrontal inactivation selectively impairs reversal learning (Stefani and Moghaddam, 2005, Chadman et al., 2006, Brigman et al., 2008, Dalton et al., 2011). There is also evidence that PAE can disrupt NMDAR expression and function (Savage et al., 1991, Hughes et al., 1998, Mameli et al., 2005, Puglia and Valenzuela, 2010, Brady et al., 2013). Although requiring further study, our data together with previous studies, indicate decreased neuron plasticity, particularly in the OFC after PAE treatment may contribute prolonged perseveration due to prolonged well-learned signaling patterns.

Similar to previous studies, our data indicates low-frequency spiked-coupling during the well-learned discrimination phase (Thorn and Graybiel, 2014) in the DS of SAC control animals. Our data, however, appears to be influenced by low frequency effects below 6 Hz, so this conclusion should be considered carefully. Intriguingly, control DS spike-field coupling clearly becomes apparent during reversal session four, between 10 to 20 Hz, and by chance reversal spikes became selectively coupled in the high (25-40 Hz) frequency range, both within the larger beta band frequency (13-30 Hz). While increased beta-band activity is largely associated with cessation of movement, in cognitive processing beta-

band is indicative of top-down processing when stimuli are ambiguous and depend on endogenous percepts (Okazaki et al., 2008, Engel and Fries, 2010). Intriguingly, the development of beta-coupling as the perseverative phase is exited in control mice may indicate that stimulus contingencies are entering an ambiguous phase where decisions are driven by top-down processing from frontal regions. However, as mice fully enter the chance and learning phase, choices become more stimulus driven as there are no longer pre-defined reward expectations and neural timing becomes associated with higher frequencies, as gamma oscillations (>30Hz) are well known to be involved in bottom-up processing (Cardin et al., 2009). These data suggest that in the DS of PAE animals, top-down control does not help promote the exit from perseveration. The failure to initiate the learning phase of reversal would promote the continuation of the previously correct behavioral action, leading to maladaptive perseveration.

Finally, the impaired ability to initiate flexible behavior in PAE animals may be due to a decreased functional connectivity between the OFC and DS on the first session of reversal. In a T-maze task, striatal and hippocampal oscillations became the most synchronous at the decision point, representing the coordinated information transfer needed to inform actions leading to a positive outcome (DeCoteau et al., 2007a). Here, during visual discrimination, while synchrony occurs during every session, it may be particularly critical when contingencies change and behavior needs to be altered, similar to previous hypotheses suggesting PFC coordinated control over goal-directed actions (Balleine et al., 2007, Forstmann et al., 2010). It is unlikely the OFC itself initiates changes in behavior, as error signaling has been shown to be a medial prefrontal cortex

(mPFC) function (Bissonette et al., 2008, Bissonette et al., 2013), but our data suggest it is a required signal, possibly as a dual signal with error signaling informing contingency changes. We hypothesize that many trials are required on a visual rodent reversal task to initiate a change in behavior, however, if the OFC is unable to signal contingency changes early in reversal due to insufficient connectivity in early reversal, as seen in PAE mice, later reversal sessions would be more greatly affected than the first session. Supporting this, reversal sessions three and four were most affected, almost exclusively across measures. Decreases in synchrony have also been seen in cocaine addicted rats and may underlie similar reversal impairments seen in this population (Goto and Grace, 2005, Calu et al., 2007, McCracken and Grace, 2013). Intriguingly, our data are in contrast to synchrony increases seen after third trimester high dose PAE in the olfactory hippocampal circuit (Wilson et al., 2011). However, differences may be attributed to circuit specific effects of alcohol or in differences in collection techniques, as previous work has been done in anesthetized animals. Together with previous studies, our data suggest that a decreased functional connectivity between the DS and OFC early in reversal is a contributing factor to maladaptive perseveration, likely through delaying the initiation of reversal behavior.

Conclusion. Contrary to our hypothesis that neuronal firing rate changes could significantly contribute to maladaptive perseveration in PAE animals, our data do not show that neuronal OFC regulation over the DS drive of repetitive behaviors is lost in PAE animals. Instead, loss of neuronal signaling coordination within and between the OFC and DS both delays the initiation of reversal and re-entry into the learning phase.

Loss of OFC coordination and spike-field coupling suggest impaired function and plasticity that impairs the ability of PAE mice to initiate a reversal. Simultaneous loss of coordination and spike-coupling, indicating a loss of top-down signaling in the DS, may impair PAE animals' ability to re-enter the learning phase, thus prolonging perseveration. Together with a loss of communication between the OFC and DS, perseveration is driven by an inability of PAE mice to properly coordinate neural activity. Our data clearly show that in PAE animals, although changes in firing rate may exacerbate maladaptive perseveration, the primary mechanism is signalizing disorganization within and between the OFC and DS after rare correct choices during early reversal. Our study identifies a challenge in studying individuals with FASD due to the more subtle effects on timing and coordination than overall neuron signaling. However, these data suggest that proper re-training of circuits to the correct timing may be a viable future treatment option for individuals with FASD. Future studies will need to more closely test this as a treatment option by manipulating portions of the circuit and identifying changes in behavior.

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DISCUSSION

The present studies have shown that moderate PAE is sufficient to selectively impair reversal learning on a translational visual touch-screen task. In this task, in optimally functioning non-treated mice, lOFC neurons increase firing rate upon reward approach during sessions when stimulus-outcome contingencies were well learned, indicating a reward expectancy signal. In addition, single units coordinate with the oscillatory activity differently across discrimination reversal in a manner that suggests propagation of signals that correlate with continuation or cessation of behaviors. After PAE, while there are alterations in firing rate and recruitment of both OFC and DS neurons, changes are relatively minor, and although they may prolong perseveration, they are unlikely to fully explain behavioral deficits. More substantial disruptions of local and inter-region coordination by oscillations occurred throughout reversal, suggesting the inability to coordinate neural signals, both within and between regions, resulted in impaired reversal. In addition, an increase in theta spike-filed coupling, primarily in the DS of PAE animals, may suggest decreased plasticity and an inability to alter previously learned responses, which may drive maladaptive perseveration. Together, our results demonstrate that moderate PAE more subtly effects neural coordination instead of single unit responses as predicted, and treatments focusing on strengthening organization would be useful in treating FASD.

5a. Reversal Behavior in PAE Treated Mice

Comparable to maze-based tasks, moderate PAE impairs reversal learning on a visual-touch screen paradigm by increasing the number of repetitive trials needed to shift behavior and re-attain chance criterion (Lee and Rabe, 1999, Allan et al., 2014, Hamilton et al., 2014). Importantly, the lack of treatment effects on initial acquisition confirms that deficits in reversal are not contaminated by deficits in learning outcome associations, as time to learn initial visual discriminations and time to re-attain criterion were not altered by PAE treatment.

In contrast to initial behavioral studies, we did not see robust effects in reversal learning deficits between treatments in recording subjects across the paradigm. However, where non-implanted SAC control animals took an average of 250 and PAE treated completed 350 correction trials to exit the perseverative phase, *both* SAC control and PAE treated *implanted* mice both took an average of 350 correction trials. Correction trials, first presentation errors and correct responses were comparable to non-implanted animals across all other time points. In addition, the time to make a touch-response in implanted animals was approximately two seconds slower, but only during the perseverative phase of reversal. Together these data suggest that electrode implantation and tethering may have affected behavior of all implanted mice, regardless of treatment, resulting in a masking effect of PAE deficits in reversal learning during the early perseverative reversal phase. However, upon closer dissection of behavior, we did see significant increases on first session reversal correction trials in single region electrode implanted animals. Some maze-based studies saw similar limited effects on early sessions of reversal learning

(Thomas et al., 2004b). This suggests that PAE treated mice with electrode implants are still impaired on reversal, but we were unable to visualize behavioral deficits due to electrode implantation altering behavior.

An altered pattern of impaired behavior in implanted animals is not surprising, as inserting an electrode unavoidably causes damage to the target region and overlying tissue, even with surgical techniques used to minimize it. By damaging either the OFC or DS, it appears we impaired flexibility resulting in an increase of perseverative trials during early reversal in control animals. However, electrode implantation and multiple recording sessions did not impact learning on any stage of the paradigm, supporting that the OFC and DS regions targeted are selectively important in reversal (Bissonette et al., 2008, Calabresi et al., 2014). Furthermore, damaging both regions at the same time, especially ipsilaterally, due to the majority of connectivity being within-hemisphere (Voorn et al., 2004, Schilman et al., 2008, Pan et al., 2010, Hoover and Vertes, 2011, Liljeholm and O'Doherty, 2012), appears to differentially affect resulting behavior as all effects of PAE treatment were obscured.

Even if visual reversal behavior was not measurably impaired after surgical implantations, underling neural changes caused by PAE are still present. Similarly in individuals with family history of alcohol abuse there are alterations in EEG measures, but behavioral impairments are only revealed with more difficult tasks (Kamarajan et al., 2006, Rangaswamy et al., 2007). Rodent PAE studies have also shown that deficits can become apparent on more challenging variants (Savage et al., 2002, Kamarajan et al.,

2006, Brady et al., 2012). Surgical implantation may have masked effects by impairing SAC control and PAE treated mice to the maximum levels on this task, however, further challenge above the current pairwise discrimination reversal may pull out behavioral impairments in future studies. Future research may consider utilizing more complex reward schedules or more stimuli for more difficult visual discriminations to truly tax the OFC (Amodeo et al., 2016, McMurray et al., 2016).

Alterations in reversal behavior may also be due to changes in motivation due to the food reward utilized in this study and others. The lOFC is anterior and slightly medial to the brain region controlling tongue protrusion (Whishaw and Kolb, 1983), which means that PAE or electrode implantation may affect consumption behavior. A similar moderate PAE paradigm in rats showed deficient tongue protrusion distance for consumption of chocolate syrup or peanut butter (Hamilton et al., 2014). Alterations in tongue protrusion could have alternatively been due to decreased sweet preference, instead of impairments in feeding behavior; however, sugar preferences have been shown to be increased, or not altered at all after PAE (Abel and Dintcheff, 1986, Sanchez Vega et al., 2013). Changes in sweet preference may depend upon exposure paradigm. Some studies suggest that either prenatal alcohol treatment or breeding for alcohol preference decreases the ability to differentiate between sucrose and noxious quinine solutions (Arias and Chotro, 2005, Crabbe et al., 2011). While sucrose preference has not been tested in our paradigm of exposure, we utilized a non-sweetened food pellet, and it is unlikely that motivation and feeding behavior were altered, as treatment did not affect time to retrieve the food reward. While neither OFC nor DS single region implants effects time to retrieve food

reward, dual region implants took significantly longer to retrieve it, but only during reversal. The time to make a choice and reward retrieval during discrimination were not altered in these mice, most likely indicating that another factor, other than motivation, caused the slowed behavior during reversal. Our data suggest that neither treatment nor implant affected food preference or consumption, however these are important factors to consider when utilizing motivational rewards to initiate behavior.

The current studies have shown that PAE specifically impairs reversal on a visual-based task with no confounds of spatial location learning deficits. Although electrode implant largely masks treatment effects, data suggest underlying neuronal activity changes are still present.

5b. Single Unit Activity – Effects of SAC and PAE

During touch-screen visual reversal lOFC neurons, in non-treated mice, increase firing rate to reward expectation in a similar manner to those seen previously in rodent maze and primate visual tasks (Thorpe et al., 1983, Schoenbaum and Eichenbaum, 1995, Schoenbaum et al., 2003). Interestingly, there were marked differences in lOFC signaling in mice exposed prenatally to SAC, the control for PAE treatment. The most striking difference is that lOFC reward expectancy signaling is present during every stage recorded, not limited to criterion stages. Even though we collapsed across correct and incorrect trial types in SAC mice since lOFC firing was dominated by win-stay and perseverative trials in non-treated mice, this does not explain the lack of change across sessions. While SAC can cross the placenta and has been shown to accumulate in the

fetus (Pitkin et al., 1971, Cohen-Addad et al., 1986), we are unaware of any studies reporting negative neural effects after prenatal SAC exposure. It is likely we captured a slightly extended population of lOFC neurons in SAC and PAE treated mice, which suggest value expectancy signaling may be encoded consistently across sessions in our paradigm. This may be due to the value encoding of the associative tone, which is learned during training and does not vary across sessions, only which stimulus it is paired with. This explanation is in agreement with another study in which the OFC began signaling value of associative cues across learning (Padoa-Schioppa and Assad, 2006). Even with differences between studies, the lOFC in SAC animals was shown to follow both reward expectancy and unexpected outcome signaling rules.

We also demonstrated that DS firing rate responses to both expected reward and unexpected error mirror those seen in the lOFC. The current study targeted the DLS, which signals habit actions and is most active once behaviors are learned (Yin et al., 2009). The activation we see to correct trials is not limited to well-learned sessions. However, this is not necessarily contradictive to its proposed function, as both DLS and DMS are concurrently active, thus, while activity is always present, it should be strongest at correct choices during well-learned phases (Thorn et al., 2010, Gremel and Costa, 2013). While activity isn't altered per se, phasic recruitment to correct choice is high during criterion stages in the DS, which is congruent with previous early gene *c-fos* activation showing peak recruitment during well-learned sessions (Brigman et al., 2013). Firing rate data suggest the DS in a dual role with lOFC in expectancy signaling, which may double as a habit performance signal based on phase recruitment.

The use of tone signaling in our paradigm allowed us to separate out stimulus verses reward responding. However, since the tone was consistent across learning sessions, we may have biased our results to only observe reward expectancy signals due to the associative tone. However, our results do suggest a difference in signaling between discrimination and reversal responses to tone, in that oscillatory coordination was only disrupted after reversal began in PAE mice, suggesting there must be a link between stimulus and tone pairing that was not evident in our recordings.

Chronic intermittent alcohol studies suggest increased amplitude and duration of firing rate to correct choices in the DS that drive habit formation (DePoy et al., 2013). While we do see earlier increases in firing rate on early and chance sessions of reversal, these are not prolonged across the trial, as seen in chronic intermittent alcohol studies. Interestingly, however, the numbers of phasic neurons are increased in PAE animals during early reversal. Without changes in firing rate however, it may be that these responses are compensatory or may be due to an inability to change response pattern, as suggested by altered theta spike-field coupling discussed in Chapter 4. Surprisingly, lOFC neuron firing rates after PAE were not decreased during correct or incorrect trials during early reversal, as hypothesized. The largest decrease in firing rate in PAE mice occurred after an incorrect choice on chance reversal. However, paired with few changes in DS firing rate, this is unlikely to disrupt reversal overall, but may prolong the perseverative period. Together, changes in firing rates of both lOFC and DS in PAE animals do not appear to explain behavioral inflexibility.

5c. Disrupted Coordinated Network Activity

As discussed in Chapter 4, decreases in coordinated activity and impaired spike-plasticity resulted in a maintenance of the well-learned behavioral pattern during early reversal in PAE mice, indicating a failure to initiate reversal in the OFC. Loss of coherence and spike-field coupling during the normal time point of perseveration decline in the DS suggests a loss of DS modulation and function at the exit of the perseverative phase supported a delay in entering the re-learning phase in PAE mice. Together with decreases in early reversal functional connectivity between OFC and DS in PAE mice, we hypothesize the more subtle loss of coordinated activity prolongs perseveration in PAE mice.

The lOFC in non-treated mice showed coupling or decoupling of spikes during particular trial types, suggesting promotion or inhibition of specific behaviors (Marquardt et al., 2017). We may not have seen a similar pattern in SAC treated animals due to collapsing across correct and incorrect trial types. However, lOFC spikes in SAC control animals couple to positive reward-expectation during discrimination criterion, which decreases during the perseverative sessions of reversal, as seen in previous studies (van Wingerden et al., 2010a, Pennartz et al., 2011). In a maze-based paradigm, DMS and DLS neurons became entrained to low-frequency ranges and were associated with learning spatial-based action patterns (Thorn and Graybiel, 2014). While we did see increases in low-frequency coupling during discrimination criterion, suggesting we captured well-learned coupling in the DS of control animals, more noticeable spike-coupling in the beta frequency band appeared late in the perseverative phase. Beta-frequency coupling may

represent the top-down control from frontal regions indicating a drive of internal contingency expectations that initiates the re-learning phase (Engel and Fries, 2010), which is largely absent in PAE mice. Impaired spike-field coupling in the OFC and DS may be exacerbated by the decreased functional connectivity during early reversal sessions in PAE mice. The lack of early communication may not only decrease the ability of the OFC to initiate reversal, but also represent inefficient signaling to the DS, thus resulting in an additional delay of entering the re-learning phase. Loss of spike-field coordination and inter-region communication support the hypothesis that inappropriate timing of signals may be the main driving factor behind maladaptive perseveration.

5d. Future Direction

The most interesting outcome from the current studies is the increased spike-field coupling in the OFC after PAE. As discussed in Chapter 4, in a visual-touch screen discrimination paradigm, this aberrant increase in low-frequency spike-field coupling in the OFC may be caused by a decrease in synaptic plastic potential as has been shown in other regions after PAE (Medina, 2011). Since the OFC has not been shown to be necessary for establishing stimulus outcome associations, and subjects with OFC inactivation or damage can eventually reverse, it may be that OFC does not inform striatal action, but alters striatal plasticity to either promote or decrease actions. Thus, loss of synaptic plasticity in the OFC may translate to decreased influence on downstream regions. In accordance with this, repeated optogenetic stimulation of the OFC – VS circuit produces obsessive compulsive-like behaviors in the mouse (Ahmari et al., 2013). In addition, DS plasticity changes have already been shown to occur during

learning in our visual discrimination reversal paradigm, with less inducible changes as behaviors become habits, but a return of plasticity in the DS after the perseverative phase of reversal (Brigman et al., 2013). While not directly tested, our current data suggest, OFC plasticity follows a similar plastic pattern, with high levels of plasticity during reversal that allow for re-setting of reward contingencies. Therefore, promoting OFC plasticity during reversal, through either increasing specific OFC to DS connections, or modulating OFC plasticity directly by tetanic stimulation should increase the rate at which subjects alter behavior after a reversal, in both SAC control and PAE animals. Tetanic stimulation or optogenetic stimulation of specific projections of the OFC, at the time point after associative tone, would cause an increase in plasticity that would be translated into altered behavior during reversal in downstream regions, like the DS. While our data does show the OFC and DS are involved in unexpected outcome signaling, the lack of oscillatory changes after PAE during incorrect trials suggests that these do not play a substantial role in altering flexibility and would not be beneficial to target.

Future work on dissecting the cortico-striatal circuit of reversal behavior must also consider the influence of other pathways. Since both the DS and OFC have such similar phase responses, it is likely that while the regions interact and directly inform one another, the timing of the oscillations are likely set by the same pace-making region to allow an efficient timed information transfer (Herreras, 2016). In this case, it is a reward driven task, and since the dopamine (DA) pathway innervates both regions (Zingg et al., 2014), DA signaling may set the timing of the local field potentials. In addition, DA has been shown to influence synaptic plasticity (Jay, 2003, Mao and Wang, 2015), and

therefore, in combination with OFC signaling may promote behavioral flexibility. It is well-established that disruption of D1-like receptors via antagonism impairs stimulus-outcome associations (Smith-Roe and Kelley, 2000, Eyny and Horvitz, 2003). D2-like receptors may have an extra role in flexibility, because although whole brain disruption impairs learning and locomotor function, targeted inactivation in prefrontal regions impairs behavioral flexibility selectively (Kruzich and Grandy, 2004, Floresco et al., 2006, Barker et al., 2013). Furthermore, PAE has been shown to effect DA receptor expression and distribution in non-human primates and rats (Druse et al., 1990, Schneider et al., 2005). If direct, or indirect targeting through OFC, is not sufficient to improve reversal, DA modulation through receptor expression or drug infusion may be a viable mechanisms to consider, particularly in PAE animals where there may be ineffective DA signaling.

5e. Conclusion

Together, our data suggest a dual role of OFC and DS in visual touch-screen discrimination reversal signaling of value expectancy and habit performance. In conflict with our hypothesis, there were no changes in single unit signaling that appeared sufficient to explain reversal impairments. Paired with decreases in regional coordination and deficient functional connectivity, our hypothesis was correct in that deficient OFC signaling was partially responsible for impaired reversal. In addition to deficient reversal initiation, delayed and decreased DS coordination may postpone entry into the re-learning phase. Further study of the cortico-striatal circuit underlying reversal learning will need to focus on plasticity mechanism and modulatory systems, like DA. I would hypothesize

that increasing synaptic potential of the OFC through stimulatory mechanisms or modulating the DA system will improve behavioral flexibility in both control and PAE animals. Therefore, treatments focusing on strengthening coordination of plasticity, like tDCS, may be useful in treating FASD executive function deficits.

APPENDICES

Appendix A. Chapter 2 Table 1. Functional Observational Battery

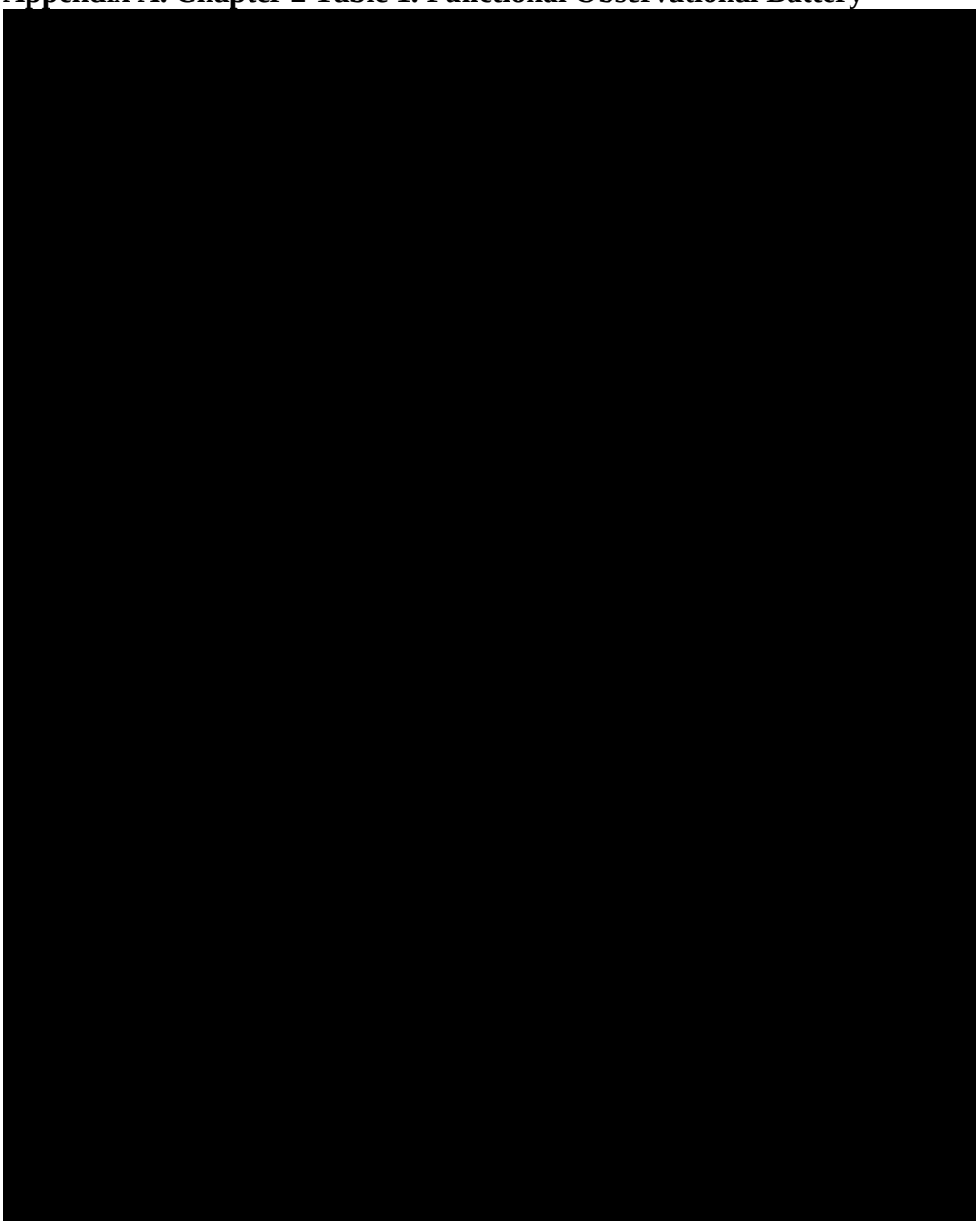


Table 1. Functional Observational Battery. PAE mice were normal on measures of physical health, sensory reflexes, neurological functions, and empty cage behaviors compared to SAC controls. Data denote the percentage of animals showing a response unless specified otherwise in parenthesis.

Appendix B. Chapter 2 Table 2. Pre-training

Table 2: PAE male and female mice demonstrated normal motivation to retrieve food reward and performance across all three pre-training stages compared to SAC control mice (data= sessions to criterion ±SEM).

Appendix C. Chapter 3 Supplementary Methods

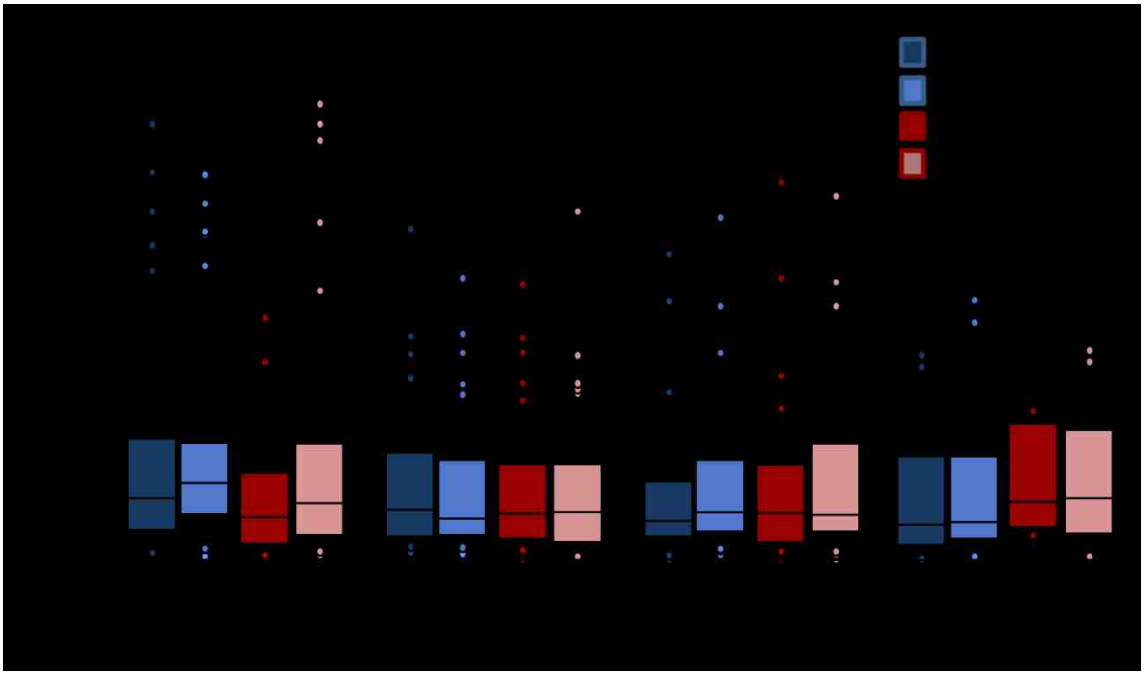
Local Field Potential Power. Time-frequency analyses were adapted to depth recordings as previously described (Cavanagh et al., 2009) and computed using custom Matlab (TheMathWorks) scripts. Power was computed for LFP data averaged across all electrodes and aligned to time of choice. Each of the defined choice types: *win-stay, lose-shift, perseverative* error and *regressive* error, was convolved separately with a set of complex Morlet wavelets, defined as a Gaussian-windowed complex sine wave: $e^{-i2\pi f} e^{-12/(2*\sigma 2)}$, where *t* is time, *f* is frequency (from 1 to 80 Hz in 80 logarithmic spaced steps to maximize lower frequency visualization), and σ is the width of each frequency band set at $4/(2\pi f)$. Instantaneous power of the time-frequency oscillations was calculated at each frequency by finding the magnitude of the resulting convolved data: $p(t) = real[z(t)]^2 + imag[z(t)]^2$, where *z(t)* was the convolved time-frequency data at each time point (*t*). Data was decibel normalized to an average baseline (1 sec pre-event), and compared across frequency bands, trial types and discrimination reversal sessions. Baseline was averaged from 250 repetitions of convolution with a combined baseline from 12 random trials from each trial type and session and a Morlet wavelet family (described above). Total trials per stage and type: *win-stay* Disc. Criterion 323, Early Reversal 35, Chance Reversal 199, Reversal Criterion 279; *lose-shift* Disc. Criterion 54, Early Reversal 104, Chance Reversal 163, Reversal Criterion 40; *perseverative* Disc. Criterion 13, Early Reversal 676, Chance Reversal 273, Reversal Criterion 12; *regressive* Disc. Criterion 54, Early Reversal 105, Chance Reversal 170, Reversal Criterion 44. Difference power graphs were calculated by subtracting each reversal session time-frequency spectrum from discrimination reversal, individually. Using a non-parametric

approach, significance of reversal difference from discrimination was determined by two standard deviations difference from randomly distributed data within trial type and session. Cluster correction was applied to remove areas of significance that were two standard deviations smaller than the mean cluster size to control for spontaneous or instantaneous power changes, which are non- meaningful outcomes.

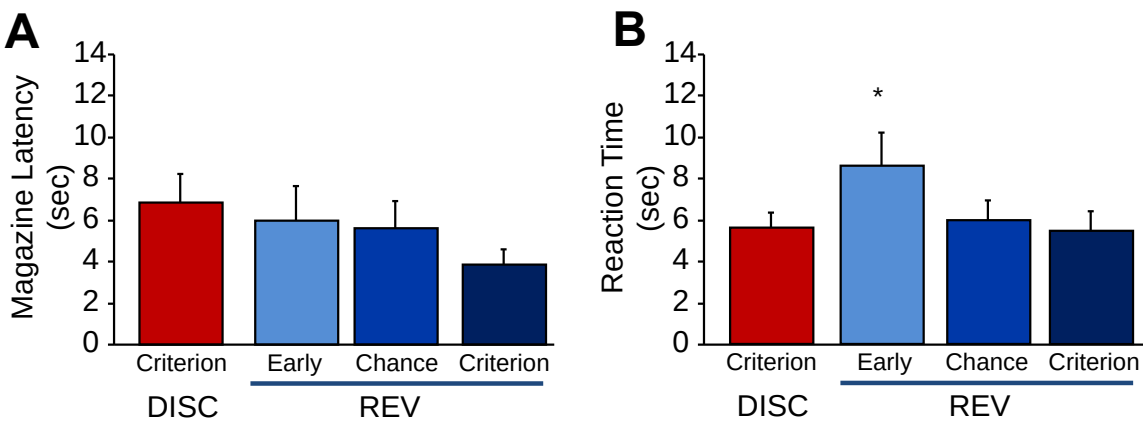
Spike Field Coupling. Epochs of oscillatory LFP data (±250ms) were set around individual spike events for each choice behavior with the spike occurrence at time point 0 (spike# avg per epoch: 1123). Spike-LFP phase angle was computed as: $A_j(f) = \arctan(\frac{imag(z_j)}{real(z_j)})$, where Z$j$ is the local field potential at every time point (j) after convolution with a complex Morlet wavelet family defined as a Gaussian-windowed complex sine wave: $e^{-i2\pi f}e^{-12/(2*\sigma 2)}$, where t is time, f is frequency set from 1 to 80 Hz in 40 linear spaced steps, and σ is the width of each frequency band set at $4/(2\pi f)$, for an average tradeoff between time and frequency precision (Cohen, 2014a). The paired phase consistency (PPC0) value (Vinck et al., 2010) was used as a measurement of angle consistency over time points to provide a measure of LFP phase coherence at the time of spike occurrence at a given time point. PPC was defined as the cosine of the angular difference between each angle within the vector: $PPC = \sum_{j=1}^{N}\sum_{k=j+1}^{N}\left|mean(e^{(i(\theta_j-\theta_k))})\right|$, where θ is the angle in radians and N is defined by length of LFP epoch and number of spikes. 1000 PPC calculation permutations were conducted on 10 randomly selected spike- locked LFPs to determine an average unbiased by spike number. A smaller number of spike- locked LFPs was selected to avoid excluding bins with low spike activity and higher variability caused by lower spike- locked LFPs resulted in a more rigorous test of

significance. (Vinck et al., 2010). Bins were combined for a final average of the 3 sec post-choice time epoch and PPC within each trial type, session and time epoch was compared to scrambled data within the same type.

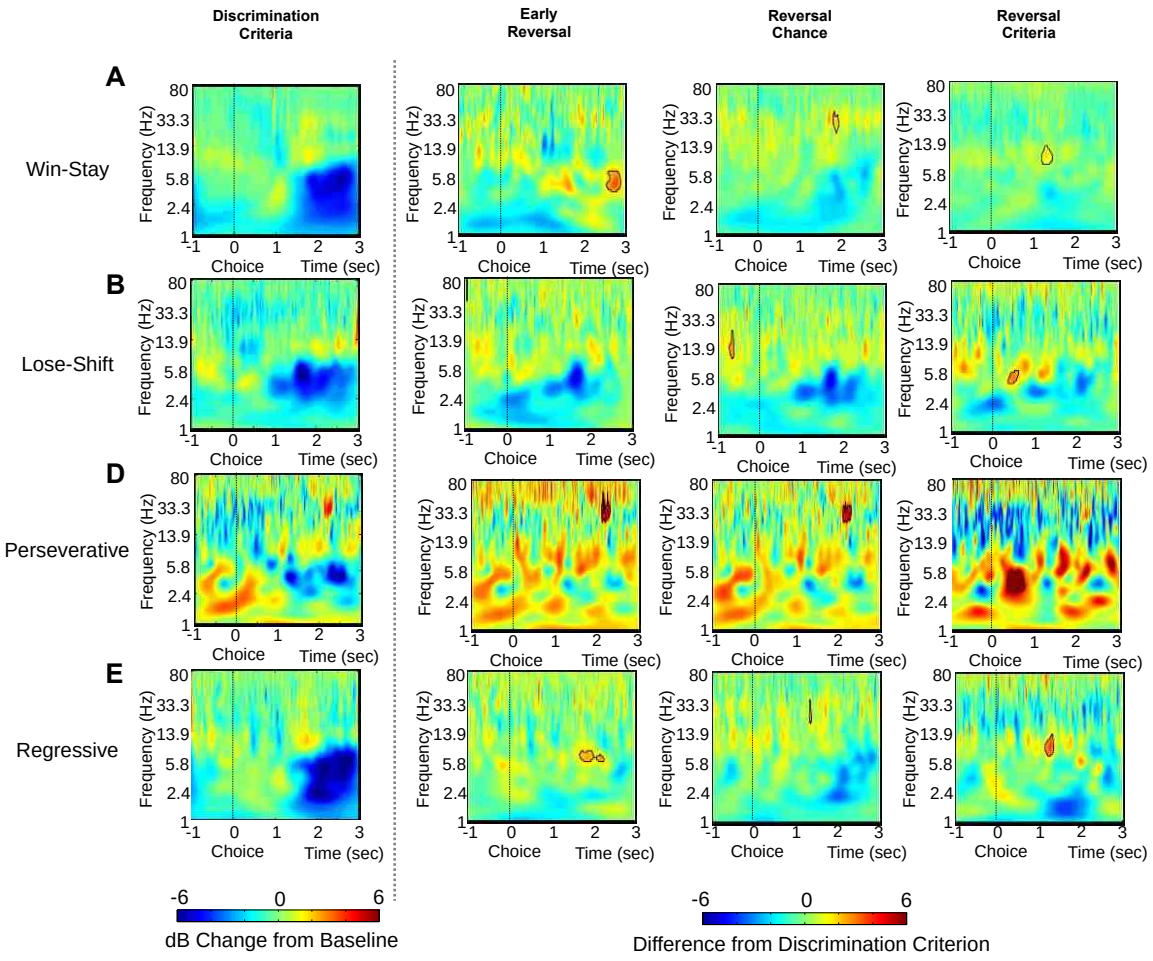
Appendix D. Chapter 3 Supplementary Figures



Supplementary Figure 1. Baseline Firing Rates. Box plots show the variation in average firing rate for 1 second prior to choice across captured neurons. Rates did not significantly change across trial types, or sessions, therefore, one second prior to choice was used as a baseline period for Z-score comparison of post-choice firing rates between trial types and sessions.

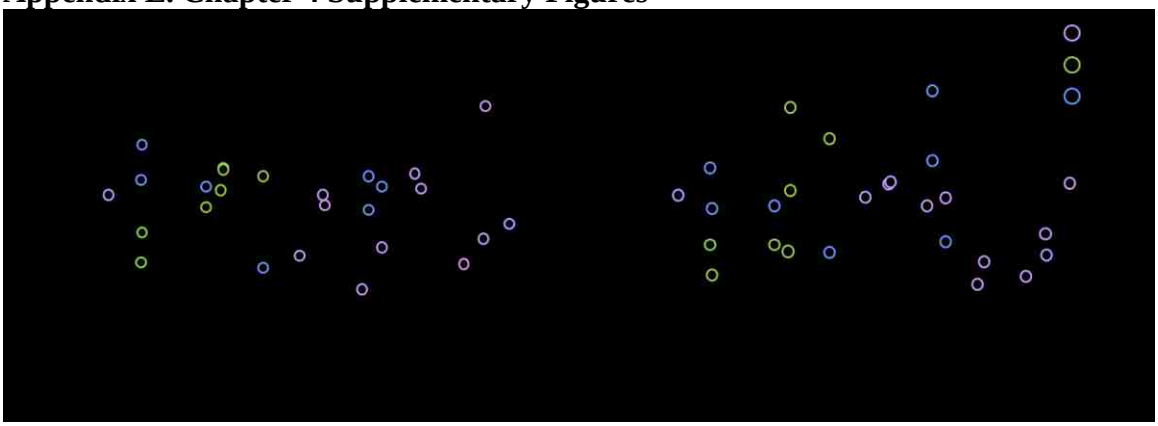


Supplementary Figure 2. Behavioral Measures. **A.** No significant differences in average time to retrieve reward, in sec, across all correct trial types, during each session of recording. **B.** Average time to make a response, in sec for correct or incorrect, after lever press initiation. Only during early reversal did mice take significantly longer to make a response. Data are means ±SEM. N=12 mice per recording session. * = P<.05, compared to all other sessions



Supplementary Figure 3. Power Changes Across Sessions. **A-E.** Discrimination criterion is power time-frequency spectra on a -6 to 6 scaled color dB change from baseline. Reversal sessions are difference from discrimination criterion (reversal – discrimination) on a -6 to 6 change color scale. Time of response is t=0 on the x-axis. Correct or incorrect response is determined by trial type indicated to at the far left of the figure. Three sec post-choice and 1 sec pre- choice was analyzed. Discrimination criterion dB changes were normalized to a combined baseline period of 1 sec pre- choice across all trial types and sessions. Frequency is indicated on the y-axis in logarithmic steps from 1 to 80 Hz. Circled areas indicate times and frequencies of significant (P<.05) difference from chance as calculated by two standard deviations away from random shuffled distribution of the data within session and trial type.

Appendix E. Chapter 4 Supplementary Figures



Supplementary Figure 1. Maternal Drinking Does Not Affect Perseveration. Neither number of correction trials on first session of reversal (left) nor reversal sessions during the perseverative phase (<50% correct; right) were effected by average maternal alcohol consumption. Each point represents an individual subject, with outline indicating implant type.

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