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THE EFFECTS OF ACUTE STRESS AND ADOLESCENT ALCOHOL EXPOSURE ON BEHAVIORAL FLEXIBILITY IN ADULTHOOD

Sarah Corrin Garr

A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies.

Department of Neuroscience

2017

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SARAH CORRIN GARR. The effects of acute stress and adolescent alcohol exposure on behavioral flexibility in adulthood. (Under the direction of L. JUDSON CHANDLER).

The prefrontal cortex (PFC) is critical for executive functions that underlie behavioral flexibility, but is especially vulnerable to environmental insults during development, which concludes after adolescence. Adolescence is a time of neural development, and is marked by increased risk-taking and impaired judgment. Adolescence is often associated with engagement in risky behaviors such as experimentation with drugs of abuse, including alcohol. Alcohol is particularly damaging to the PFC, and leads to negative impacts on executive functions. Traumatic stress has also been shown to negatively impact executive functions, and alcohol use and stress disorders frequently occur co-morbidly. Additionally, deficits in executive functions following adolescent alcohol or traumatic stress exposure in rats may differentially affect different strains of rats. This dissertation addressed the overarching hypothesis that binge-like adolescent alcohol (AIE) and a model of traumatic stress (SPS) negatively impact executive functions in adulthood, and that two strains of rats (Long-Evans, LE, and Sprague-Dawley, SD) may respond differentially to these exposures. First, the effects of AIE and SPS in adulthood on probabilistic reversal learning (PRL) were examined. AIE impaired discrimination learning with probabilistic reinforcement in LE rats on day one of the PRL task, and led to decreased reward and negative feedback sensitivity in SD rats over extended testing. SPS exposure following AIE led to increased negative feedback and reward sensitivity in LE rats. The second component of this dissertation addressed the effects of

AIE and SPS on the probabilistic decision-making task. AIE led to increased choice latency and impaired mastery of the task in SD rats during initial training sessions. SPS exposure following AIE led to decreased risky choice compared to SPS exposure alone in SD rats. The third component of this dissertation addressed the effects of AIE and SPS on fear-related behaviors. AIE and SPS exposure led to faster acquisition of associative fear conditioning in LE rats, and increased resistance to extinction. Taken together, this dissertation demonstrates that AIE leads to persistent deficits in behavioral flexibility in adulthood, and that SPS exacerbates these deficits.

CHAPTER 1

BACKGROUND & SIGNIFICANCE

ALCOHOL ABUSE

Alcohol is one of the most commonly abused drugs in the United States, and its misuse leads to effects including impaired driving and car crashes, liver disease, increased risk for certain types of cancers, and familial dysfunction (NIAAA, 2015a). Alcohol misuse has been estimated to cost the US approximately 250 billion dollars, and three-quarters of that cost is due to binge drinking. As defined by the National Institutes of Health (NIH), binge drinking consists of consuming 5 or more standard alcoholic drinks in one sitting (NIAAA, 2015b). Heavy use of alcohol, especially early in life such as during adolescence, is associated with an increased risk for alcohol use disorders later in life (NIAAA, 2017a).

Alcohol use in adolescents is relatively common with more than one-third of individuals by age 15, and two-thirds by 18 years of age, reporting that they have tried alcohol. Additionally, when teens and young adults drink alcohol, they typically consume it in a binge-like fashion (NIAAA, 2017b). Young people drink more than 90% of their alcohol in this binge-like fashion. In fact, 13.4% of those aged 12-20 reported engaging in binge drinking within the past month, and an additional 3.3% reported heavy alcohol use within the same timeframe (NIAAA,

2015b). While drinking such a large quantity of alcohol at a young age can have acute effects – including impaired judgment and increased risk-taking manifesting as drinking and driving, physical and sexual assault, and unprotected sex – long term effects of this type of alcohol consumption can lead to persistent cognitive deficits that impact an individual well into adulthood. These include reduced regional blood flow (Suzuki, Oishi, Mizutani, & Sato, 2002), impaired visuospatial abilities and mental flexibility (Fein, Bachman, Fisher, & Davenport, 1990b), and decreased attention (Fein, Torres, Price, & Sclafani, 2006b). However, the full extent of these deficits and how long impairments may last is poorly understood. Animal models of binge-like ethanol exposure during adolescence enable detailed studies of behavioral, neural, and molecular mechanisms underlying these deficits that would not otherwise be possible with human subjects.

STRUCTURE & FUNCTION OF THE PFC

As the evolutionarily most recent addition to the brain, the prefrontal cortex (PFC) is mostly composed of associative cortex. That is, it has reciprocal connections throughout the rest of the brain, as well as independent inputs from – and outputs to – other neural areas including sensory, motor, memory, and other association cortices. The anatomical boundaries of the prefrontal cortex are by no means a settled matter, but one commonly accepted cross-species definition is the area of frontal cortex that has stronger reciprocal connections with the mediodorsal (MD) nucleus of the thalamus than with other thalamic

nuclei (H. M. Uylings & Van Eden, 1990). Functionally, it has been hypothesized that the overall function of the PFC is to, “select and generate behavior patterns.” This function is supported by the fact that the PFC uses its own “working memory subsystem but that it uses a long-term memory store that is largely a function of the medial temporal regions” (H. Uylings, Groenewegen, & Kolb, 2003). Within this anatomically and functionally defined area, the PFC has several distinct regions, including the dorsal anterior cingulate cortex (dACC), the dorsolateral PFC (dlPFC), the ventromedial PFC (vmPFC), the ventrolateral PFC (vlPFC), the orbitofrontal cortex (OFC), and some of the insular cortex. Studies in primates have shown that damage to the anterior cingulate region may result in difficulty initiating movements and a reduced pain response, while damage to orbitofrontal areas results in spontaneity, impaired olfactory and gustatory information processing, and changes in social and emotional behaviors. Lesions of the dorsolateral frontal areas results in impaired working memory, specifically as it relates to supervising and planning behavior (H. Uylings et al., 2003). The vmPFC mediates affective and economic value as well as some aspects of social cognition (Delgado et al., 2016), while the vlPFC is critical for behavioral control and tasks that involve working memory. Although each area of the PFC is thought to contribute independently to an organism’s planning and behavior, the overall result of this cognitive symphony is executive control. Specifically, the PFC is responsible for processing the various types of information brought through the cortico-cortical connections to the PFC while taking into account

information from working and long-term memory to efficiently select the most beneficial course of action for the organism.

While humans are thought to have the most developed prefrontal cortex, there are anatomical and functional correlates in non-human primates as well as other mammals. It is easiest to make these comparisons using the cytoarchitectural classification of Brodmann's areas. The prefrontal cortex in the human brain is comprised of Brodmann's areas (BA) 8, 9, 10, 11, 12, 13, 14, 44, 45, 46, and 47, all of which are dysgranular or granular regions of cortex; that is they contain a rudimentary (dysgranular) or well-developed (granular) layer IV of the cortex (Wallis, 2013), with small, round cells that appear like granules. A common non-human primate research subject, the macaque monkey has parallel areas in its prefrontal cortex (BA 9-14). However, it is much more difficult to find parallel regions of cortex in the rat, as its prefrontal cortex is significantly smaller and less developed than that of primates; therefore, it is more accurate to find functionally parallel cortical regions, such as the ACC, insular cortex, OFC, and vmPFC (Wallis, 2013). Interestingly, Preuss & Kass (1999) hypothesized that the dlPFC may be a specialization of primates. However, recent studies have reported cortex anatomically and functionally similar to dlPFC in rodents (H. Uylings et al., 2003), and at least one research group has shown cortex that is anatomically similar to the primate dlPFC in dogs (Markow-Rajkowska & Kosmal, 1987; Stepniewska & Kosmal, 1986).

Adolescence & the PFC

During adolescence, the PFC undergoes significant cortical refinement via synaptic pruning (Petanjek et al., 2011), and myelination of cortico-cortical and cortico-subcortical connections. The brain matures primarily in a caudal-rostral direction with the visual cortex among one of the first areas to reach maturity, and the PFC as one of the last to mature after adolescence. In addition, the striatum reaches maturity prior to the PFC, which results in a bias towards striatal influence over decision-making and behavior during this time due to the absence of top-down inhibitory control by the PFC. The striatum consists primarily of the caudate, putamen, and nucleus accumbens. The dorsal striatum (encompassing the caudate and putamen) is primarily responsible for motor function and stimulus-response learning, whereas the ventral striatum (including the nucleus accumbens shell and core) mediates reward-learning and motivation. The relatively stronger influence of the striatum in relation to the PFC in adolescent decision-making is thought to partially contribute to a phenotype of increased risk-taking. This exemplifies behaviors that are typical during adolescence: impaired judgment, increased exploration and independence, and increased risk-taking (L. P. Spear, 2000). This behavioral drive can lead to experimentation with alcohol and other drugs of abuse.

EFFECTS OF ETHANOL ABUSE

Alcohol – specifically ethanol – has acute effects as an anxiolytic, a muscle relaxant, a sedative, and can lead to impairment in muscle coordination.

The effects of chronic ethanol abuse include stroke, high blood pressure, hepatitis, pancreatitis, and cancer of the liver, breast, and throat, just to name a few (NIAAA & NIH, 2016). Ethanol's broad cognitive and behavioral effects are due to its relatively promiscuous interactions with a broad range of neurotransmitter systems and receptor types in the central nervous system (CNS). Its anti-convulsant, anxiolytic, sedative, and ataxic effects are due in part to ethanol's facilitation of the γ-amino-butyric acid (GABA) receptor's activity. Its anti-convulsant, sedative, and subjective intoxication cues are associated with its inhibition of the glutamatergic N-methyl-D-aspartate (NMDA) receptor's activity. In contrast, the stimulant effect of low doses of ethanol may relate to its actions at serotonergic and dopaminergic receptors. Finally, the rewarding effects of ethanol are associated with actions on the dopaminergic reward pathways as well as on the endogenous opioid system. Symptoms of withdrawal are also mediated by these same pathways: seizures are associated with a lack of ethanol's effects at GABA and NMDA receptors, and aversive effects (withdrawal) are mediated in part by the dopaminergic, serotonergic, and opioidergic systems of the brain. While effects of ethanol can be rewarding (and anxiolytic) throughout the life span of an organism, adolescence is a window of vulnerability, especially for the prefrontal cortex.

The prefrontal cortex is especially vulnerable to the impacts of ethanol during adolescence (Bava et al., 2009b). As the last major region of the brain to reach maturity, environmental insults such as repeated binge-like ethanol intoxication could permanently alter the developmental trajectory of the prefrontal

cortex and its cortical and subcortical targets. Additionally, the prefrontal cortex appear to be more susceptible to both short- and long-term damage from chronic ethanol exposure compared to other areas of the brain, such as the hippocampus (Fowler et al., 2014). After three weeks of monitored abstinence, ethanol-dependent human adolescents displayed impaired visuospatial function and verbal skills, which is similar to the performances of patients with mild frontal-subcortical dysfunction (Brown, Tapert, Granholm, & Delis, 2000).

Effects of adolescent ethanol abuse

Several studies have shown deficits in executive functions following adolescent ethanol abuse in humans, such as impaired inhibitory control (Schweinsburg et al., 2004; Tapert et al., 2007) and decision-making (Uekermann & Daum, 2008; Wilcox, Dekonenko, Mayer, Bogenschutz, & Turner, 2014). Adolescent ethanol abuse in humans has also been shown to lead to accelerated cortical thinning in frontal and temporal areas involved with, “visual object recognition and language comprehension,” (Squeglia, Jacobus, & Tapert, 2014). These data highlight the vulnerability of the PFC to environmental insults such as ethanol exposure during adolescence. It is hypothesized that ethanol abuse during this window of vulnerability to developmental insult can permanently alter the developmental trajectory of the prefrontal cortex and lead to persistent cognitive deficits extending into adulthood. It has been reported that human adolescents with a history of binge drinking had functionally impaired white matter tracts across the brain compared to adolescents without a history of

binge drinking (Bava et al., 2009b; McQueeny et al., 2009). A more recent study showed that human adolescents who went on to initiate drinking (eventually averaging ~5 drinks per occasion 4 times per month) showed increased thinning of the right frontal cortex and diminished white matter development in initiators (those who started drinking) compared to non-users at follow-up compared to baseline (Luciana, Collins, Muetzel, & Lim, 2013). In addition to increased white matter, non-users also displayed greater increases in fractional anisotropy (FA) over the two-year follow-up compared to initiators. FA is a scalar measure of uniformity of diffusion. Smaller values indicate that diffusion is uniform in all directions, whereas larger values indicate that diffusion is completely anisotropic, or along only one axis. Therefore, it is reasonable that the developmental increase in white matter seen in non-users is accompanied by an increase in FA. Additionally, the lack of increase in FA in adolescents who initiated drinking ethanol in the study from Luciana et al. (2013) indicates the possibility of microstructural abnormalities in the white matter of initiators. Squeglia et al. (2015) replicated these findings and showed that human adolescents who initiated heavy drinking had diminished white matter development during follow-up scans over a period of up to eight years. However, a 2014 study from Pfefferbaum et al. demonstrated that measurements of white matter microstructure (such as FA) in adult human alcoholics could recover and move towards normality with increasing time in sobriety (Pfefferbaum et al., 2014).

Effects of ethanol abuse on the OFC

As a part of the prefrontal cortex, the OFC reaches maturity after adolescence. As such, it is also especially vulnerable to the effects of environmental insults during adolescence, such as binge-like ethanol exposure (Bava et al., 2009a; Fowler et al., 2014). McMurray and colleagues (McMurray, Amodeo, & Roitman, 2016) reported that binge-like consumption of ethanol gelatin during adolescence in rats led to an increased preference for large, risky rewards over small, certain rewards in adulthood. Using *in vivo* electrophysiology, they also found that increased ethanol consumption was, "associated with increased risk preference," (McMurray et al., 2016) and that, "higher risk preference was associated with reduced responses to rewards in one identified population of neurons [in the OFC]." They further hypothesized that the population of neurons referenced earlier may be inhibitory interneurons that modulate output to other reward-related areas of the brain such as the nucleus accumbens and the ventral tegmental area. However, it is important to note that the blood ethanol concentrations (BECs) reported by McMurray et al. were significantly lower than the threshold for binge-like ethanol exposure defined by the National Institute on Alcohol Abuse and Alcoholism (NIAAA). Interestingly, Coleman and colleagues (Coleman, Liu, Oguz, Styner, & Crews, 2014) found that adolescent binge-like ethanol administration lead to increased brain volume in the OFC in male rats, which correlated with findings in alcoholic adolescent human males (Medina et al., 2008). This could be due to a relative increase in white matter compared to controls, which also parallels the finding from Medina

and colleagues: an enlarged volume of the vlPFC of alcoholic adolescent human males, which “was associated with a 7% increase in white matter,” (Coleman et al., 2014). It is important to note that neurodevelopmental milestones in human males occur later than in females, and that white matter volume generally increases over the course of adolescence (Lenroot & Giedd, 2006). However, this area of the brain in humans (the vlPFC, which contains the OFC) typically decreases in volume as it matures throughout the late teen years and early 20s (Gogtay et al., 2004).

Effects of adolescent ethanol abuse on brain volume

Heavy drinking during human adolescence has also been shown to lead to decreases in the volume of the left ventral diencephalon (which includes the thalamus and hypothalamus), the left caudate, the left middle and inferior temporal gyri, and the brain stem (Squeglia, Rinker, et al., 2014). This volume reduction may partially reflect increased pruning of synaptic connections; while normative adolescent development involves decreasing grey matter due to neural pruning (Petanjek et al., 2011), and increasing white matter due to myelination, a sharper decline in grey matter volume – as demonstrated in this study – may indicate pathological changes in normative development. The authors note that, “These volumetric changes were positively correlated with lifetime ethanol use and peak number of drinks on an occasion in the past year, suggesting a dose-dependent effect of substance use on cortical thinning,” (Squeglia, Rinker, et al., 2014). Additionally, they note that other longitudinal

studies with functional MRI (fMRI) analysis have demonstrated increased brain activation in human adolescents who are heavy drinkers; this increased activation during tests of visual working memory (Squeglia et al., 2012) and response inhibition (Wetherill, Squeglia, Yang, & Tapert, 2013) may represent neural compensation for decreased grey matter seen in heavy drinkers observed in each of these studies.

Other studies have shown decreased hippocampal volume in human adolescents with alcohol use disorder (AUD) either in both hemispheres (De Bellis et al., 2000), or in the left hippocampus alone (Nagel, Schweinsburg, Phan, & Tapert, 2005). De Bellis et al. showed that total hippocampal volume was correlated negatively with duration of AUD, and was correlated positively with age of onset; this suggests that earlier initiation of drinking and longer durations of AUD correlate with smaller hippocampal volumes after diagnosis. Assays of hippocampal function in human adolescents who abuse ethanol have shown that those with a history of binge drinking showed deficits in spatial orientation compared to non-binge drinkers (Blankenship, Blackwell, Ebrahimi, Benson, & Wallace, 2016), and deficits in spatial working memory with more neural activation, both compared to light drinkers (Tapert et al., 2004).

ANIMAL MODELS OF ADOLESCENT ETHANOL ABUSE

Heavy drinking during human adolescence can lead not only to a higher incidence of ethanol abuse in adulthood, but to persistent cognitive deficits that last well into abstinence and adulthood (Fein, Bachman, Fisher, & Davenport,

1990a; Fein, Torres, Price, & Sclafani, 2006a). However, it is difficult to differentiate between pre-existing differences and consequential changes in human adolescent binge drinkers. One way to control for some of the variables that are innately present in a cohort of human subjects is to use an animal model of binge-like ethanol exposure. Following adolescent ethanol exposure, adult rats exhibit several persistent cognitive deficits parallel to those seen in human subjects. Specifically, adult rats show deficits in behavioral flexibility as assessed via a set-shifting task (Gass et al., 2014), a spatial reversal learning task (Coleman et al., 2014), risky decision-making tasks (McMurray et al., 2016; Schindler, Tsutsui, & Clark, 2014), and extinction of ethanol seeking behaviors (Gass et al., 2014). Together, these results in rodent models of adolescent ethanol abuse indicate a phenotype of behavioral inflexibility and impaired cognitive function, which parallels the findings of research conducted in humans.

There are several binge-like ethanol exposure models, encompassing multiple methods of ethanol delivery and duration of exposure during adolescence. Binge-like ethanol exposure is defined by NIAAA as a pattern of drinking that results in BECs of 80mg/dl or more, although many studies report BECs of 150-200mg/dl, which parallels those reported from late adolescent humans in field studies (Day, Celio, Lisman, Johansen, & Spear, 2013; L. Spear, 2016). Models of binge-like ethanol exposure can broadly be separated into self-administered and passive exposure models. Self-administration models have the advantage of taking into account the motivational aspects of the development of ethanol dependence, but the ethanol exposure of individual subjects cannot be

as tightly controlled as in passive exposure models. Accordingly, these models do not always result in binge-like levels of ethanol exposure for every subject, especially in rodent models. These models include self-administered drinking studies such as intermittent bottle access, drinking in the dark, and two-bottle choice, as well as the ethanol-laced gelatin consumption model. Passive exposure models, as stated earlier, do not account for the motivational aspects of the development of ethanol dependence, but can control individual subjects' ethanol exposure both in terms of timing as well as achieving high blood ethanol levels. These models include experimenter-administered ethanol via intra-gastric (IG, or gavage) dosing or intraperitoneal (IP) injections. However, both the IP and IG routes of forced administration can be associated with stress due to experimenter handling for the animal, depending on the speed and experience of the experimenter administering the dose. An alternative passive ethanol exposure model is the vaporized ethanol exposure procedure. This involves chambers of tightly controlled concentrations of vaporized ethanol, into which subjects can be placed for ethanol exposure, usually overnight for 12-16 hours. For adolescent binge-like exposure, a pattern of two nights on, two nights off is usually followed. This is called adolescent intermittent ethanol (AIE) exposure, and it models the common pattern of repeated binge exposure and withdrawal in which adolescent humans engage. While the method of drug delivery (inhalation and absorption through the lungs) is not a common method of ethanol use in humans, it is a method of experimenter-controlled passive ethanol exposure that allows for carefully regulated ethanol exposure, and repeated intoxication and

withdrawal. In addition to tightly regulated concentrations of ethanol vapor in the chambers, periodic blood samples to measure BECs as well as regular assessments of behavioral intoxication upon removal from the chambers can also ensure that the ethanol exposure is safe yet sufficient to qualify as a binge-like exposure. Finally, the timing of the ethanol exposures allows for repeated episodes of withdrawal, which have been reported to be critical for the development of dependence-like behavior in adulthood (Crews, Vetreno, Broadwater, & Robinson, 2016). Behavioral tasks to assess cognitive function are carried out in adulthood.

BEHAVIORAL TESTS OF COGNITIVE FLEXIBILITY

The PFC mediates many of the higher order behaviors including executive functions (i.e. working memory and strategic planning) and behavioral flexibility. This requires extensive connections to, from, and within the PFC including the visual, motor, and association cortices; the thalamus, striatum, hippocampus and other subcortical structures; and other areas of the prefrontal cortex (Fuster, 2015). Behavioral flexibility requires a balance between focus on the task at hand and the ability to flexibly update strategies in order to optimize outcomes (Floresco, 2013).

There are many tasks that are used to assess various aspects of behavioral flexibility, but they can be separated into three broad categories: set-shifting, reversal learning, and extinction learning (Hamilton & Brigman, 2015). Set-shifting includes tasks that require the subject to first learn a response rule

and then update that rule to account for new information either within the same or in a different dimension (visual, tactile, olfactory, or spatial); these are useful for assessing working memory span and endurance, attentional set formation, and attention shifting. One example of such a task is the operant set shifting procedure, in which a response rule is established (i.e. respond on the right lever for reward, a rule in the spatial dimension), and then shifted extra-dimensionally to require the subject to attend to a light cue above a lever to ascertain the rewarded response (a rule within the visual dimension). Reversal learning, in which tasks require the subject to learn a response rule and then directly reverse it, is useful for assessing many of the same aspects of behavioral flexibility as set-shifting tasks, albeit in a slightly different manner; these include working memory span and endurance, the ability to update response values, and inhibition of previously rewarded responses. Finally, extinction learning tasks require the subject to use negative feedback for a learned association in order to extinguish the previously learned association; these kinds of tasks are useful for assessing inhibition of previously learned associations and updating the value of outcomes and/or associations. One example is fear conditioning and extinction, in which a tone (a conditioned stimulus, CS) is associated with a foot shock (an unconditioned stimulus, US). This association is then extinguished by exposing the subject to the tone without the foot shock. Of these three broad categories of tasks that assess behavioral flexibility, the current project utilized reversal learning and extinction learning tasks.

Probabilistic reversal learning task

Reversal learning is thought to be mediated by a network of brain regions including the orbitofrontal cortex (OFC), the medial PFC (mPFC) to a lesser extent, and the necessary sensory cortices (visual, olfactory, auditory, tactile, and/or spatial) for the specific task. There are several types of reversal learning tasks: 1) visual discrimination tasks, which usually utilize two or more visual cues, 2) olfactory discrimination tasks, which utilize two or more odor cues, 3) spatial tasks, which utilize mazes including the Morris water maze, the plus maze, the T-maze, and the radial arm maze with 5 or more arms, and 4) operant tasks, which utilize visual, auditory, and spatial stimuli (Hamilton & Brigman, 2015). All of these tasks form associations between stimuli, cues, or spaces, and reward. The rodent OFC has been thought to be essential for reversal learning, specifically for updating reward value (Gallagher, McMahan, & Schoenbaum, 1999; Hornak et al., 2006) and maintaining an accurate representation of an internal "task state" (Sharpe, Wikenheiser, Niv, & Schoenbaum, 2015). A "task state" is the neural representation of the abstract (and physical) context in which a given action is being considered or executed; often this representation involves unobservable components, such as the many possible results (and combinations thereof) of running a red light (Sharpe et al., 2015). Additionally, while past studies have shown that the OFC is critical for reversal learning, other recent work in rodents has shown that the OFC is critical for initial reversal learning, but not serial reversals (Boulougouris, Dalley, & Robbins, 2007). Interestingly, Boulougouris & Robbins (2009) demonstrated this principle by using excitotoxic,

bilateral OFC lesions after initial reversal training, and there were no differences between control and treatment groups in the number of trials to reach criterion during retention (of pre-surgical training) or reversal phases of the task.

One paradigm that assesses behavioral flexibility, outcome value, reward sensitivity, and negative feedback sensitivity is the probabilistic reversal learning (PRL) task. The rodent operant version of this task (Gemma L. Dalton, Phillips, & Floresco, 2014) involves a “correct” lever, which is reinforced with an 80% reward probability, and an “incorrect” lever, which is reinforced 20% of the time. After initially discriminating the correct lever, the identity of the levers is reversed. This pattern of discerning the correct lever followed by a lever identity reversal continues until the end of the session, allowing evaluation of probabilistic discrimination and within-session reversal learning. Brain regions involved in this task include the nucleus accumbens shell (NAcs) and core (NAcc), the OFC, and – to a lesser extent – the mPFC. Regional inactivation studies in rodents from the Floresco lab using this task confirm earlier studies demonstrating that the NAcc facilitates reward approach and the NAcs plays a critical role in probabilistic reinforcement learning (Gemma L. Dalton et al., 2014). Additionally, the same lab used the same task and inactivation of prefrontal cortices to further determine that the medial OFC (mOFC) is critical for learning with probabilistic reinforcements, and that the lateral OFC (lOFC) is important for efficient approach to reward-related stimuli. In a later report, the same authors go on to contribute to the data implicating the lOFC in early but not late serial reversal learning (G. L. Dalton, Wang, Phillips, & Floresco, 2016). Interestingly, the same

study showed that mPFC inactivation improved PRL performance, most likely by increasing both reward and negative feedback sensitivity to yield an increased number of reversals completed per session. Neither inactivation of the infralimbic (IL) PFC or the anterior cingulate (ACC) significantly impaired PRL performance, although ACC inactivation slowed choice latencies (G. L. Dalton et al., 2016).

Fear conditioning & extinction

Extinction learning can be conceptualized as a type of inhibitory learning, as extinction involves "behavioral modifications that occur as a result of negative contingencies between a stimulus and a response," (Hamilton & Brigman, 2015), which is an inhibition of previously learned behavioral associations. Before extinction can occur, these types of tasks must include an "excitatory learning history," (Hamilton & Brigman, 2015), during which an initial association is formed via Pavlovian or fear conditioning. Pavlov's initial hypothesis of extinction learning was that extinction was not the erasure of previous memory, but the formation of a new memory that could inhibit the previously learned association (Pavlov, 1927; Quirk, Garcia, & González-Lima, 2006); this hypothesis has been supported with recent work that also serves to elaborate on the specific neural bases of extinction learning. The brain regions that are currently thought to underlie extinction learning, especially extinction of fear conditioning, include the mPFC, the OFC, the basolateral (BLA) and central (CeA) nuclei of the amygdala, the intercalated cells (ITC) of the amygdala, and sensory cortices specific to the learning paradigm (visual, olfactory, auditory, tactile, and/or spatial) (Quirk et al.,

2006). Within the frontal cortex, activity in the OFC has been shown to be crucial for initial extinction learning, whereas coordinated activity in the prelimbic (PL) and infralimbic (IL) medial prefrontal cortices has been shown to be critical for consolidation of extinction learning. For example, in the rodent OFC, excitotoxic lesions of the lateral and ventral OFC have been shown to impair initial extinction learning (West, Forcelli, McCue, & Malkova, 2013; Zelinski, Hong, Tyndall, Halsall, & McDonald, 2010), whereas recordings in the mPFC have shown an increase in IL activity (Francois et al., 2014) and excitability (Santini, Quirk, & Porter, 2008) during extinction. Additionally, high frequency stimulation of the IL immediately after fear conditioning in rodents facilitated extinction learning (Maroun, Kavushansky, Holmes, Wellman, & Motanis, 2012), whereas low frequency stimulation of the IL impaired extinction learning (Shehadi & Maroun, 2013). Extinction has also been shown to decrease the efficacy of neurotransmission between the mPFC and the BLA while also shifting the balance of excitation and inhibition towards inhibition (Cho, Deisseroth, & Bolshakov, 2013). It is this increase in "top-down" prefrontal control of the amygdala that contributes to the newly formed extinction memory inhibiting the previously learned fear conditioning memory.

Probabilistic decision-making task

As discussed earlier, adolescence is a time of impaired judgment, increased exploration and independence, and increased risk-taking (L. P. Spear, 2000). The probabilistic decision making task (PDT) is an animal model of risky

choice that can be used to assess probability discrimination, efficiency of decision-making, risk tolerance, negative feedback sensitivity, and reward sensitivity (Onge & Floresco, 2009). It is an operant task comprised of blocks of trials in which the subject chooses between a “risky” lever associated with a large reward, and a “certain” lever associated with a small reward. The risky lever is associated with a decreasing probability of reward across five blocks (100%, 50%, 25%, 12.5%, and 6.25%), whereas the certain lever always has a 100% rate of reward. Each block of reward probability for the risky lever includes forced choice trials at the beginning to establish the new reward probability, and free choice trials afterward to test the subject’s preference for the risky lever. However, this task has not been used exclusively; other models of risky choice include tasks with intermittently introduced novel stimuli associated with unknown reward probabilities (Costa, Tran, Turchi, & Averbeck, 2014), and delay discounting tasks (Mar, Walker, Theobald, Eagle, & Robbins, 2011; Stopper, Green, & Floresco, 2014), which, it could be argued, are more suited to assessing impulsive than risky choice. Interestingly, acute administration of ethanol (0.5-1.5 mg/kg) had no effect on performance in the PDT (Mitchell, Vokes, Blankenship, Simon, & Setlow, 2011) despite obvious motor impairments. In literature examining human subjects, the Balloon Analogue Risk Task (BART) is commonly used to assess risky decision making by simulating the inflation of a balloon where each pump of air is paired with a reward. Following each air pump, the subject can collect their reward(s) or continue with the task, but an exploded balloon yields no rewards. Reynolds et al. (2006) found no effect of acute ethanol

administration on young adult healthy social drinkers, but Claus et al. (2017) found that human adolescents who frequently used ethanol and marijuana showed “reduced differentiation of increasing risk,” in the dorsal ACC, insula, striatum, and superior parietal lobe. This means that with decreased activation of these areas, especially the dACC, which has been closely associated with appraisal of risk, the subject is expected to demonstrate more risk-taking (Claus et al., 2017). However, pre-clinical studies in rats have shown that reversible inactivation of the anterior cingulate cortex has no effect on PDT performance (Onge & Floresco, 2010). In the same study, OFC inactivation increased choice latency in the later blocks (25% and 12.5%) without affecting risky choice. Finally, prelimbic inactivation in rodents led to an increase in choice latency, an increase in risky choice when the probability of reward decreased across blocks, and a decrease in risky choice when the probability of reward increased. St. Onge & Floresco concluded that the mPFC serves an “updating function,” by integrating environmental, temporal, and cue-related information to optimize reward acquisition (Onge & Floresco, 2010). They note that the mPFC of the rat has anatomical connections analogous to the ventral ACC of primates, performs functions similar to the primate dorsolateral PFC, and ultimately may be similar to both regions in anatomical and functional manners.

RAT STRAIN DIFFERENCES

There are many rat strains available for almost any experimental purpose, but the two that are used in the studies discussed below are the pigmented Long-

Evans (LE) rats, and the albino Sprague-Dawley (SD) rats. Historically, pigmented rat strains (such as LE rats) have been selected for tasks involving visual discrimination, as albinism (such as in SD rats) has been documented to impair visual acuity. Additionally, many studies have compared various rat strains for performance on spatial memory and learning tasks, acquisition of operant tasks, measures of attention and impulsivity, differences in drug response or metabolism, and differences in response to stress. It is important to note that there are two broad types of rat strains relative to genetics: inbred and outbred. Inbred rats have been developed via inbreeding for specific traits (such as P-rats, or ethanol-preferring rats), while outbred rats (such as Long-Evans rats) have been bred to maintain relative genetic diversity within a distinct strain.

The Long-Evans (LE) rat was initially created by crossing the outbred, albino Wistar rat with a wild, gray rat in 1915 by Drs. Long & Evans. The Sprague-Dawley (SD) rat was created by Sprague-Dawley farms in 1925 as an outbred, multipurpose, albino rat that was especially easy to handle. Long-Evans rats are commonly used in studies of cognitive performance, but multiple studies have shown that different strains of rats, including the LE and SD rats, perform similarly on behavioral tasks with minimal additional training. For example, Auclair et al. (2009) assessed performance in the five-choice serial reaction time test (5-CSRTT) in SD and LE rats. The 5-CSRTT involves five choices of nose poke holes; after a brief visual stimulus at one of the holes, the rat must nose poke in the indicated hole for a food reward. Fewer trials were required for SD rats to acquire the task, but LE and SD rats performed similarly after task

acquisition (Auclair et al., 2009). However, a separate study demonstrated that SD rats required more training on an operant task to both initiate trials as well as complete a reversal of the task compared to LE rats (Turner & Burne, 2014). Similarly, an older study from Andrews et al. (1995) showed that LE rats acquired an operant procedure faster than SD rats. Interestingly, Andrews et al. (1995) also showed that LE and SD rats performed similarly in a swim maze task, but that SD's showed increased latency to choose compared to LE rats. Turner and colleagues used the acoustic startle response (ASR) to measure response to a loud noise, and revealed that LE rats showed increased habituation to the stimuli compared to SD rats (Turner & Burne, 2014), which may indicate that LE rats handle stress more readily than SD rats. Similarly, Faraday (2002) demonstrated that chronic restraint stress increased the ASR of SD but not LE rats. Clearly there is no clear consensus from the literature as to a "better" or "worse" strain for performance on behavioral tasks, but perhaps LE rats handle stress better than SD rats (Faraday, 2002; Turner & Burne, 2014).

Unpublished data from our lab has shown that AIE differentially affected anxiety-like behavior as assessed with the light/dark box. Rodents naturally prefer dark and/or enclosed spaces, so the open, light side of the light/dark box is relatively aversive to them. Following AIE, LE rats spend significantly more time in the light side, whereas SD rats spend significantly more time in the dark side of the light/dark box. This could be interpreted as decreased anxiety-like behavior in LE rats, but increased anxiety-like behavior in SD rats, following AIE exposure.

Similar to the lack of consensus on performance in behavioral tasks, the literature on behavioral and metabolic responses to drugs is murky as well. A study from Horowitz et al. (1997) revealed that cocaethylene (the metabolic product of cocaine and ethanol) administration significantly increased locomotion and exploratory behavior in SD but not LE rats; however, concomitant administration of fluoxetine (an SSRI) with cocaethylene increased the locomotion and exploratory behaviors of LE rats to levels comparable to SD rats. A follow-up study from the same group found that there was no difference between LE and SD rats in dopamine or serotonin neurotransmission in the striatum or cingulate, and hypothesized that transmitter release or post-synaptic receptor sensitivity may instead account for the differences in response to cocaethylene in LE versus SD rats (Baumann, Horowitz, Kristal, & Torres, 1998). Another study from Horowitz and colleagues (2002) demonstrated that fluoxetine administration led to increased immediate early gene expression (as assessed with Fos immunohistochemistry) in the striatum of LE but not SD rats. Faraday et al. (1999) used increasing doses of nicotine to show that it increased ASR in SD rats, but impaired it in LE rats. Additionally, they noted that LE rats appeared to develop tolerance to the nicotine by day 12 of a 14-day administration, whereas SD rats did not display this. Finally, phencyclidine (PCP, an NMDA receptor antagonist) administration led to increased latency and omissions in SD but not LE rats on the 5-CSRTT (Auclair et al., 2009). If there are any generalities to take away from the above summary of studies, it is that SD rats may be more prone to displaying increased choice latency, either in a spatial water maze (Andrews et

al., 1995) or on the 5-CSRTT after PCP administration (Auclair et al., 2009), and that there are definite but complex differences in serotonin neurotransmission and/or serotonin receptor sensitivity in LE and SD rats (Baumann et al., 1998; Horowitz et al., 2002; Horowitz et al., 1997).

STRESS & ETHANOL ABUSE

Stress and adolescent binge-like ethanol exposure can lead to many of the same symptoms of cognitive dysfunction: deficits in cognitive flexibility (Park, Wood, Bondi, Arco, & Moghaddam, 2016) and impaired attention and working memory (Arnsten, 1998; Goldman-Rakic, 1999). Unfortunately, the effects of stress can also increase the vulnerability to substance abuse, especially ethanol. Of the patients who survive a traumatic situation, up to 75% of them subsequently report problems with drinking (V.A., 2016). Additionally, approximately 70% of veterans seeking treatment for a stress disorder have an AUD (V.A., 2016). People with an AUD have an increased likelihood of also being diagnosed with an anxiety disorder, with PTSD being the most common anxiety disorder (Petrakis, Gonzalez, Rosenheck, & Krystal, 2002). Symptoms of PTSD include mentally re-experiencing the trauma, avoiding triggers associated with the trauma, social avoidance, negative affect, and hyperarousal (V.A., 2015). One animal model of acute stress that models some of the behavioral and neurological aspects of PTSD is the single prolonged stress (SPS) model. This consists of (consecutively) 2 hours of restraint stress, a 20 minute forced swim in 24°C water, 15 minutes of recovery, and ether anesthesia until loss of the toe

pinch reflex (Liberzon, Krstov, & Young, 1997). After this exposure, the rat is left undisturbed in its home cage for 7 days; these 7 days of relative isolation have been shown to be a “consolidation phase,” that is important for the development of behaviors and neurochemical changes that model those seen in PTSD (Knox, Nault, Henderson, & Liberzon, 2012; Liberzon et al., 1997; Liberzon, Lopez, Flagel, Vazquez, & Young, 1999).

SPS exposure leads to increased negative feedback of the hypothalamic-pituitary-adrenal (HPA) axis (Liberzon et al., 1997; Liberzon et al., 1999), which parallels the impaired cortisol response to stress seen in patients with PTSD (Yamamoto et al., 2009). Additionally, animals exposed to SPS display increased arousal and an exaggerated response to both trauma-related (Khan & Liberzon, 2004) and trauma-unrelated cues and/or contexts (Knox et al., 2011), similar to patients with PTSD (Grillon, 2002; Grillon, Morgan, Davis, & Southwick, 1998). Knox et al. (2012) specifically demonstrated that SPS exposure led to impaired extinction retention for context and cued fear; however, it did not affect the acquisition or extinction processes. This parallels the deficits in extinction of fear conditioning seen in PTSD, which is thought to be due to an inability to learn that something that was once dangerous is now safe (Jovanovic, Kazama, Bachevalier, & Davis, 2012; Liberzon & Abelson, 2016). A myriad of hippocampal abnormalities have also been seen following SPS exposure in rodents, including decreased NMDA receptor density (Yamamoto et al., 2009), increased BDNF mRNA and TrkB receptors (Takei et al., 2010), and increased glucocorticoid receptors (GRs) in the dorsal hippocampi of female but not male rats (Keller,

Schreiber, Staib, & Knox, 2015). It has been shown that patients with PTSD have reduced hippocampal volumes (Karl et al., 2006; Kitayama, Vaccarino, Kutner, Weiss, & Bremner, 2005), although some studies assert that reduced hippocampal volumes may be a pre-existing risk factor for developing PTSD (Gilbertson et al., 2002). No study to date has confirmed if SPS exposure also leads to reduced hippocampal volumes. Chronic treatment with selective serotonin reuptake inhibitors (SSRIs) can remedy some of these symptoms in both PTSD patients and SPS exposed animals (Berger et al., 2009; Takahashi, Morinobu, Iwamoto, & Yamawaki, 2006); administration of SSRIs prior to and continuing after SPS exposure can also prevent the increase in contextual fear and anxiety behaviors (Yamamoto et al., 2009).

Stress and alcohol use disorders are, “inextricably intertwined,” with such frequency that many patients must be treated for both concurrently (Kofoed & Friedman, 1993). While the diagnosis of PTSD preceded an AUD diagnosis in a sample of veterans from WWII, an AUD diagnosis preceded a PTSD diagnosis in a separate sample of Vietnam veterans (Davidson, Kudler, & Saunders, 1990). This demonstrates the frustratingly complex relationship between stress disorders and ethanol abuse. Of note is the considerable overlap in circuitry between drug addiction and stress: both involve decreased prefrontal activity (Arnsten, Raskind, Taylor, & Connor, 2015; Goldstein & Volkow, 2011; McMurray et al., 2016), which is due at least in part to increased catecholaminergic activity (Arnsten et al., 2015). Following this dysfunction of the prefrontal cortical “brakes” of the brain, “a vicious cycle that maintains primitive circuits in control of

behavior," is initiated so that decreased prefrontal cortical activity is perpetuated in both drug addicted and stressed states (Arnsten et al., 2015). Kushner et al. (2000) framed this problem of comorbid ethanol use and anxiety as a "feed forward cycle" in which initial ethanol use can relieve symptoms of anxiety, but longer-term ethanol use actually generates more anxiety. Several recent pre-clinical studies have examined the interaction between ethanol administration and stress. Varlinskaya et al. (2016) demonstrated that AIE followed by stress exposure (90 minutes restraint stress for 4 days in adulthood) led to a lack of habituation in the corticosterone (CORT) response to repeated restraint stress over the 4 days of stress exposure; normally, adult rodents display a habituated CORT response to repeated stress whereas adolescents do not display this habituation. The lack of CORT habituation in AIE animals during repeated stress suggests a dysregulation of the HPA axis, which can also be seen in SPS exposed animals (Liberzon et al., 1999). Other studies utilizing an adult model of chronic ethanol exposure (chronic intermittent ethanol exposure, CIE) have demonstrated that repeated episodes of forced swim stress (FSS) led to increased escalation of ethanol intake compared to ethanol-dependent, unstressed mice (Rodberg et al., 2017).

COMT, PTSD, & ethanol abuse

Dopaminergic neurotransmission in the PFC is critical for many executive functions, as well as working memory. Studies have shown that dopamine receptor signaling is critical for behavioral flexibility (Floresco, Magyar, Ghods-

Sharifi, Vexelman, & Tse, 2005), risk-based decision-making (Onge, Abhari, & Floresco, 2011), and working memory (Sawaguchi & Goldman-Rakic, 1991, 1994). However, the dopamine transporter (DAT) is not significantly expressed in the PFC, so catechol-O-methyltransferase (COMT) is the major pathway through which dopamine is enzymatically cleared from the synaptic cleft. Interestingly, the Val158Met polymorphism of COMT gene in humans plays an important role in both PTSD and drug abuse. The substitution of methionine for valine at amino acid 158 in the COMT protein leads to a significant decrease in the ability of COMT to catabolize catecholamines. Compared to val/val and val/met allele carriers, met/met carriers (those with relatively decreased COMT activity and increased baseline catecholamine concentrations) display an increased incidence of PTSD (Arnsten et al., 2015), and increased BOLD response in the OFC following a reward (Dreher, Kohn, Kolachana, Weinberger, & Berman, 2009). Additionally, in a gambling task that investigated the effect of how a situation is presented (the framing effect), Met allele carriers were more susceptible to negative framing and thus gambled more than val/val allele carriers (Gao et al., 2016).

SUMMARY

The frontal lobe is one of the last areas of the brain to reach maturity, and the prefrontal cortex undergoes significant development during adolescence. Additionally, adolescence is a time of increased risk-taking and impaired behavioral inhibition; it is also a time when experimentation with drugs can initiate

life-long addictive behavioral patterns. One of the most commonly abused drugs during adolescence is ethanol; the prefrontal cortex is particularly vulnerable to neurotoxic effects of this drug compared to other brain regions. Adolescent ethanol use typically follows a pattern of binge-like episodes of use and withdrawal, which human neuroimaging studies have shown to lead to abnormal developmental trajectories of both white and gray matter. A variety of cognitive assessments in humans have also shown that adolescent ethanol abuse leads to impaired behavioral flexibility.

Ethanol use disorders and stress or anxiety disorders are frequently comorbid, with several studies showing that a diagnosis of either type of disorder may precede (and possibly predispose the patient to) the other. There is also considerable overlap in the circuitry of stress reactivity and drug addiction, notably a significant decrease in prefrontal activity. However, the complicated development of these disorders makes it difficult to determine if a condition is pre-existing or a pathological change. Therefore, carefully controlled studies using animals are necessary to elucidate the mechanisms involved with both disease states.

STATEMENT OF PROBLEM & SPECIFIC AIMS

Adolescent ethanol abuse leads to demonstrable developmental changes and cognitive impairments in adulthood. Various developmental and cognitive deficits have been reported in the human as well as animal literature. Additionally, many human and animal studies have shown the deleterious effects

of traumatic stress on cognitive function. However, relatively few studies have attempted to determine the combined effects of adolescent binge-like ethanol exposure as well as traumatic stress. The following experiments use the adolescent intermittent ethanol (AIE) model of adolescent binge-like ethanol exposure, as well as the single prolonged stress (SPS) model of some symptoms of PTSD to aim to address the overarching question: do AIE, SPS, or AIE and SPS lead to cognitive deficits in adulthood? Cognitive deficits were assessed via reversal learning, risky decision-making, and extinction learning procedures. The overall hypothesis being tested was that **binge-like ethanol exposure during adolescence would lead to impaired behavioral flexibility, and that SPS would interact with AIE to further facilitate these deficits.** This hypothesis was tested via the following three specific aims:

SPECIFIC AIM 1: TEST THE HYPOTHESIS THAT AIE AND SPS WILL RESULT IN IMPAIRMENT OF PERFORMANCE ON AN OPERANT PROBABILISTIC REVERSAL LEARNING PROCEDURE IN ADULT RATS.

Adolescent binge-like exposure to ethanol has been shown to impair spatial reversal learning in male C57BL/6 mice (Coleman et al., 2014) and operant set-shifting performance in male Long-Evans rats (Gass et al., 2014). Additionally, single prolonged stress (SPS) has been shown to lead to impaired behavioral flexibility in a reversal learning task as well as a set-shifting task in male Sprague-Dawley rats (George et al., 2015). However, preliminary data suggested that AIE exposure did not lead to deficits in reversal learning in male Long-Evans

rats. Therefore, the hypothesis of this aim was that AIE exposure would facilitate the effects of SPS exposure in adulthood on performance of the probabilistic reversal learning procedure in male Sprague-Dawley but not Long-Evans rats.

SPECIFIC AIM 2: TEST THE HYPOTHESIS THAT AIE AND SPS WILL RESULT IN INCREASED RISKY CHOICE ON AN OPERANT PROBABILISTIC DECISION-MAKING TASK IN ADULTHOOD.

Binge-like exposure to ethanol during adolescence has been reported to result in increased risky choice in adulthood (McMurray et al., 2016; Nasrallah, Yang, & Bernstein, 2009; Schindler et al., 2014). Additionally, binge-like adolescent ethanol exposure has been shown to increase the dopaminergic response of the mesolimbic dopamine system to risk but not reward (Nasrallah et al., 2011). Therefore, the hypothesis of this aim was that SPS exposure in adulthood would facilitate the effects of AIE exposure in adolescence on performance of the probabilistic decision-making procedure in adulthood.

SPECIFIC AIM 3: TEST THE HYPOTHESIS THAT SPS WILL EXACERBATE AIE-INDUCED ALTERATIONS OF FEAR-RELATED BEHAVIORS IN ADULT LONG-EVANS RATS.

Adolescent binge-like ethanol exposure has been shown to lead to impairments in extinction of ethanol-seeking behavior (Gass et al., 2014) as well as deficits in extinction of fear conditioning (unpublished observations from the Gass lab). SPS exposure has been shown to impair extinction retention of cued fear without affecting fear conditioning or extinction (Knox et al., 2011). Therefore, the

hypothesis of this aim was that AIE exposure would facilitate the effects of SPS exposure in adulthood on extinction of fear conditioning as well as extinction retention of cued fear.

CHAPTER 2

ADOLESCENT ETHANOL VAPOR EXPOSURE & SINGLE PROLONGED STRESS MODELS

As discussed briefly in the previous chapter, the methods and results in this chapter detail the adolescent intermittent ethanol (AIE) exposures for subsequent data chapters. There are several methods of ethanol administration in rodents that mimic different parameters of human use of ethanol, and each have their advantages and disadvantages. Voluntary ethanol consumption in rodents can be achieved by providing free access to a solution containing ethanol either with or without concomitant water access (Rossi & Zucoloto, 1977; Sarles, Lebreuil, Tasso, & Figarella, 1971). While this model has the highest face validity in regards to how humans ingest ethanol, it is problematic in that the experimenter has no control over the amount of ethanol consumed by each rat. Subsequently, there may only be a small portion of a given cohort of rats that meets the criteria for intoxication (80mg/dl)(NIAAA, 2015a), or that reaches substantial levels of intoxication similar to what is seen in human alcoholics. Experimenter-administered ethanol, while it is not representative of the way most humans use ethanol, can resolve the problem of varying levels of ethanol consumption between cohorts, as intoxication and blood ethanol concentrations (BECs) can be tightly controlled. Consequently, much higher BECs can be

achieved with these types of methods in rodents than with voluntary ethanol self-administration.

Involuntary ethanol administration can be achieved with intraperitoneal (IP) injection of ethanol, intragastric (IG) gavage with ethanol, or inhalation of vaporized ethanol. IP administration of ethanol (Seitz et al., 1990; Siegmund, Haas, Schneider, & Singer, 2003) involves injecting an ethanol solution directly into the intraperitoneal cavity. This method is useful for examining the acute metabolic and behavioral effects of ethanol on animals, as it avoids first-pass metabolism. This enables the experimenter to account for variation between animals in metabolic rates, but the stress and inflammation associated with multiple injections in a chronic ethanol administration paradigm can be a confound. IG gavage of ethanol (Lieber, DeCarli, & Sorrell, 1989; Siegmund et al., 2003), while it is involuntary, has a greater degree of face validity. It utilizes the same route of administration as human consumption of ethanol, but allows the experimenter to regulate the dose and monitor the effects of ethanol administration so as to minimize variability between animals. However, repeated gavage administrations can be associated with stress due to experimenter handling, which may confound any results seen with chronic IG ethanol administration. Finally, ethanol vapor inhalation (Rogers, Wiener, & Bloom, 1979) may be used for involuntary ethanol administration by placing an animal in a sealed chamber of vaporized ethanol to inhale the gas. This results in BECs similar to those seen with other involuntary administration methods, and intoxication can be tightly regulated by adjusting the ethanol vapor concentration.

This method is not similar to human ethanol use, as very few humans inhale ethanol (Glatter, 2013; Press, 2006), and inhaled ethanol is not processed metabolically the same way as ethanol metabolized through the gastrointestinal tract. However, there is relatively less handling compared to other ethanol administration methods, and thus presumably less handling stress associated with chronic ethanol vapor exposure. Therefore, although it is not physiologically relevant to the manner in which humans consume ethanol, it is useful for examining the effects of longer term, experimenter administered ethanol in rodents.

The characterization of exposure parameters includes studies in both Sprague-Dawley (SD) and Long-Evans (LE) rats. Additionally, the initial hypothesis that two different outbred strains of rats would display differential effects of AIE exposure on behavioral flexibility in adulthood was based on data, reported here, of differences in anxiety-like behavior in adult male LE versus SD rats following AIE exposure.

AIE VAPOR EXPOSURE & MEASUREMENT OF BECS

Cross-fostered litters of 8 male and 2 female rats with a dam were ordered from Harlan (now Envigo) to arrive at PD22, and the litters were weaned at PD24 after 2 days of habituation to the animal facility after shipping. Male rats were then divided into control and experimental groups and pair-housed in standard polycarbonate cages. Pairing was always done within a litter and there was always a within litter control and experimental group. Access to food and water in

the home cage was continuous throughout the experiment except for the weeks of operant training (see below). The animal colony room was maintained on a reverse 12:12 light-dark cycle with lights off at 0900. All experimental procedures were conducted during the dark cycle, when rodents are most active, between 0900 and 1800. All procedures were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina, and within guidelines set forth by the National Research Council's Guideline for the Care and Use of Mammals in Neuroscience and Behavioral Research (2003).

The AIE exposure procedure used in the present study involved intermittent binge-like exposure to ethanol by vapor inhalation as previously described in Gass et al. 2014. AIE exposure encompassed post-natal days (PD) 28 through 44, and involved 5 cycles of 2 consecutive episodes of ethanol vapor inhalation, with each exposure consisting of 14-hrs in the vapor chambers followed by 10-hrs out of the chambers. Rats were exposed to ethanol on PD28 & 29 (cycle 1), PD31 & 32 (cycle 2), PD35 & 36 (cycle 3), PD38 & 39 (cycle 4), PD42 & 43 (cycle 5) (**Figure 2-1**).

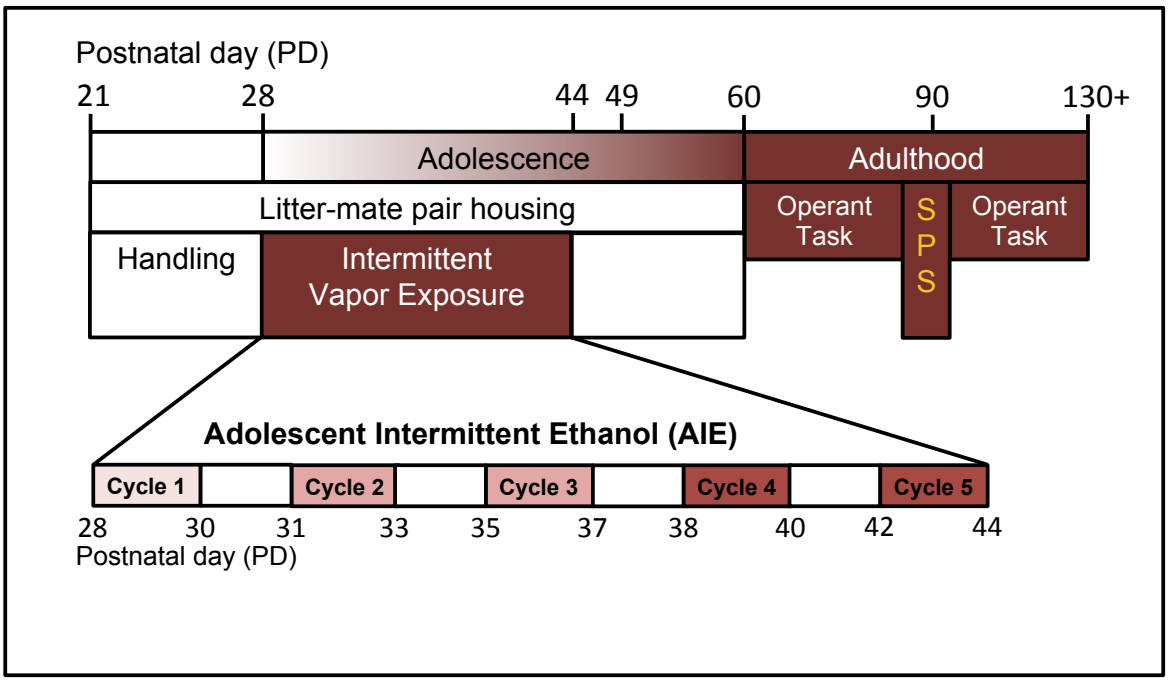

Figure 2-1. Experimental timeline of AIE exposure, pair housing, and operant testing

A 5-point behavioral intoxication rating scale was used to provide an index of the level of intoxication that was achieved during each of the exposure cycles. The rats were scored according to the following behaviors: 1 = No signs of intoxication; 2 = Slightly intoxicated (slight motor impairment); 3 = Moderately intoxicated (obvious motor impairment but able to walk); 4 = Highly intoxicated (severe motor impairments, loss of righting reflex); 5 = Extremely intoxicated (loss of righting reflex for at least 30 seconds and loss of eye blink reflex) (**Table 2-1**).

Table 2-1. Behavioral intoxication ratings for AIE exposures

Intoxication Rating	**1**	**2**	**3**	**4**	**5**
Behavioral Markers	No signs of intoxication	Slight motor impairment	Obvious motor impairments, but able to walk	Severe motor impairments, loss of righting reflex	Loss of righting reflex and loss of eye blink reflex

The target intoxication level was slight to moderate intoxication, which corresponded to an intoxication rating of 2 to 3, respectively. This level of intoxication is similar to that observed after binge-drinking in adolescent humans (NIAAA, 2015b). In addition to providing a measure of the level of intoxication, the rating also provided immediate information that was used to make adjustments in the level of ethanol vapor in the chambers in order to maintain a consistent level of intoxication across exposure days. Tail vein blood was obtained at the end of each of the 2-day ethanol vapor exposure cycles. Immediately following the collection of blood from the tail vein, the blood was centrifuged at 10,000 X g for 10 minutes to obtain plasma supernatant, which was then stored at -20 °C until assayed. Next, 10 µl of plasma was used for determination of ethanol levels using a colorimetric enzymatic assay as described previously (Prencipe, Iaccheri, & Manzati, 1987) for all of the Long-Evans rats and the Sprague-Dawley rats used in the light/dark box assessment, or using enzymatic oxygen rate detection via an AM1 Alcohol Analyser from Analox (Analox-AM1) for the Sprague-Dawley animals used in the probabilistic decision-making and probabilistic reversal learning tasks.

Single Prolonged Stress (SPS) exposure

The SPS paradigm is an animal model of traumatic stress that reliably reproduces some of the key symptoms of post-traumatic stress disorder (PTSD). In humans, PTSD is marked by several categories of symptoms: re-experiencing symptoms, avoidant behavior, negative affect, and hyperarousal. While it is currently not possible to ascertain if an animal has re-experiencing symptoms such as nightmares, flashbacks, or intrusive thoughts, the other symptom categories have been modeled in animals. Avoidant behavior in humans is characterized by eschewing crowds or cues that elicit memories of the original traumatic event (V.A., 2015); similar parallel behaviors in animals exposed to SPS include avoidance of trauma-related cues (Khan & Liberzon, 2004) and decreased social and novelty preference (Eagle, Fitzpatrick, & Perrine, 2013). Negative affect in humans is characterized by anhedonia, depressive symptoms, and misanthropic behaviors (V.A., 2015); parallel behaviors in SPS-exposed animals involve social avoidance (Eagle et al., 2013), decreased sucrose preference, and decreased cocaine preference (Enman, Arthur, Ward, Perrine, & Unterwald, 2015). Finally, symptoms of hyperarousal in humans include increased startle response, difficulty sleeping and concentrating, and increased negative feedback of the hypothalamic-pituitary-adrenal (HPA) axis (V.A., 2015; Yehuda et al., 1993). In animals, SPS has been shown to lead to increased contextual fear-related behaviors (Takahashi et al., 2006), increased startle reflex (Khan & Liberzon, 2004), and increased negative feedback of the HPA axis (Liberzon et al., 1997; Liberzon et al., 1999). Increased negative feedback of the

HPA axis manifests as decreased plasma levels of adreno-corticotrophic hormone (ACTH). Corticotropin releasing hormone (CRH), released by the hypothalamus, stimulates the anterior pituitary to release ACTH, which then stimulates the adrenal cortex to release glucocorticoids (primarily cortisol in humans, and corticosterone in rodents). Glucocorticoid release from the adrenal cortex regulates hormone release from the pituitary and the hypothalamus via negative feedback, so decreased ACTH following SPS exposure indicates enhanced negative feedback of the HPA axis (Liberzon et al., 1997). Additionally, PTSD has been shown to lead to deficits in extinction learning (Milad et al., 2008), which has been replicated in animals (Knox et al., 2011).

PTSD and ethanol use disorders are frequently comorbid, with many patients being treated concurrently for both (Petrakis et al., 2002). Additionally, those with PTSD have been reported to have problems with ethanol use both before and after diagnosis (Kofoed & Friedman, 1993). Therefore, preclinical experiments addressing the intersection of these two disorders are needed to disentangle the causes of and solutions to both PTSD and alcohol use disorders. Interestingly, disorders of stress (such as PTSD) and alcohol use disorders share many symptoms. For example, it has been reported that AIE leads to a lack of habituation of the corticosterone response, which is a marker of HPA axis dysregulation (Varlinskaya et al., 2016). AIE has also been shown to lead to deficits in extinction learning (Bergstrom, McDonald, & Smith, 2006; Broadwater & Spear, 2013), decreased reward preference, and decreased social preference

(Varlinskaya et al., 2016). Therefore, it was hypothesized that SPS exposure would exacerbate the effects of AIE.

In studies contained in this dissertation that involve SPS, animals that were subjected to exposure were PD90 or older. The SPS procedure is based on previous work by Liberzon et al. (Liberzon et al., 1997; Liberzon et al., 1999). On the day of the procedure, the control group was brought out of the animal facility to be kept in a lighted area outside of the exposure room in order to match the disturbance in light cycle that the SPS group would experience but without experiencing the smells and sounds from the stressed rats in the SPS group. Rats assigned to the SPS group were exposed to three successive stressors: 2 hours of restraint stress in a clear, acrylic cylinder; 20 minutes of forced swim in 23-25°C water that was deeper than the length of the rat; 15 minutes for recovery in a clean cage on a heating pad; followed by ether anesthesia to unconsciousness (approximately 5 minutes of exposure). Following this final stressor, the rats were allowed to recover in a clean cage on a heating pad until they were dry (approximately 2 hours), and then returned with the control group to the animal facility where they were left undisturbed for 7 days. Behavioral testing resumed on the 8^{th} day after control or stressor exposures. Each of these exact stressors, in the order in which they are presented here, with the consolidation phase of 7 days after the stressors, have been shown to be vital to the development of behaviors which model some symptoms of PTSD in humans such as hyperarousal, extinction retention deficits, and enhanced negative

feedback of the hypothalamic-pituitary-adrenal (HPA) axis (Knox et al., 2012; Liberzon et al., 1997; Liberzon et al., 1999).

The rats used in these studies (n = 27 AIE, 26 Control Long-Evans; n = 27 AIE, 32 Control Sprague-Dawley) were all male, and were separated within litters into pair-matched groups of Control or AIE exposed animals, which were put through AIE exposure, operant training and testing, SPS exposure, and then re-testing on the operant task. Intoxication scores measured at the end of each 14-hour ethanol vapor exposure period were averaged across all five cycles within each rat strain cohort. Across the five cycles of ethanol vapor exposure, there were minor but statistically significant differences between rat strains. A comparison via a 2-way ANOVA (strain by cycle number) revealed that, for the scores on Day 1, there was a significant interaction between strain and cycle number ($F_{(5,502)}$ = 9.967 $p < 0.0001$). Additionally, there was a significant difference due to cycle number ($F_{(5,502)}$ = 14.27 $p < 0.0001$) and strain ($F_{(1,502)}$ = 7.01 $p = 0.0084$). For the scores on Day 2, there was only a significant difference due to cycle number ($F_{(5,502)}$ = 3.063 $p = 0.0098$). These results demonstrate a significant difference due to cycle number for both Days 1 & 2 of each exposure cycle, regardless of strain, but a significant effect of strain on BEC for Day 1 only (**Figure 2-2**).

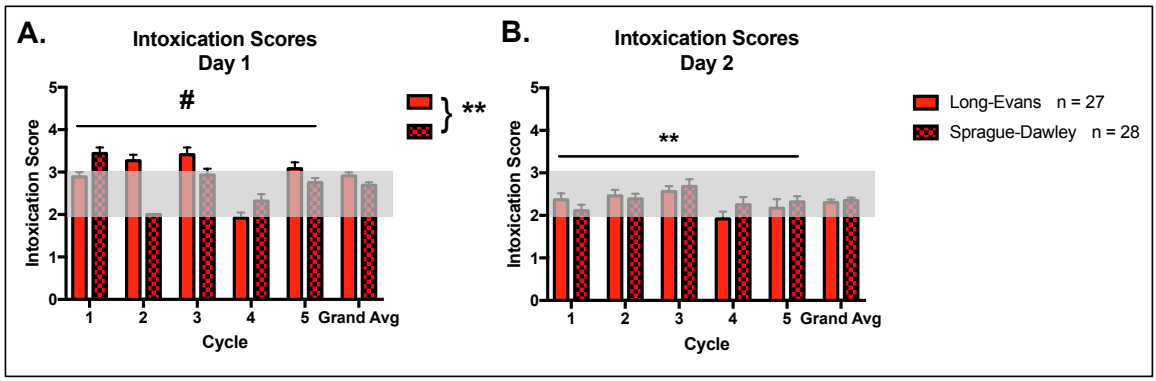

Figure 2-2. Strain of rat is not associated with persistent or consistent differences in intoxication scores across five cycles of ethanol exposure. A) Intoxication scores collapsed across rat strain for day 1 of each of five cycles of intoxication, and a grand average collapsed across all five cycles of ethanol exposure. B) Intoxication scores collapsed across rat strain for day 2 of each of five cycles of intoxication, and a grand average collapsed across all five cycles of ethanol exposure. ** $p < 0.01$ # $p < 0.0001$

The grand averages, computed from the intoxications scores from the respective days of each of the five exposure cycles, for Day 1 were 2.91 ± 0.08 for LE rats, and 2.69 ± 0.07 for SD rats; an unpaired t-test revealed no significant differences between strains with respect to this measure. The grand averages for Day 2 were 2.30 ± 0.07 for LE rats, and 2.35 ± 0.07 for SD rats; a separate unpaired t-test revealed no significant differences between strains with respect to this measure, either. Finally, the grand average for both days across all cycles were 2.60 ± 0.06 for LE rats, and 2.52 ± 0.05 for SD rats (**Figure 2-3**). A 2-way ANOVA (strain by day of cycle) revealed that there was a significant difference due to day of cycle ($F_{(1,250)}$ = 40.98 $p < 0.0001$), but no significant difference between strains. Additionally, an unpaired t-test of intoxication scores collapsed across and all cycle numbers and both days of the cycle revealed that there was no significant difference between the strains ($t_{(490)}$ = 0.698 $p = 0.486$).

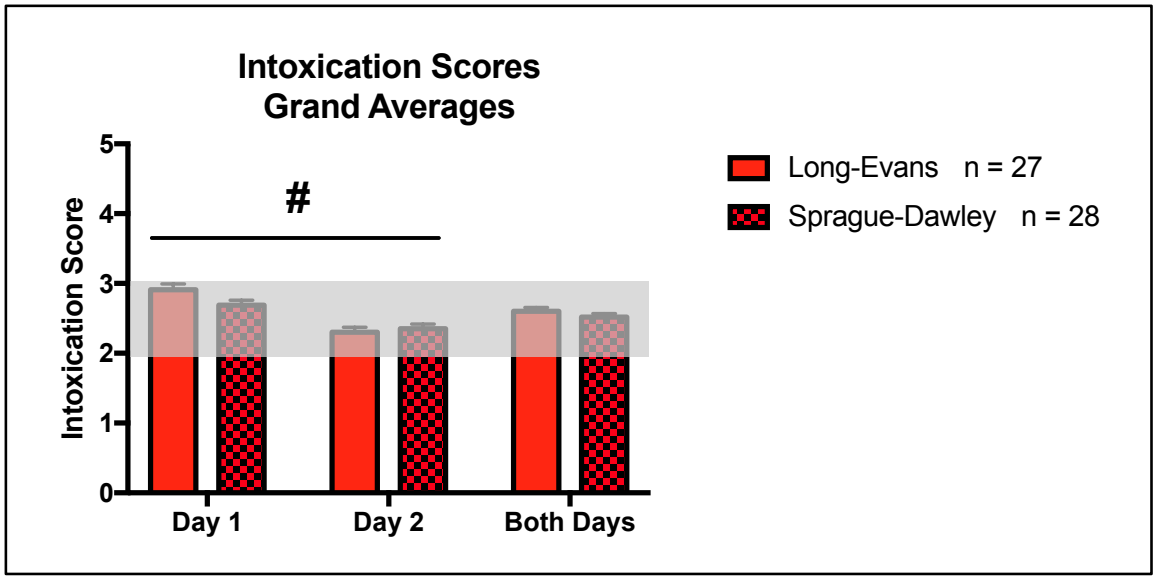

Figure 2-3. Strain of rat is not associated with differences in average intoxication scores. Grand average intoxication scores for Days 1 & 2 across five cycles of binge-like ethanol exposure, and collapsed into a grand average for both days across the five cycles. **** $p < 0.0001$

Tail vein blood drawn at the end of each of the 2-day ethanol vapor exposure cycles was used to measure blood ethanol concentrations (BEC). A 2-way ANOVA revealed a significant difference due to strain ($F_{(1,247)} = 86.08$ $p < 0.0001$), and a significant interaction between strain and cycle number ($F_{(4,247)} = 3.866$ $p = 0.0046$), but no significant difference due to cycle number alone ($F_{(4,247)} = 2.396$ $p = 0.051$) (**Figure 2-4A**). Additionally, an unpaired t-test of the grand average of BECs, collapsed across all 5 exposure cycles within strain, revealed a significant difference between strains ($t_{(252)} = 8.488$ $p < 0.0001$) (**Figure 2-4B**).

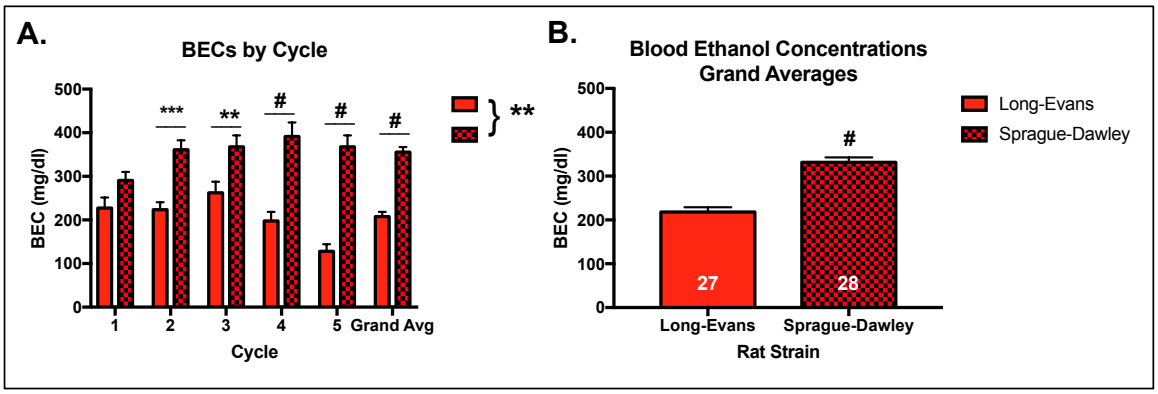

Figure 2-4. Sprague-Dawley rats display significantly higher BECs compared to Long-Evans rats throughout five cycles of ethanol vapor exposure. A) Average BECs by cycle and rat strain for cycles 1-5, with B) a grand average collapsed across all five cycles, all displaying a significantly increased BEC in SD compared to LE rats. ** $p < 0.01$ *** $p < 0.001$ # $p < 0.0001$

As both the intoxication scores and BECs were revealed to be significantly different due to strain across exposure cycles, an analysis was conducted of the correlation of the average BECs at each intoxication score within rat strain. The average BEC at each intoxication score was significantly correlated for both LE ($p = 0.0006$) and SD ($p = 0.0087$) rats (**Figure 2-5**). Additionally, a 2-way ANOVA (strain by intoxication score) revealed that there was a significant effect of both strain ($F_{(1,242)} = 43.39$ $p < 0.0001$) and intoxication score ($F_{(3,242)} = 37.09$ $p < 0.0001$), but no interaction between the two ($F_{(3,242)} = 0.963$ $p = 0.411$). Additionally, multiple comparisons with Sidak's correction revealed that the BEC values for each strain were significantly different from each other at each intoxication score: 1 ($t_{(242)} = 2.6$ $p = 0.39$), 2 ($t_{(242)} = 7.62$ $p < 0.0001$) 3 ($t_{(242)} = 5.642$ $p < 0.0001$), and 4 ($t_{(242)} = 2.53$ $p = 0.047$). There were insufficient samples ($n = 3$ SD, $n = 0$ LE) to conduct analyses at an intoxication score of 5 (**Figure 2-5**).

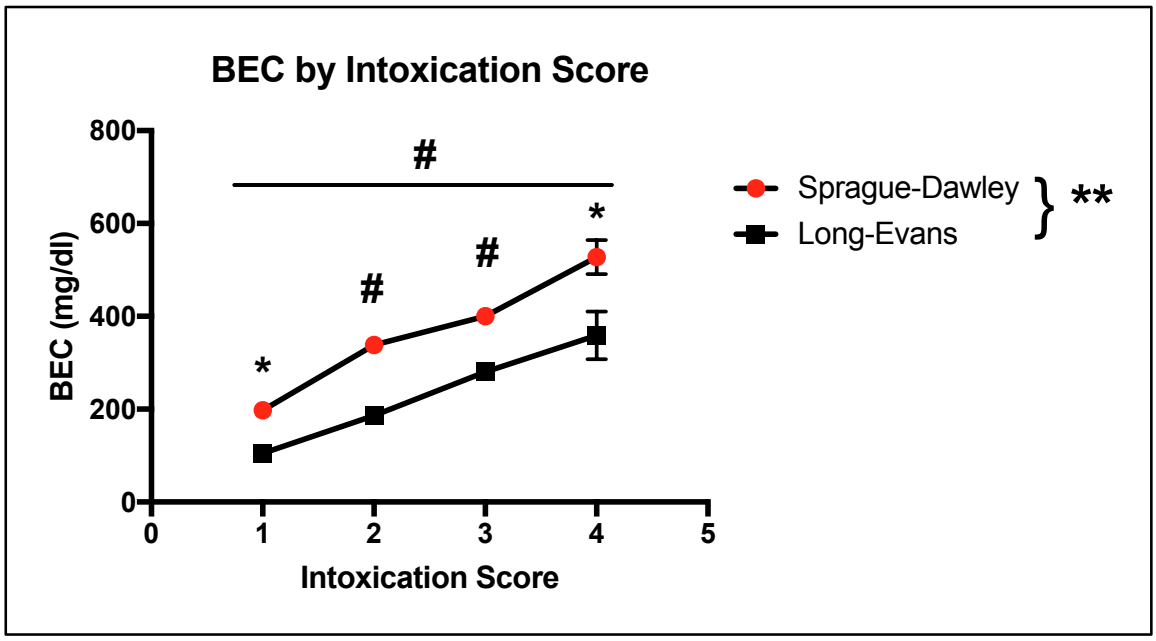

Figure 2-5. Sprague-Dawley rats display significantly increased BEC values at each intoxication score compared to Long-Evans rats. n = 15-20 BEC values for 1, n = 40-70 BEC values for 2, n = 47 BEC values for 3, n = 4-6 BEC values for 4, n = 3 BEC values for 5 * $p < 0.05$ ** $p < 0.01$ # $p < 0.01$

Analysis of the two methods used to calculate BECs in these studies (colorimetric assay and enzymatic oxygen rate detection) revealed that there was no significant difference in the BECs generated from each measure (unpublished observation from our lab). Therefore, it seems reasonable to conclude that, at a given level of intoxication determined by behavioral observation, SD rats display an increased BEC compared to LE rats. While this is a potentially interesting observation, additional studies are required to more fully define these differences. To this end, additional studies involving much larger sample numbers are ongoing in the lab to correlate BECs with intoxication scores according to age, strain, method of BEC analysis, and researcher.

EFFECT OF AIE ON ANXIETY IN ADULTHOOD

Following AIE exposure, rats were allowed to mature into early adulthood (after PD60), and then assessed for anxiety-like behavior with the light/dark box. This task assesses anxiety-like behavior in rodents by taking advantage of two conflicting drives: the exploratory drive, as the apparatus and environment are novel, and the drive for safety and security, as rodents usually prefer dark, enclosed spaces. Increased time spent in the dark side of the box would indicate an increased drive for safety and a decreased exploratory drive, which could be interpreted as increased anxiety-like behavior. In contrast, increased time spent in the light side of the box is typically interpreted as a display of decreased anxiety-like behavior.

Light/dark box: Identical boxes of opaque, glossy black or white PVC were constructed and joined together with a doorway tall and wide enough for a standard adult rat. The white/light box was constructed without a ceiling whereas the black/dark box had a ceiling of the same black PVC material. The light/dark box apparatus had no floor, so it was set on the same white, matte, wood-grained PVC flooring as the open field. The testing room was illuminated by overhead fluorescent bulbs, and a white noise machine – turned to “white noise” – had the volume set to 76 dB. In the light side of the box, the fluorescent bulbs produced an illumination of approximately 300 lux. Following a 5-minute acclimation period, the test subject was removed from the home cage and placed

in the light box facing away from the doorway to the dark box. The subject was left alone in the testing room for 5 minutes. At the end of the test the room lights were turned on and the test subject was returned to the home cage. The assessment was recorded from directly above at 60 frames per second for later analysis in Ethovision. Between each test subject the entire apparatus and the PVC flooring were wiped down with Cavicide and allowed to air dry. At a later time, the video files were analyzed in Ethovision for the amount of time spent in the light side of the box as well as number of entries into the light side of the box. These values were averaged across treatment groups, and differences between groups were assessed via a 2-way ANOVA for exposure (AIE or Control) and stress (SPS or Control) with a significance threshold of $\alpha=0.05$. This task was never administered more than once for each test subject in order to reduce any confounds due to environmental acclimation.

Following AIE exposure, both Long-Evans and Sprague-Dawley AIE and Control groups were assessed for anxiety-like behavior using the light/dark box task. The data were analyzed using paired t-tests within each rat strain, as the time spent in the light side of the box was calculated as the time during the session in which the rat was not in the dark side of the box; that is, it is the same data expressed in different ways. This analysis revealed that the Long-Evans AIE group spent more time in the light side of the box compared to the Control group ($t_{(11)} = 2.42$ $p = 0.034$), whereas the Sprague-Dawley AIE group spent more time in the dark side of the box ($t_{(7)} = 3.47$ $p = 0.010$) (**Figure 2-6**).

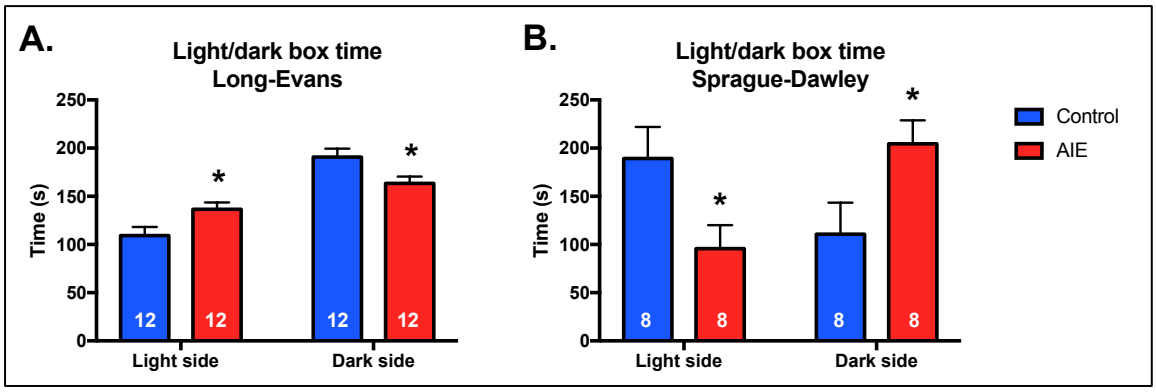

Figure 2-6. AIE is associated with differential anxiety-like behavior in Long-Evans versus Sprague-Dawley rats. AIE led to decreased anxiety-like behavior in Long-Evans (A) but increased anxiety-like behavior in Sprague-Dawley rats (B). * $p < 0.05$

This indicates decreased anxiety-like behavior for LE rats, but increased anxiety-like behavior for SD rats following AIE exposure. This decreased anxiety-like behavior in LE rats may be interpreted as an increase in disinhibition as has previously been observed in our lab in adult rats exposed to AIE. The light/dark box takes advantage of the conflicting drives in the rodent to explore a novel environment (spend time in the light side of the box) and to avoid brightly lit areas (spend time in the dark side of the box). Increased disinhibition could manifest similarly to decreased anxiety-like behavior, as a decreased drive for safety and an increased drive to explore. Literature assessing anxiety-like behavior after AIE exposure shows mixed results, with some studies showing increases in anxiety-like behavior in adulthood (Coleman et al., 2014; Pandey, Sakharkar, Tang, & Zhang, 2015; Sakharkar et al., 2016; Vetreno, Broadwater, Liu, Spear, & Crews, 2014), and others showing decreased anxiety-like behavior, or increased disinhibition (Ehlers, Criado, Wills, Liu, & Crews, 2011; Gass et al., 2014; Gilpin,

Karanikas, & Richardson, 2012). In order to clarify these results from tasks designed to assess anxiety, the modified open-field conflict task could be useful as an additional measure. This task uses food placed in the middle of a brightly lit open field, and the rat's contact with the food may be interpreted as a measure of its disinhibitory drive. Animals tested in adulthood after AIE using this procedure were reported to spend significantly more time exploring and eating the food, consistent with the suggestion that AIE leads to an increased disinhibitory drive (Ehlers et al., 2011). This increase in disinhibition in adulthood following AIE exposure may be mediated by PFC dysfunction, as inactivation of the PFC led to increased time spent in the open arms of the elevated plus maze (Shah, Sjovold, & Treit, 2004).

It is important to note that rat strain and AIE exposure method may be an important factor contributing to these mixed results of anxiety-like behavior in adulthood after AIE. Some studies that used SD rats showed that AIE led to increased anxiety-like behavior in adulthood (Pandey et al., 2015; Sakharkar et al., 2016), whereas studies that used LE rats showed an increase in disinhibition after AIE (Ehlers et al., 2011; Gass et al., 2014). Additionally, studies that used intragastric or intraperitoneal administration of ethanol are the same studies that reported increased anxiety-like behavior, and those that reported an increase in disinhibitory drive administered ethanol in vapor chambers. It is possible that increased stress from injections or gavage of ethanol may combine with the effects of binge-like ethanol exposure to produce increased anxiety-like behavior in adulthood, whereas the relatively lower stress of ethanol vapor inhalation

would not lead to this increase in anxiety-like behavior (Crews et al., 2016). Finally, it should be noted that the significant increase in the amount of time spent on the light side of the box for LE rats may be statistically significant but not functionally meaningful, as the difference in time spent between each side is 27.25 seconds out of a total of 300 seconds. Further studies replicating this result would confirm if this is a functionally meaningful difference in the LE AIE compared to Control groups.

In conclusion, both SD and LE rats' behavioral intoxication scores were within the target range of the AIE exposure. Interestingly, the BECs of the SD rats were significantly higher than those of the LE rats for a given level of behavioral intoxication. The objective of the vapor exposure procedure was to maintain a consistent level of intoxication not only across cycles, but also between strains. This resulted in higher BEC values for SD rats as a group. This may be due to several factors, including rat strain. However, further analysis with different cohorts (as is currently ongoing in the lab) is necessary to confirm these differences. Following AIE exposure, LE rats displayed decreased anxiety-like behavior (or increased disinhibition) on the light/dark task, whereas SD rats displayed increased anxiety-like behavior. These differences in performance on a task used to measure anxiety-like behavior may demonstrate inherent differences between SD and LE rats' response to AIE exposure, as several studies using SD rats have demonstrated increased anxiety-like behavior following AIE, and studies using LE rats have demonstrated increased disinhibition, similar to what is reported here. However, as differences in ethanol

sensitivities were not the focus of this project, further testing is needed to clarify whether LE rats show decreased anxiety-like behavior or increased disinhibitory behavior, possibly with the modified open-field conflict task.

CHAPTER 3

ADOLESCENT BINGE-LIKE ETHANOL EXPOSURE DIFFERENTIALLY AFFECTS PROBABILISTIC REVERSAL LEARNING IN LONG-EVANS VERSUS SPRAGUE-DAWLEY RATS

BACKGROUND & SIGNIFICANCE

The prefrontal cortex (PFC) is the seat of executive control. As such, it takes in information from sensory, motor, and association cortices; processes it along with information from long-term and working memory storage; assesses the values of various stimuli and possible actions; and selects appropriate behavioral responses. This includes functions such as value assessment (thought to depend at least in part on the orbitofrontal cortex, OFC), positive and negative feedback sensitivity (mediated in part by the medial PFC, mPFC), and working memory (through recurrent loops of excitatory neurotransmission within the cortex). Each of these functions are important for behavioral flexibility, or keeping the balance between focus on the current task and the ability to flexibly update strategies in order to optimize outcomes (Floresco, 2013).

Although the PFC is responsible for executive control over all cerebral activity, it is one of the last areas of the brain to reach maturity. Accordingly, it is especially vulnerable to pathological changes during its developmental trajectory, which includes adolescence. The PFC undergoes thinning of gray matter and development of white matter throughout adolescence. However, adolescence is

behaviorally typified by increased risk-taking, such as abuse of ethanol. More than 90% of ethanol consumed by individuals under the age of 21 is in the form of binge drinking, or more than four drinks within two hours as defined by the National Institutes of Health (NIAAA, 2015b). It has been reported that binge-like ethanol exposure during adolescence in humans leads to increased thinning of gray matter as well as decreased integrity of white matter in the frontal cortex (Luciana et al., 2013; Squeglia, Rinker, et al., 2014). It has also been reported that the PFC is more vulnerable to insult by ethanol than other brain regions (Bava et al., 2009a).

Multiple studies have shown that adolescent binge-like ethanol exposure leads to functional impairments and anatomical abnormalities in cortical areas critical for reversal learning. Reduced responses to reward were seen in OFC neurons in rats following adolescent binge-like ethanol exposure (McMurray et al., 2016). An increase in OFC volume was seen in rats following a binge-like ethanol exposure in adolescence (Coleman et al., 2014), which parallels findings in a clinical study of adolescent males who abused ethanol (Medina et al., 2008); this appears to be pathological, as orbitofrontal and ventrolateral PFC areas typically decrease in volume throughout adolescence (Gogtay et al., 2004). Adolescent binge-like ethanol exposure has also been shown to impair mPFC function as assessed via operant set-shifting (Gass et al., 2014). Additionally, a study by Coleman et al. (2014) in rats demonstrated that adolescent binge-like ethanol exposure led to deficits in behavioral flexibility as measured via spatial reversal learning. However, this study used a reversal task dependent on spatial

learning and memory, which is mediated in relatively larger part by the hippocampus. The probabilistic reversal learning (PRL) task used in the following experiments is an operant procedure, so it is not as dependent on hippocampal function. Additionally, the procedure in the following experiments utilizes probabilistic reinforcement, which more robustly engages working memory and value assessment, both mediated by prefrontal cortical areas.

While these are effects of a rat model of binge-like ethanol exposure in adolescence (adolescent intermittent ethanol, AIE), both stress and AIE exposures lead to similar deficits. Both types of exposures lead to impaired behavioral flexibility as assessed via reversal learning and set-shifting tasks (George et al., 2015), as well as decreased prefrontal engagement ("hypofrontality") in the PFC and OFC during a set-shifting task (Park et al., 2016). Additionally, both AIE and a model of post-traumatic stress disorder, the single prolonged stress (SPS) exposure, led to impaired habituation of the corticosterone (CORT) response to repeated stress; adult animals typically respond to repeated stress with successively lower concentrations of CORT in the blood (habituation of the CORT response), but AIE (Varlinskaya et al., 2016) and SPS (Liberzon et al., 1997) both lead to impaired habituation of this response.

All of the studies mentioned previously comparing the effects of stress and AIE used Sprague-Dawley (SD) rats, an outbred albino rat strain. However, as noted in chapter 2, Long-Evans (LE) rats displayed reduced anxiety-like behavior whereas SD rats displayed increased anxiety-like behavior as assessed via the

light/dark box following AIE exposure. Previous work in our lab has also demonstrated that AIE led to decreased anxiety-like behavior as assessed via the elevated plus maze (Gass et al., 2014). Taken together, this led to the hypothesis that LE and SD rats may be differentially affected by AIE and/or SPS exposure, and that they may perform differently on the reversal learning task used in the following experiments. Specifically, it was hypothesized that greater deficits would be observed following AIE and/or SPS exposure in Sprague-Dawley compared to Long-Evans rats in the reversal learning task.

MATERIALS & METHODS

Animal care, AIE exposure, blood ethanol concentration (BEC) analysis, and SPS exposure were presented in chapter 2. The specific timeline of AIE exposure, PRL testing, SPS exposure, and PRL re-testing for the studies in this chapter is schematically presented in **Figure 3-1**.

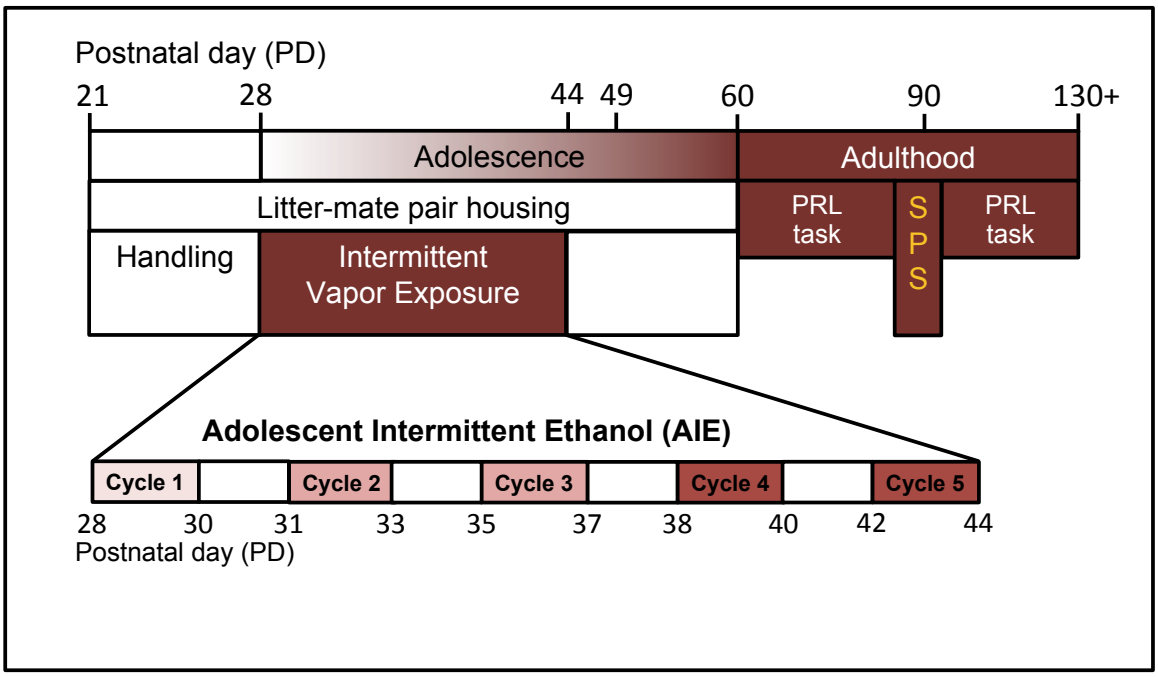

Figure 3-1. Experimental timeline of AIE exposure, pair housing, and operant testing

Operant Behavior

One week prior to the beginning of any operant training, rats were single housed and restricted to 85-90% of their *ad libitum* feeding weight. All operant training was started at PD70 or later. Additionally, rats were habituated to the process of moving to the behavior room for a minimum of two days before being placed in the operant box. Animals were also accustomed to the reward that would be used for operant training by receiving ~20 mL of 20% sucrose in their home cage Monday through Friday the week before operant training began. For the first day of operant training, a program was used that dispensed 10 µl of 20% sucrose at random intervals over the course of 30 minutes; 60 total reinforcements were dispensed. This was to habituate the rats to the sound of

the syringe pump turning on and off to deliver the liquid reward, and to associate the reward delivery well with a reward.

Operant boxes: Plexiglas operant boxes (Med Associates, St. Albans, VT) used in the present study measured 32 cm W x 25 cm D x 11 cm H and were located in melamine sound attenuating cubicles. Each cubicle was equipped with an exhaust fan to provide air circulation and mask external noise. Mounted on one wall of the self-administration chamber were two response levers that flanked a liquid receptacle connected to a single speed syringe infusion pump with polyethylene tubing. Located above the active lever was a 2.5 cm diameter stimulus light that was turned on during various phases of testing. Located at the top the chamber was a house light and a Sonalert speaker that emitted a tone (2900 Hz, ~65 dB) that were activated during various phases of testing. Chambers were interfaced to a PC computer that controlled experimental sessions and recorded data using commercially available software (MED Associates, MED-PC IV).

Probabilistic Reversal Learning (PRL) Task

The PRL task was based on previous work as detailed in Bari et al. (2010) and Dalton et al. (2014). The operant training consisted of 3 distinct phases: a Training phase in which rats were trained to lever press, a Probability Habituation phase in which rats learned to respond on a lever within a fixed amount of time (a trial; this phase also included the rats learning that each lever had a 50%

probability of delivering a reward), and a probabilistic reversal testing phase, where the levers changed probability so that the “correct” lever had an 80% probability of delivering a reward and the “incorrect” lever had a 20% probability of delivering a reward. Additionally, reversals of the levers were also incorporated into the program (i.e. the left lever changes from being “correct” to “incorrect”) so that the lever identities were reversed following 7 responses on the “correct” lever regardless of reward delivery.

Training phase: During this phase, rats were trained to respond to a lever with a lever press, on a fixed-ratio 1 (FR1) schedule of reinforcement. At the beginning of the first training session (30 min), one of the levers was extended and the house and receptacle lights were illuminated. Each lever press activated a syringe pump for 1.5 sec to deliver approximately 45 µl of 20% sucrose into the reward receptacle. Rats received daily 30 min sessions until stable responding was achieved, which was defined as receiving at least 50 reinforcements during a session for two consecutive training days. Training then proceeded to an FR1 schedule of reinforcement of whichever lever was not reinforced at first. Stable responses (receiving at least 50 reinforcements during a session for two consecutive training days) in this phase were required before progressing to the next phase of the training.

Probability Habituation phase: This phase served to train the rats to respond with a lever press within a discrete period of time (a trial), as well as to establish

probabilistic reinforcement of lever presses. It consisted of 3 programs of decreasing trial length time. The first and second programs had an inter-trial interval (ITI) of 40 seconds, and the third and final program had an ITI of 15 seconds. Each program had the exact same order of other components. At the start of each trial the house light would illuminate and both levers would extend. If a lever was selected then there was a 50% probability of activating a syringe pump for 1.5 seconds to deliver approximately 45ul of 20% sucrose. There were 90 trials in each of the first two programs with 20 seconds and 10 seconds during which a lever could be selected, respectively for the first and second training program. There were 100 trials in the last program with the shortest ITI time and 10 seconds in which a lever could be selected. Each rat had to omit fewer than 10 trials on 2 successive training days to progress from one program to another. For the third and final program, each rat had to omit fewer than 10 trials for 3 successive training days in order to progress to the Probabilistic Reversal testing phase.

Probabilistic Reversal Learning (PRL) testing phase: This phase introduced differential probabilistic reinforcement of lever pressing, in which each lever had different probabilities of reward. Additionally, this phase was the testing phase. It consisted of 1 day of testing on the First Day PRL program and 15 days of testing on the probabilistic reversal program. For the First Day PRL program, there were 240 trials with an ITI of 16 seconds. Each trial was identical to the final program in the Probability Habituation phase, except that the probability of

reward for each lever was adjusted so that the “correct” lever had an 80% probability of delivering a reward when pressed, and the “incorrect” lever had a 20% probability of delivering a reward; the “correct” and “incorrect” lever identities were randomly assigned at the start of each training day. For the first 80 trials there were no reversals (no changes in lever identity from “correct” to “incorrect” or vice versa). Reversals of the lever identities were suspended for the first 80 trials to ensure that the rat was adequately familiar with the differential probabilistic reinforcement associated with each lever. Starting on the 81st trials, 7 consecutive responses on the “correct” lever caused a reversal; that is, the identities of the levers reversed (i.e. the right lever changes from being the “incorrect” lever to now being the “correct” lever). This pattern continued until the 240th trial; there was no limit set on the number of reversals that could be completed. For the PRL program on days 2 through 16 of testing, there were only 200 trials total. Additionally, reversals could be completed starting with the first trial; that is, responses on the “correct” lever for the first 7 trials of this program would lead to a reversal of lever identities on the 8th trial. Reversals were allowed immediately in the program used on days 2 through 16 as the rat was familiarized with the differential probabilistic reinforcement of the levers with the First Day program. On the 8th day after SPS exposure, test subjects were re-started on the same PRL program that was used for days 2 through 16 of testing prior to SPS for 4 days.

Data Analysis: For the first day of PRL testing (with the First Day program), the following 15 days of PRL testing, and the 4 days of PRL testing following SPS exposure, the following parameters were assessed: number of completed reversals, number of omissions, average latency to lever press, number of errors committed in the 1st discrimination and 1st reversal, the win-stay ratio, the lose-shift ratio, number of trials to meet criterion for the 1st discrimination and 1st reversal, corrected trials to meet criterion for the 1st discrimination and 1st reversal, and reversals per 100 trials (**Table 3-1**).

Table 3-1. Parameters assessed during PRL testing

Parameter	Definition	Notes
Criterion	Seven correct responses without errors	Minimum number of correct responses to cause a reversal of lever identities
Reversals completed	Sum of the number of times the subject met criterion in one session	
Omissions	Trials without a lever press	
Reversal per 100 trials	Reversals per session averaged per 100 trials	Accounts for large discrepancies between subjects in number of reversals per session
Average latency to lever press	The time between lever presentation and lever press, averaged across all trials for one session	
Errors in discrimination	Incorrect responses before the first reversal was completed	
Errors in 1st reversal	Incorrect responses after the first reversal and before the second reversal was completed	
Win-stay ratio	The proportion of trials where a correct, rewarded response was followed by another correct response	
Lose-shift ratio	The proportion of trials where a correct, unrewarded response was followed by an incorrect response	
Trials to criterion for discrimination	The number of trials needed to meet criterion before the first reversal was completed	
Trials to criterion for 1st reversal	The number of trials needed to meet criterion after the first reversal but before the second reversal was completed	
Corrected trials to criterion for discrimination	Trials to criterion for discrimination minus omissions	Accounts for omissions
Corrected trials to criterion for 1st reversal	Trials to criterion for first reversal minus omissions	Accounts for omissions

The number of completed reversals was the number of times the subject met criteria (7 correct selections without errors) within a session. The number of omissions was the number of trials in which the subject did not select a lever within the given time limit of a trial. The average latency to lever press was the average time between the initiation of a trial (lever extension) and when a lever press occurred. The trials encompassed in the 1st discrimination were determined by the number of trials needed to reach criterion (7 correct selections) the first time; this was also the value for the number of trials to meet criterion for the 1st discrimination. For the First Day program it was possible to meet criterion before the 81st trial, but the lever identities would not be reversed until the 81st trial; the corrected trials to meet criterion for the 1st discrimination and the 1st reversal accounted for omissions by subtracting them from the number of trials needed to meet criterion. The number of trials needed to reach criterion for the second time determined the trials encompassed in the 1st reversal; this was also the value for the number of trials to meet criterion for the 1st reversal. For the First Day program, this was computed by taking the total number of trials (240) minus the number of trials in the 1st discrimination. The errors for either the 1st discrimination or the 1st reversal were the number of incorrect selections within those trials encompassed in each section. The win stay ratio was the proportion of trials where a correct, rewarded selection is followed by another correct selection. The lose shift ratio was the proportion of trials where a correct, unrewarded selection is followed by an incorrect selection. Reversals per 100 trials were computed using the number of reversals completed in a session,

divided by the number of trials in a session (the total number of trials minus the number of omissions from that day). Differences between AIE and Control groups were assessed using Students' t-tests, and the threshold for significance was set at $p < 0.05$. Differences between AIE-SPS, AIE-Control, Control-SPS, and Control-Control groups were assessed using ANOVA tests, MANOVA tests, and/or Students' t-tests, with the threshold for significance again being set at $p < 0.05$ or less. Data are presented as mean ± SEM.

RESULTS

The rats used in this set of studies (n = 45) were separated within litters into pair-matched groups of Control or AIE exposed animals. The LE cohort was put through AIE exposure, the PRL task, SPS exposure, and then re-started on the PRL task. After completion of these studies using LE rats, these studies were repeated with SD rats; therefore, the PRL studies in LE and SD rats were run in series and not in parallel. These studies were not run in parallel due to logistical and spatial constraints. Both LE and SD rats were used in order to assess differences in response to AIE and/or SPS exposure by strain, as preliminary data (detailed in chapter 2) from our lab indicated that LE and SD rats displayed decreased and increased anxiety-like behaviors, respectively, after AIE exposure.

In the first set of experiments, differences in operant reversal learning with probabilistic reinforcement were assessed using the probabilistic reversal learning (PRL) task (Gemma L. Dalton et al., 2014), wherein rats chose between

a correct lever that was usually reinforced (rewarded 80% of presses) and an incorrect lever that was not usually reinforced (rewarded 20% of presses). The mOFC is critical to probabilistic reinforcement learning, and the lOFC is important for early but not late serial reversal learning as well as efficient approach to reward-related stimuli (G. L. Dalton et al., 2016). Interestingly, inactivating the mPFC improves performance on this task; an increase in reversals per session may be achieved through this inactivation by increasing both reward and negative feedback sensitivity (G. L. Dalton et al., 2016).

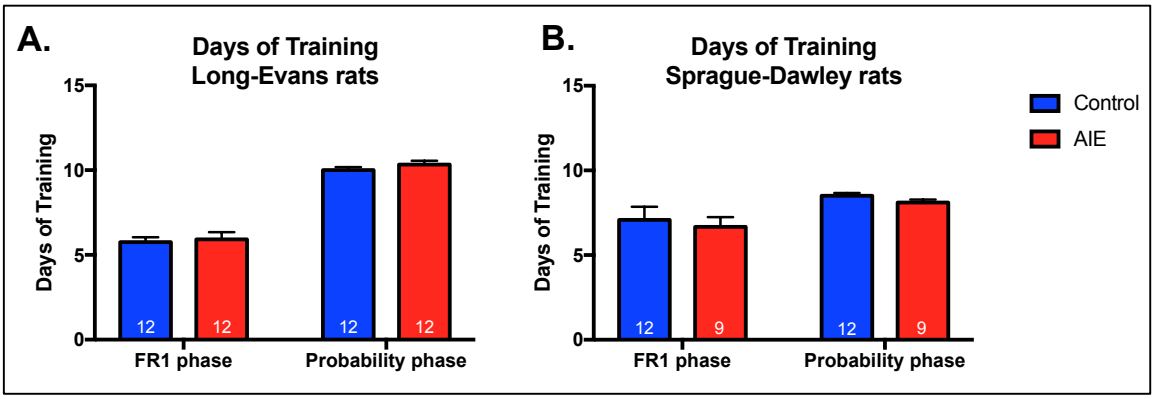

Figure 3-2. AIE is not associated with a deficit in operant learning in either Long-Evans or Sprague-Dawley rats. Average days of operant training for A) Long-Evans rats and B) Sprague-Dawley rats. Training was separated into two phases: an initial phase using an FR1 schedule of reinforcement to associate lever pressing with a reward, and a probability phase using a 50% probability of reward to introduce the concept of probabilistic reinforcement. Additionally during the probability phase, three operant programs with a 50% probability of reward had progressively decreasing inter-trial intervals to decrease response times in preparation for performance of the PRL task.

There was no evidence of a difference in learning ability between AIE and Control groups in either the Long-Evans or Sprague-Dawley cohort, as detailed in **Figure 3-2**. A 2-way ANOVA (AIE exposure by training phase) revealed that there were no differences between AIE and Control groups in number of days of training for either Long-Evans ($F_{(1,44)}$ = 0.719 p = 0.401) of Sprague-Dawley

($F_{(1,38)}$ = 0.583 p = 0.449) rats. This was not unexpected, as previous studies have not shown impairment in operant learning ability following AIE exposure (Fernandez & Savage, 2017; Gass et al., 2014).

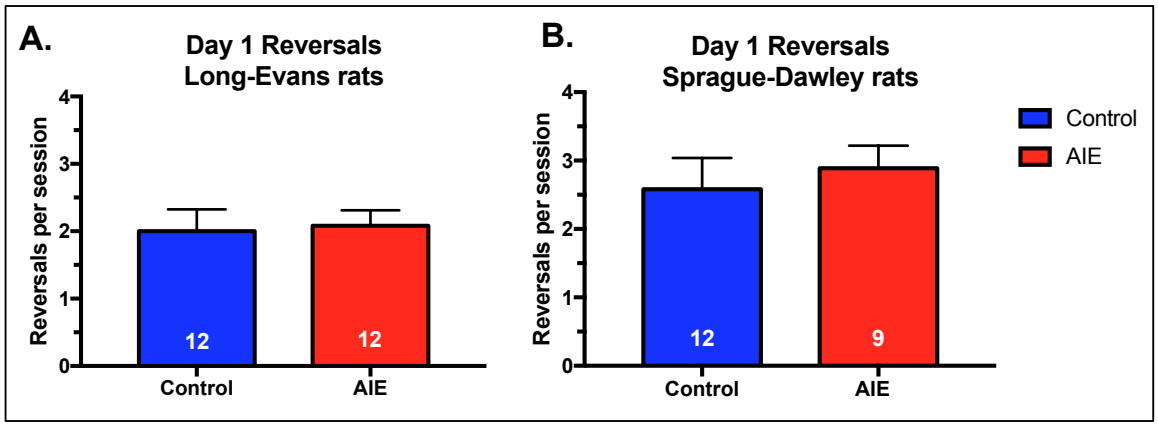

Figure 3-3. AIE is not associated with impairment of reversal learning on day one of the PRL task in either Long-Evans or Sprague-Dawley rats. Average reversals per session on day one of the PRL task for A) Long-Evans rats and B) Sprague-Dawley rats

Contrary to our original hypothesis, there was no difference in the average number of reversals completed per session on the first day of PRL performance between AIE and Control rats in either Long-Evans ($t_{(11)}$ = 0.233 p = 0.820) or Sprague-Dawley ($t_{(8)}$ = 0.229 p = 0.824) rats (**Figure 3-3**). There was also no difference in the average number of omissions per session on the first day of PRL performance between AIE and Control rats in either Long-Evans ($t_{(11)}$ = 1.615 p = 0.135) or Sprague-Dawley ($t_{(8)}$ = 0 p > 0.999) rats (**Figure 3-4**).

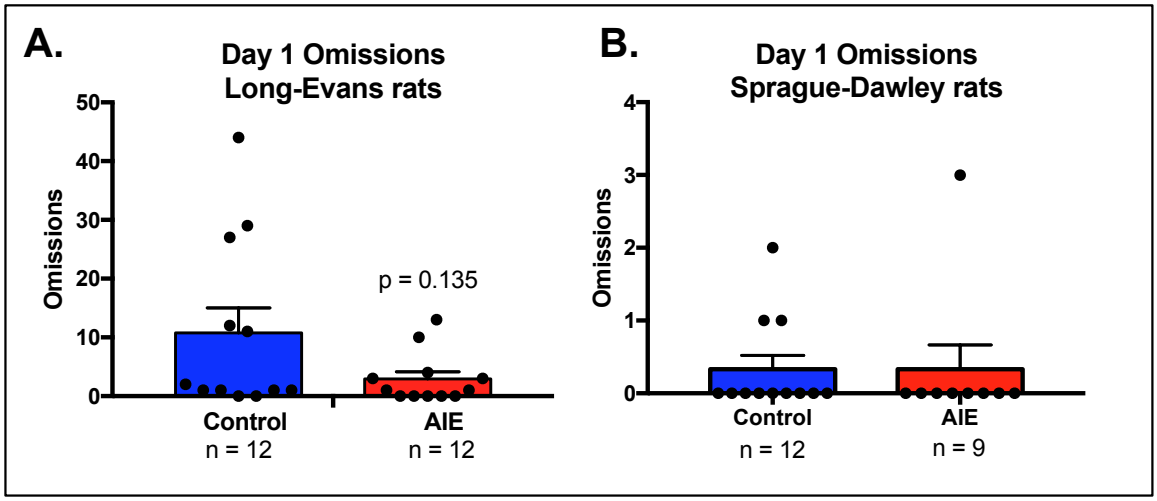

Figure 3-4. AIE is not associated with an increase in omissions on day one of the PRL task in either Long-Evans or Sprague-Dawley rats. Average omissions on day one of the PRL task for A) Long-Evans rats and B) Sprague-Dawley rats. Note the differences in scale between the graphs.

After subtracting omissions from the number of trials needed to reach criterion for each reversal, the number of reversals per session could be computed. Following that, the number of reversals completed per 100 trials can be computed by dividing the number of completed reversals by the total number of trials. This assessment can account for large discrepancies in the number of completed reversals between experimental groups, if present. There were no differences between AIE and Controls in the number of reversals completed per 100 trials in either Long-Evans ($t_{(11)}$ = 0.032 p = 0.975) or Sprague-Dawley ($t_{(8)}$ = 0.222 p = 0.8298) rats (**Figure 3-5**).

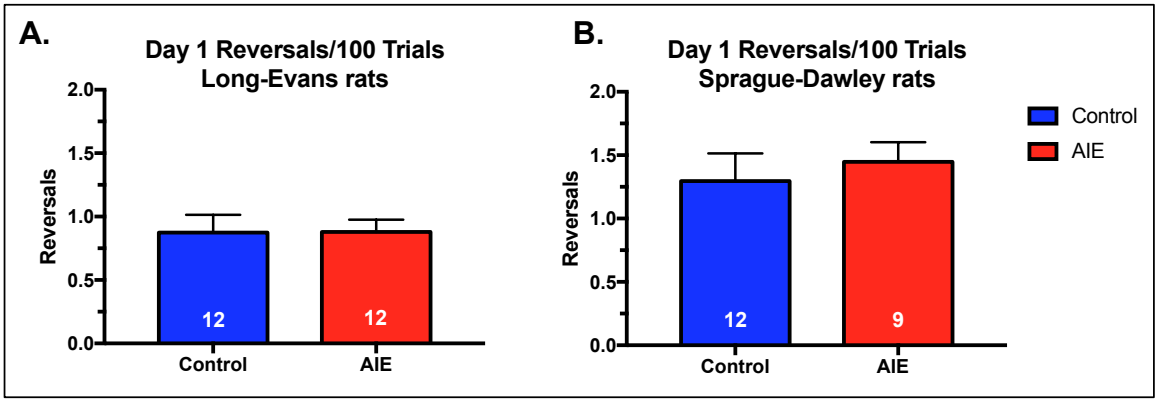

Figure 3-5. AIE is not associated with an altered number of reversals compared to Controls in either strain of rat. Average reversals per 100 trials on day one of the PRL task for A) Long-Evans rats and B) Sprague-Dawley rats

There were no differences between Control and AIE groups in Long-Evans rats on day one of the PRL task in the number of errors committed during the discrimination ($t_{(11)}$ = 1.63 p = 0.13) or first reversal phases ($t_{(11)}$ = 1.081 p = 0.302) (**Figure 3-6**), or in the win-stay ($t_{(11)}$ = 0.858 p = 0.41) or lose-shift ($t_{(11)}$ = 1.484 p = 0.166) ratios (**Figure 3-7**). Neither were there any differences between Control and AIE groups in Sprague-Dawley rats on day one of the PRL task in the number of errors committed during the discrimination ($t_{(8)}$ = 0.693 p = 0.508) or first reversal phases ($t_{(8)}$ = 0.232 p = 0.822) (**Figure 3-6**), or in the win-stay ($t_{(8)}$ = 0.634 p = 0.544) or lose-shift ($t_{(8)}$ = 0.499 p = 0.631) ratios (**Figure 3-7**).

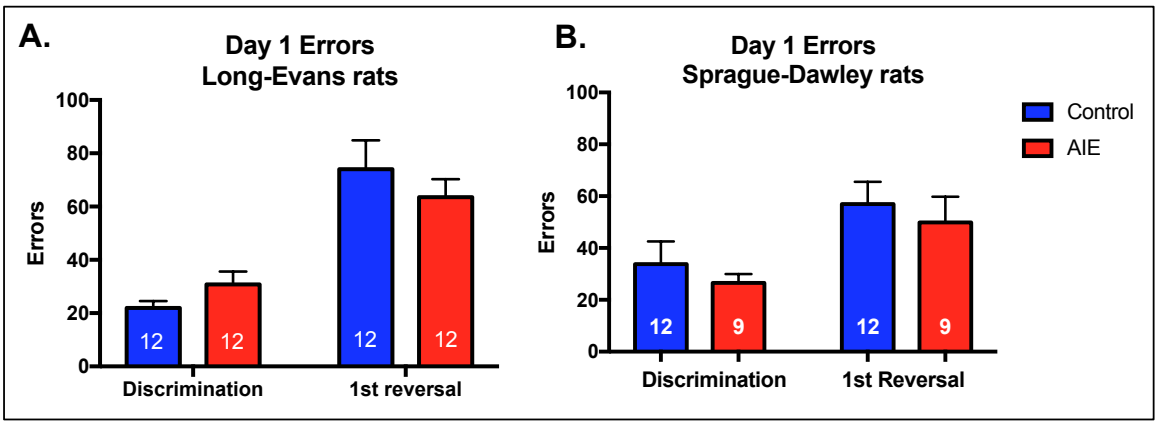

Figure 3-6. AIE is not associated with an alteration in the number of errors on the first day of PRL testing compared to Controls in either strain of rat Average errors during the first discrimination or first reversal on day one of the PRL task for A) Long-Evans rats and B) Sprague-Dawley rats

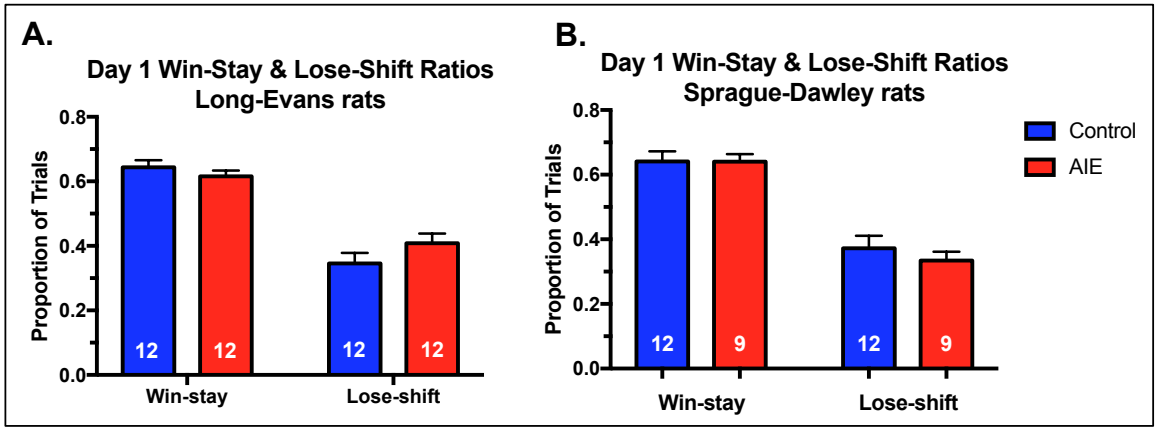

Figure 3-7. AIE is not associated with differences in either the win-stay or lose-shift ratios on the first day of the PRL task in either strain of rat. Average win-stay and lose-shift ratios for day one of the PRL task for A) Long-Evans rats and B) Sprague-Dawley rats

However, AIE Long-Evans rats did require more trials to reach criterion in the discrimination phase ($t_{(11)}$ = 2.613 p = 0.024) compared to Control Long-Evans rats on day one of the PRL task; there was no difference in the number of trials needed to reach criterion in the first reversal phase ($t_{(11)}$ = 0.509 p = 0.621). There were no statistically significant differences in the number of trials needed to reach criterion in Sprague-Dawley AIE compared to Control rats in either the

discrimination ($t_{(8)}$ = 0.602 p = 0.564) or first reversal ($t_{(8)}$ = 1.081 p = 0.311) phases on day one of the PRL task (**Figure 3-8**).

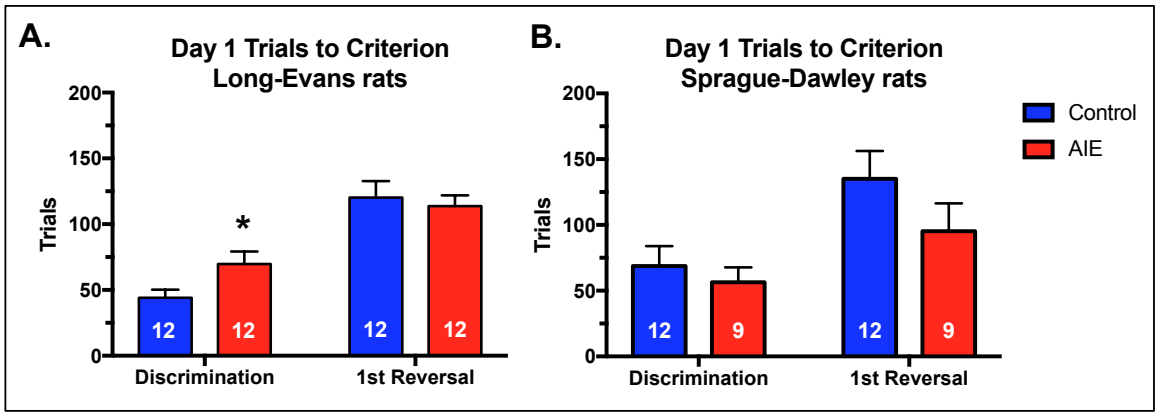

Figure 3-8. AIE resulted in a significant increase in the number of trials needed to reach criterion in the discrimination phase on the first day of the PRL task in Long-Evans rats. Average trials to criterion for the first discrimination or first reversal phases on day one of the PRL task for A) Long-Evans rats and B) Sprague-Dawley rats. * p < 0.05

There was no difference between AIE and Control groups in choice latency on day one of the PRL task in either Long-Evans ($t_{(11)}$ = 0.526 p = 0.607) or Sprague-Dawley ($t_{(8)}$ = 0.377 p = 0.716) rats (**Figure 3-9**).

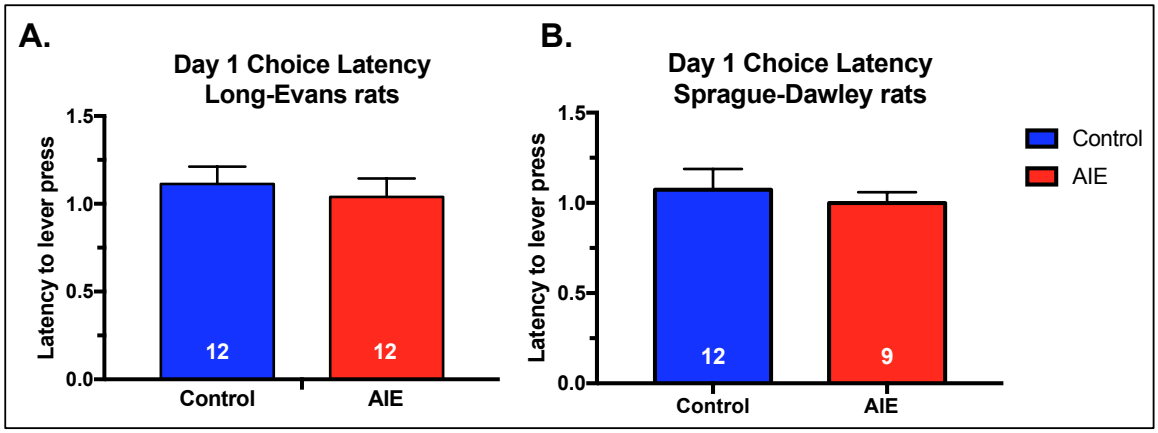

Figure 3-9. AIE is not associated with differences in choice latency on day one of the PRL task in either Long-Evans or Sprague-Dawley rats. Average latencies to lever press on day one of the PRL task for A) Long-Evans and B) Sprague-Dawley rats.

Performance of AIE and Control animals were assessed for 15 additional days on the PRL task, for a total of 16 days of PRL testing in both Long-Evans and Sprague-Dawley rats. A 2-way ANOVA (AIE exposure by days of training) revealed a significant effect of days of training, in that both Long-Evans ($F_{(15,352)}$ = 6.448 $p < 0.0001$) and Sprague-Dawley rats ($F_{(15,304)}$ = 10.51 $p < 0.0001$) exhibited a significant increase in the number of reversals completed per session over 16 days of PRL testing. However, there was an only an effect of AIE exposure in the Sprague-Dawley rats ($F_{(1,304)}$ = 9.982 $p = 0.0017$), and visual examination revealed that the AIE group completed fewer reversals than the Control rats (**Figure 3-10**). There was no difference in the number of reversals completed by Long-Evans AIE compared to Control rats ($F_{(1,352)}$ = 1.393 $p = 0.239$).

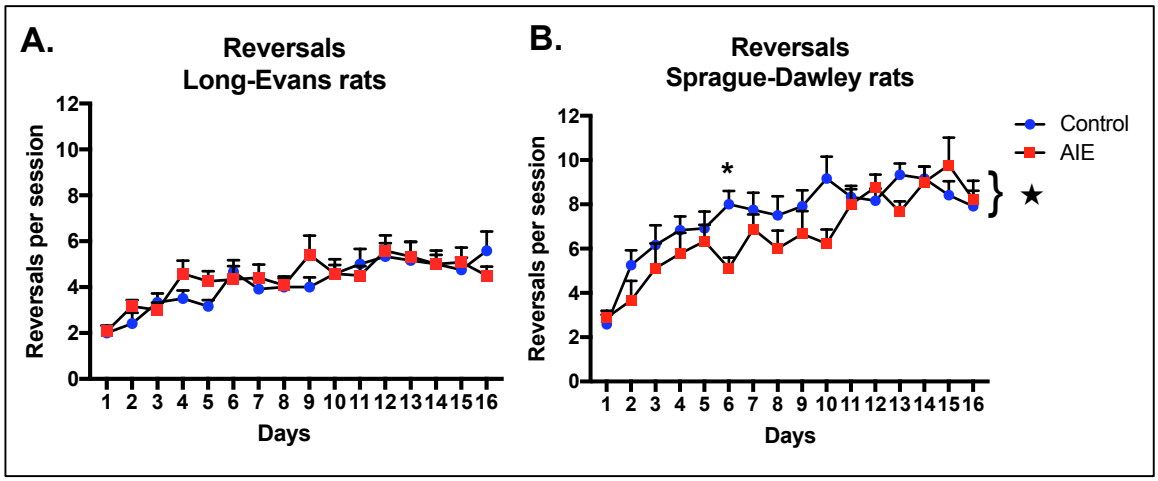

Figure 3-10. AIE resulted in a deficit in the number of reversals completed per session over 16 days of PRL testing in Sprague-Dawley but not Long-Evans rats. Average reversals per session over 16 days of PRL training for A) Long-Evans or B) Sprague-Dawley rats. * $p < 0.05$ ★ main effect of exposure n = 9-12 per group

Over 16 days of PRL testing, there was a significant effect of AIE exposure on the number of omissions per session in Long-Evans rats ($F_{(1,352)}$ = 7.511 p =

0.0064), but there was no effect of days of training ($F_{(15,352)}$ = 0.6951 p = 0.789). Neither AIE ($F_{(1,304)}$ = 0.042 p = 0.838) nor days of training ($F_{(15,304)}$ = 0.577 p = 0.892) had a significant effect on the number of omissions per session for Sprague-Dawley rats (**Figure 3-11**). Note, however, that the lack of effect in Sprague-Dawley rats may be due to a floor effect, as a 2-way ANOVA (rat strain by days of training) revealed that Sprague-Dawley rats committed significantly fewer omissions over 16 days of PRL testing compared to Long-Evans rats ($F_{(1,41)}$ = 16.86 p < 0.0001).

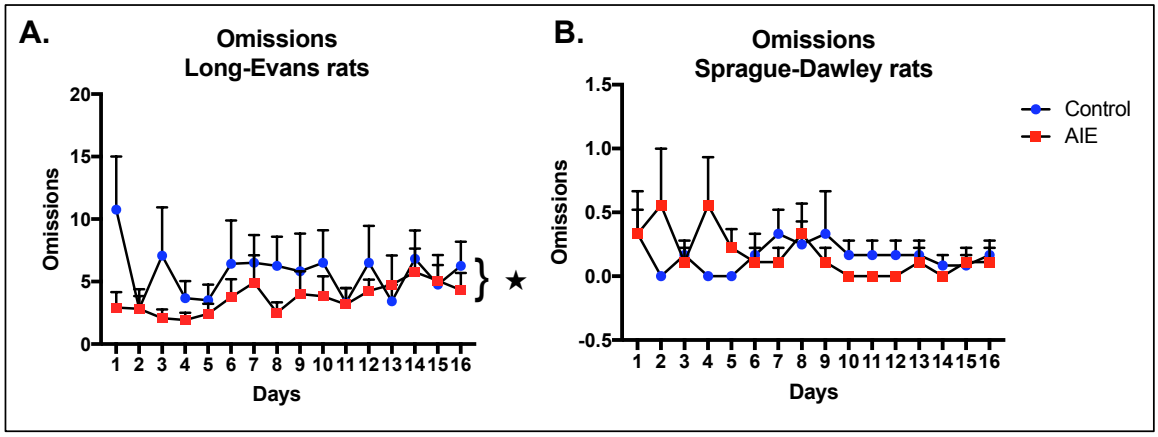

Figure 3-11. AIE resulted in a decrease in omissions compared to Control rats in Long-Evans but not Sprague-Dawley rats. Average omissions per session over 16 days of PRL training for A) Long-Evans or B) Sprague-Dawley rats. Note the differences in scale between the graphs. ★ main effect of exposure n = 9-12 per group

A series of 2-way ANOVAs (AIE exposure by days of training) revealed that there was a significant effect of days of training, in that there were significantly fewer errors committed over 16 days of PRL testing in both the discrimination and first reversal phases for both Long-Evans (discrimination: $F_{(15,352)}$ = 3.178 p < 0.0001, first reversal: $F_{(15,352)}$ = 15.25 p < 0.0001) and Sprague-Dawley (discrimination: $F_{(15,304)}$ = 6.295 p < 0.0001, first reversal: $F_{(15,304)}$ = 16.25 p < 0.0001) rats. However, there was no effect of AIE exposure, in that neither Long-Evans

(discrimination: $F_{(1,352)}$ = 0.731 p = 0.393, first reversal: $F_{(1,352)}$ = 1.568 p = 0.211) nor Sprague-Dawley (discrimination: $F_{(1,304)}$ = 1.354 p = 0.246, first reversal: $F_{(1,304)}$ = 0.604 p = 0.438) rats exhibited a difference in errors committed during the discrimination or first reversal phases between AIE and Control groups over the 16 day testing period (**Figure 3-12**).

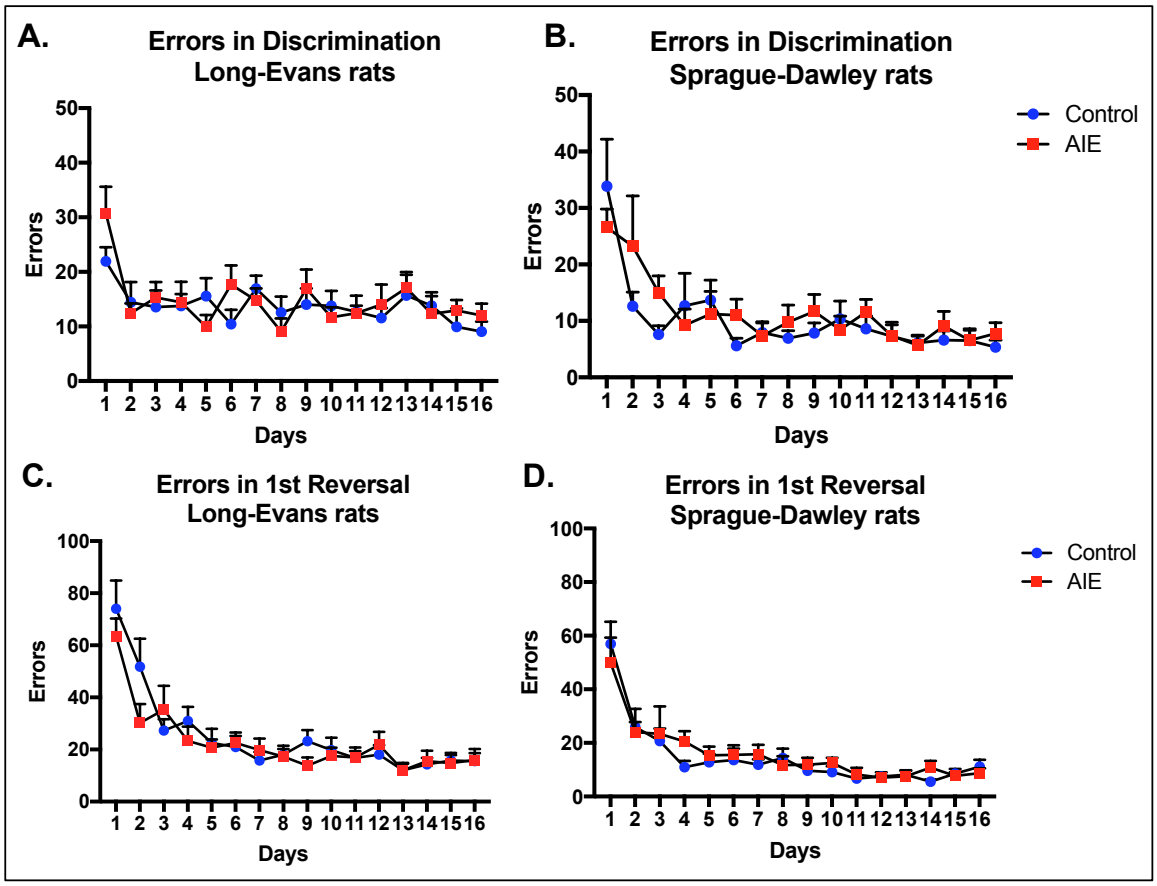

Figure 3-12. AIE is not associated with an alteration in the number of errors committed in either the discrimination or first reversal phases of the PRL task over 16 days of testing. Average errors in first discrimination phase (A & B) or first reversal phase (C & D) over 16 days of PRL training for Long-Evans (A & C) or Sprague-Dawley (B & D) rats. n = 9-12 per group

There was a significant effect of days of testing on win-stay ratios for both Long-Evans ($F_{(15,352)}$ = 10.59 p < 0.0001) and Sprague-Dawley ($F_{(15,304)}$ = 22.93 p <0.0001) rats, as well as a significant effect of days of testing on lose-shift ratios

for Sprague-Dawley ($F_{(15,304)}$ = 7.379 p < 0.0001) but not Long-Evans rats ($F_{(15,352)}$ = 1.895 p = 0.23). However, only Sprague-Dawley rats exhibited a significant effect of AIE exposure on both win-stay ($F_{(1,304)}$ = 31.02 p < 0.0001) and lose-shift ratios (F(1,304) = 14.35 p = 0.0002) over 16 days of PRL testing. Long-Evans rats exhibited no effect of AIE exposure on either win-stay ($F_{(1,352)}$ = 3.741 p = 0.054) or lose-shift ratios ($F_{(1,352)}$ = 2.942 p = 0.087) over 16 days of PRL testing (**Figure 3-13**).

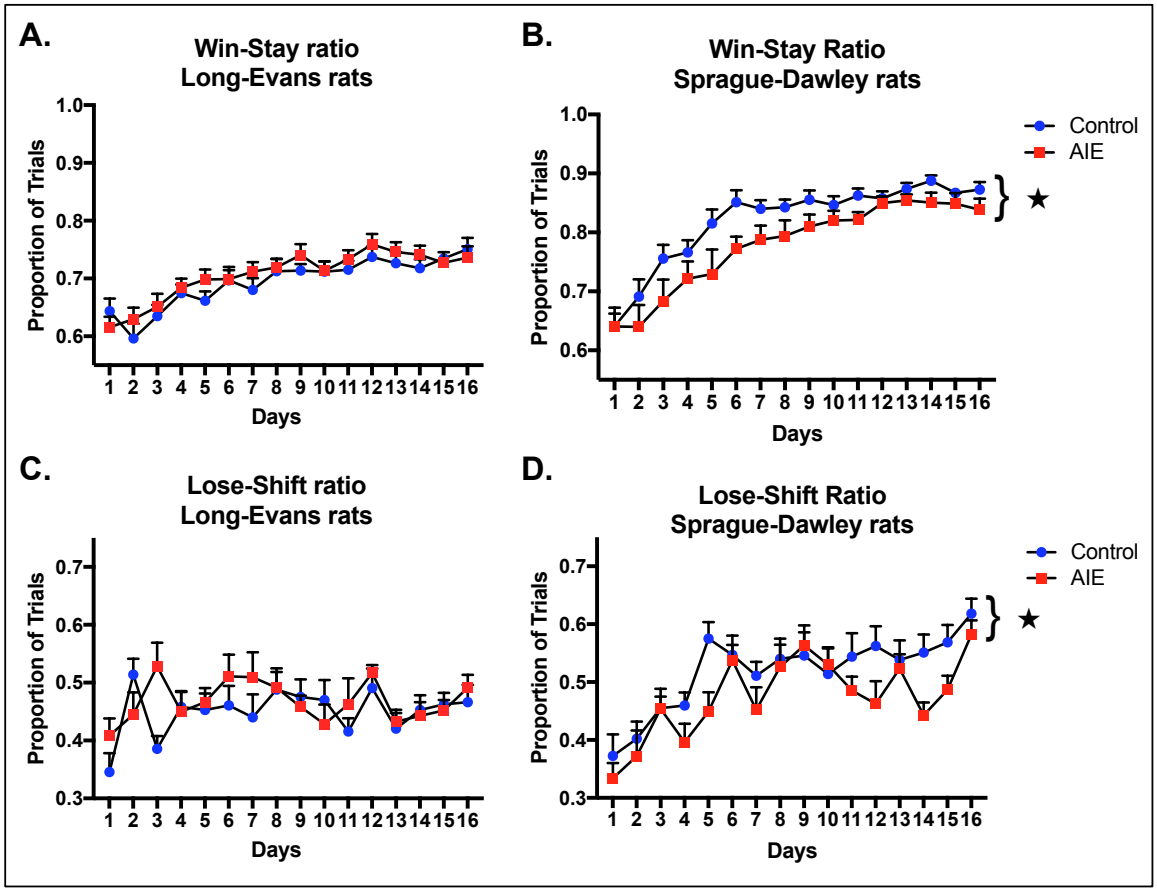

Figure 3-13. AIE resulted in decreased win-stay and lose-shift ratios over 16 days of PRL testing in Sprague-Dawley but not Long-Evans rats. Average win-stay ratios (A & B) or lose-shift ratios (C & D) over 16 days of PRL training for Long-Evans (A & C) or Sprague-Dawley (B & D) rats. ★ main effect of exposure n = 9-12 per group

A series of 2-way ANOVAs (AIE exposure by days of training) revealed a significant effect of days of training, in that Long-Evans rats exhibited a significant decrease in the number of trials needed to reach criterion over 16 days of PRL testing in the first reversal phase ($F_{(15,352)}$ = 2.138 p = 0.008) but not the discrimination phase ($F_{(15,352)}$ = 1.311 p = 0.192). However, with Sprague-Dawley rats there was a significant decrease in the number of trials needed to reach criterion in both the discrimination ($F_{(15,304)}$ = 4.358 p < 0.0001) as well as the first reversal phases ($F_{(15,304)}$ = 8.546 p < 0.0001) of the PRL task over 16 days of training. Neither strain exhibited any significant effect of AIE over the same testing period in either the discrimination (Long-Evans: $F_{(1,352)}$ = 0.047 p = 0.828, Sprague-Dawley: $F_{(1,304)}$ = 1.64 p = 0.201) or first reversal phases (Long-Evans: $F_{(1,352)}$ = 0.603 p = 0.438, Sprague-Dawley: $F_{(1,304)}$ = 1.388 p = 0.239) (**Figure 3-14**).

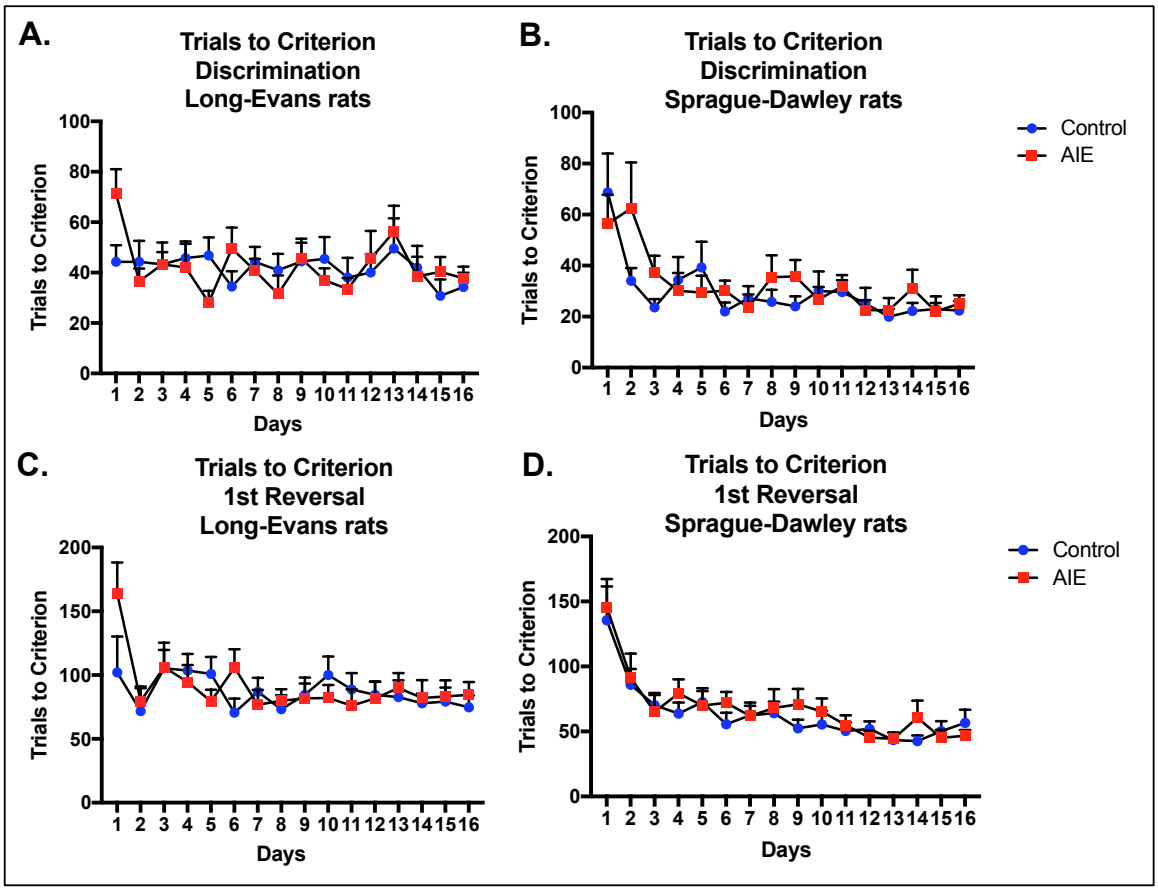

Figure 3-14. AIE is not associated with a deficit in the number of trials needed to reach criterion in the discrimination or first reversal phases over 16 days of the PRL task in either Long-Evans or Sprague-Dawley rats. Average trials to criterion for the first discrimination phase (A & B) or the first reversal phase (C & D) of the PRL task over 16 days of training for Long-Evans (A & C) or Sprague-Dawley (B & D) rats. n = 9-12 per group

Interestingly, Long-Evans rats exhibited no effect of either AIE ($F_{(1,352)}$ = 0.928 p = 0.336) or days of training ($F_{(15,352)}$ = 0.423 p = 0.972) on choice latency over 16 days of PRL testing. However, Sprague-Dawley rats exhibited a significant increase in choice latency in AIE compared to Control rats ($F_{(1,304)}$ = 42.74 p < 0.0001) and a decrease in choice latency over 16 days of PRL testing ($F_{(15,304)}$ = 10.15 p < 0.0001), but no interaction between the two factors ($F_{(15,304)}$ = 1.249 p = 0.234), detailed in **Figure 3-15**.

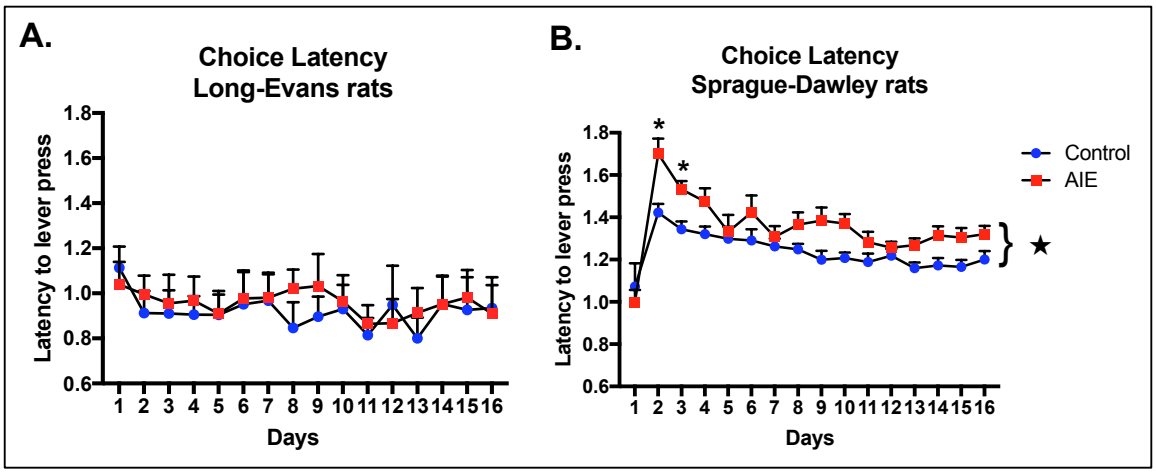

Figure 3-15. AIE resulted in an increase in choice latency in Sprague-Dawley but not Long-Evans rats over 16 days of PRL testing. Average latencies to lever press over 16 days of PRL training for A) Long-Evans or B) Sprague-Dawley rats. * $p < 0.05$ ★ main effect of exposure n = 9-12 per group

In order to accurately assess any changes in performance on the PRL task after SPS exposure, baseline performance was assessed by averaging the last four days of PRL testing (days 13-16). Behavioral testing was resumed on the 8th day after stressor exposure, and the performance over the first four days of PRL testing (days 1-4 post-SPS) were averaged together to compare with baseline performance (see below).

Contrary to the original hypothesis, a 2-way ANOVA (AIE exposure by SPS exposure) revealed that neither AIE nor SPS significantly impaired PRL performance as assessed via the number of reversals completed per session in the four days following SPS exposure in either Sprague-Dawley ($F_{(3,17)} = 0.435$ $p = 0.731$) or Long-Evans ($F_{(3,20)} = 0.093$ $p = 0.963$) rats (**Figure 3-16**). However, there was a trend, irrespective of rat strain, after SPS exposure for Control-SPS and AIE-Control groups to complete fewer reversals per session, but this effect was nullified in the AIE-SPS group. A 2-way ANOVA of the main effects of AIE or

SPS exposure (irrespective of strain), or the interaction between them, of reversals completed per session after SPS revealed that there was no effect of AIE exposure ($F_{(1,41)}$ = 0.217 p = 0.644) or SPS exposure ($F_{(1,41)}$ = 0.171 p = 0.702), but there was a trend of an interaction between AIE and SPS exposure ($F_{(1,41)}$ = 3.490 p = 0.069).

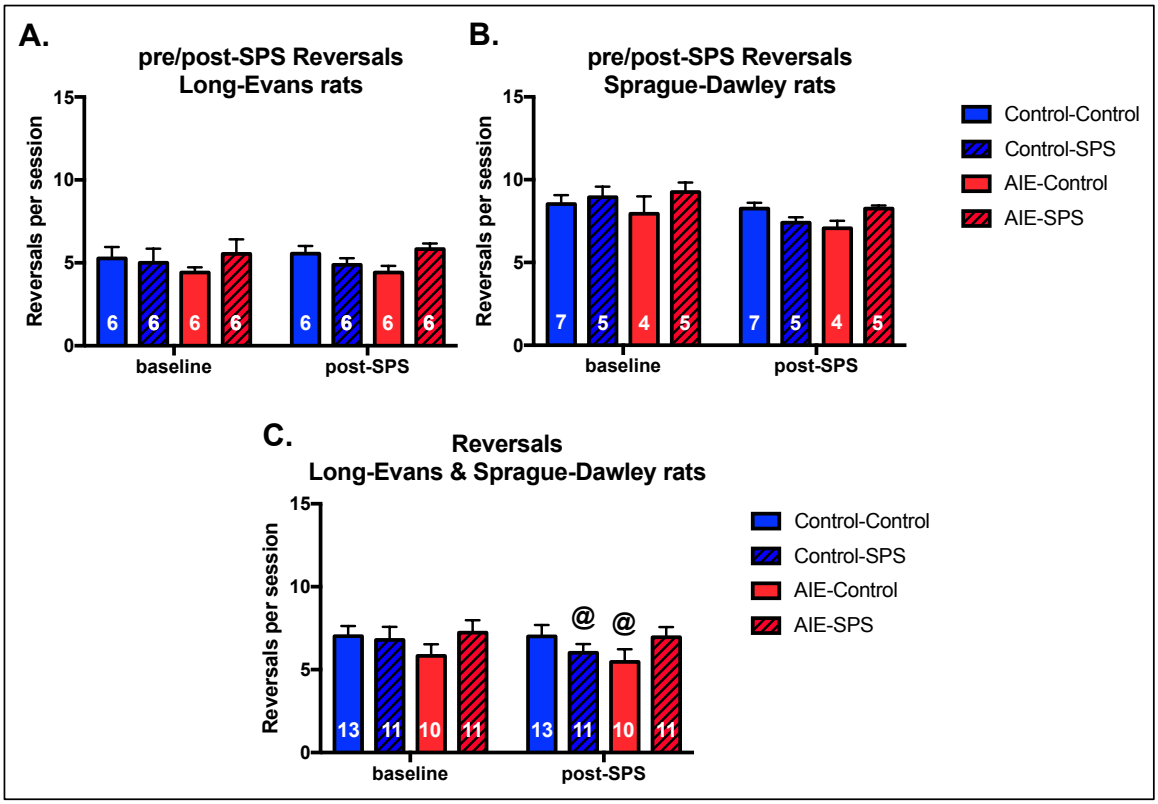

Figure 3-16. Neither AIE nor SPS affected the number of reversals completed per session in either Long-Evans or Sprague-Dawley rats. Average reversals per session over 4 days of PRL training before and after SPS exposure for A) Long-Evans rats B) Sprague-Dawley rats, and C) Long-Evans and Sprague-Dawley rats combined. @ p = 0.069 compared to Control-Control post-SPS

There were no differences seen between exposure groups in number of omissions either before or after SPS exposure, in Long-Evans ($F_{(1,40)}$ = 0.124 p = 0.727) or Sprague-Dawley rats ($F_{(1,34)}$ = 2.34 p = 0.135). Once again, a 2-way ANOVA (AIE exposure by rat strain) revealed that Sprague-Dawley rats

committed fewer omissions overall compared to Long-Evans rats ($F_{(1,41)}$ = 7.358 p = 0.010), as detailed in **Figure 3-17**.

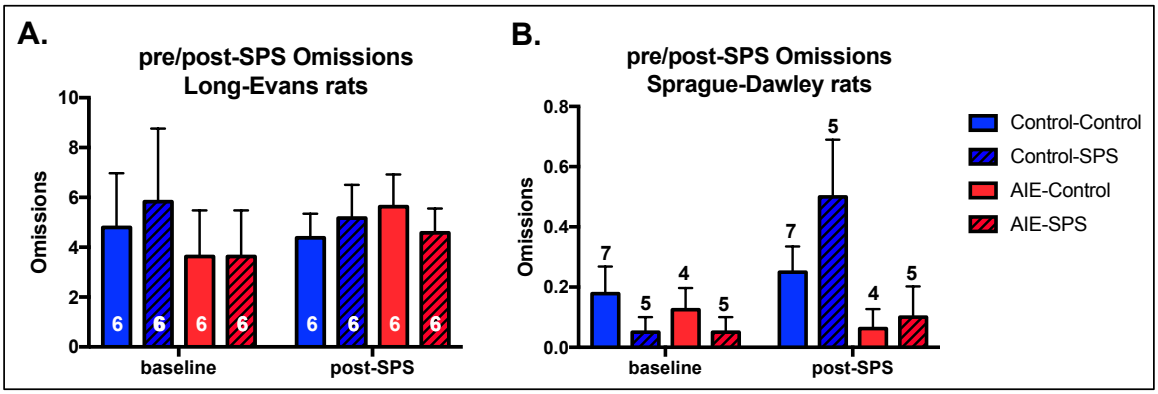

Figure 3-17. Neither AIE nor SPS affected the number of omissions per session in either Long-Evans or Sprague-Dawley rats. Average omissions per session over 4 days of PRL training before and after SPS exposure for A) Long-Evans or B) Sprague-Dawley rats. Note the differences in scale between the graphs.

In Long-Evans rats, there were no differences in baseline performance in either the number of errors during the discrimination or first reversal phases of the PRL task. A 3-way ANOVA revealed that there was no significant interaction between AIE or Control groups, SPS or Control groups, and performance before or after SPS exposure ($F_{(3,20)}$ = 0.267 p = 0.848). Follow-up analysis with a series of 2-way ANOVAs (AIE by SPS exposure, or AIE exposure by task phase) revealed that there were no interactions between AIE exposure and performance before or after SPS exposure ($F_{(3,20)}$ = 0.546 p = 0.657), or between discrimination or first reversal phases and AIE exposure ($F_{(3,20)}$ = 0.844 p = 0.486).

In Sprague-Dawley rats, there were also no differences in baseline performance in either the number of errors during the discrimination or first reversal phases of the PRL task. A 3-way ANOVA revealed that there was no significant interaction between AIE or Control groups, SPS or Control groups,

and performance before or after SPS exposure ($F_{(3,17)}$ = 1.424 p = 0.270). Follow-up analysis with a series of 2-way ANOVAs (AIE by SPS exposure, or AIE exposure by task phase) revealed that there were no interactions between AIE exposure and performance before or after SPS exposure ($F_{(3,17)}$ = 1.169 p = 0.351), or between discrimination or first reversal phases and AIE exposure ($F_{(3,17)}$ = 0.158 p = 0.923) (**Figure 3-18**).

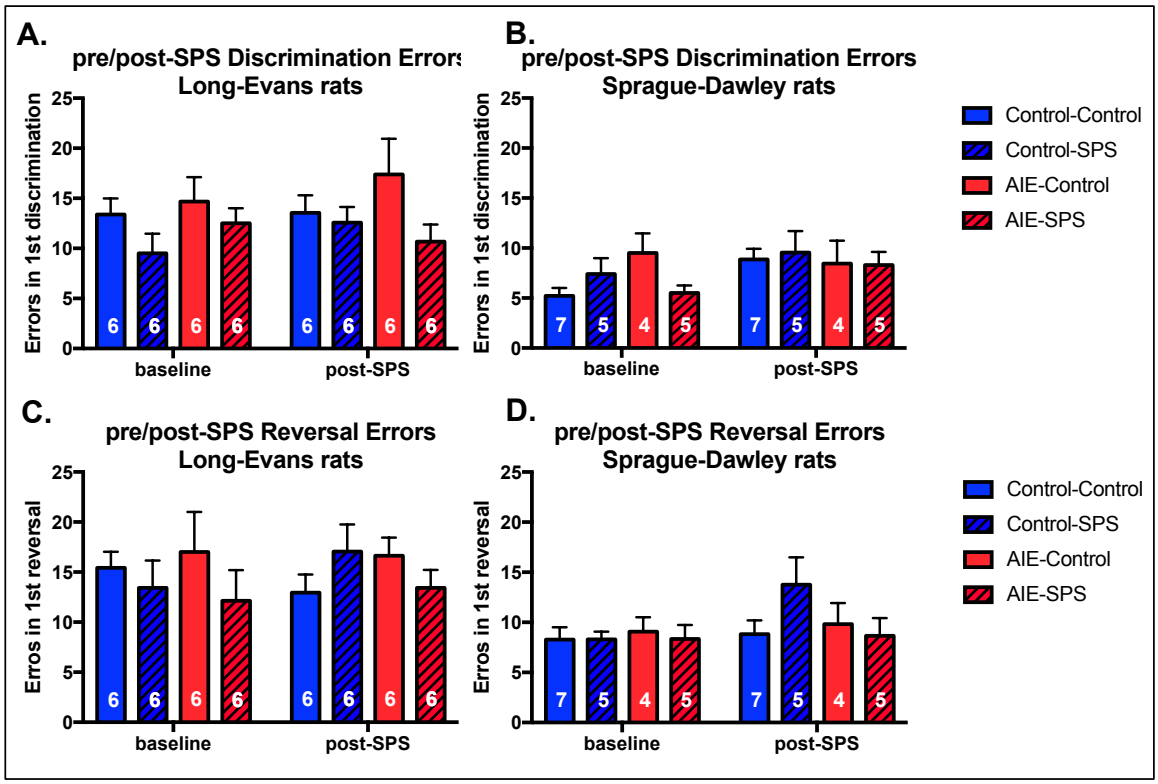

Figure 3-18. Neither AIE nor SPS affected the number of errors during either the discrimination or first reversal phases of the PRL task in either Long-Evans or Sprague-Dawley rats. Average reversals per session over 4 days of PRL training before and after SPS exposure for A) Long-Evans or B) Sprague-Dawley rats. Average errors during the first discrimination phase (A & B) or the first reversal phase (C & D) of the PRL task over 4 days before and after SPS for Long-Evans (A & C) or Sprague-Dawley (B & D) rats.

While there were no differences between exposure groups in baseline win-stay or lose-shift ratios in Long-Evans rats, a 1-way ANOVA (average ratio by

exposure group) of the average win-stay/lose-shift ratio post-SPS did reveal a significant effect of SPS exposure ($F_{(3,20)}$ = 5.168 p = 0.008). A follow-up 1-way ANOVA (lose-shift ratio by exposure group) revealed that this effect was primarily driven by an increase in the lose-shift ratio ($F_{(3,20)}$ = 5.000 p = 0.010), or increased negative feedback sensitivity. A 2-way ANOVA (average ratio by exposure group) revealed that there were no differences between exposure groups in baseline win-stay or lose-shift ratios in Sprague-Dawley rats ($F_{(3,17)}$ = 0.248 p = 0.861). However, a follow-up 2-way ANOVA (average ratio by SPS exposure) revealed that there were also no differences in win-stay or lose-shift ratios before or after SPS exposure ($F_{(3,17)}$ = 0.066 p = 0.977) (**Figure 3-19**).

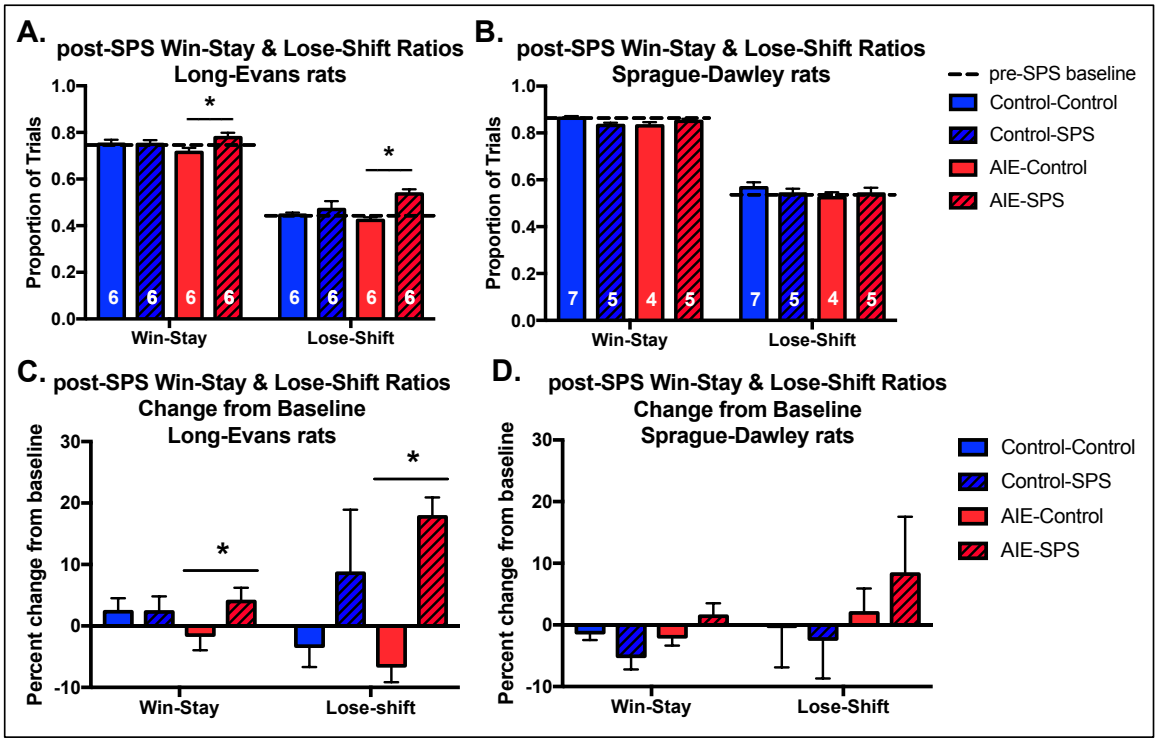

Figure 3-19. SPS resulted in increased negative feedback sensitivity in AIE-exposed Long-Evans rats. Average win-stay and lose-shift ratios over 4 days of PRL training before and after SPS exposure for A) Long-Evans or B) Sprague-Dawley rats. Percent change from baseline (before SPS exposure) for C) Long-Evans or D) Sprague-Dawley rats. * $p < 0.05$

In Long-Evans rats, a 2-way ANOVA (SPS by AIE exposure) revealed that there were no differences between exposure groups in choice latency before or after SPS exposure ($F_{(1,40)}$ = 0.006 p = 0.938). However, there were differences between exposure groups at baseline in Sprague-Dawley rats ($F_{(3,34)}$ = 4.931 p = 0.006). Subsequent comparisons using multiple t-tests with Holm-Sidak corrections revealed that the baseline choice latency of the Control-SPS group was significantly different than that of the AIE-SPS group ($t_{(8)}$ = 2.662 p = 0.029). There were no significant differences between exposure groups after SPS as assessed using multiple t-tests with Holm-Sidak corrections. A 2-way ANOVA of Sprague-Dawley choice latency before and after SPS exposure revealed that, although there was no interaction between AIE and SPS exposure, both AIE ($F_{(3,34)}$ = 4.931 p = 0.006) and SPS exposure ($F_{(1,34)}$ = 729.3 p < 0.0001) significantly affected choice latency after SPS, with SPS exposure accounting for the majority of the total variation in the sample (91.18%). Additionally, further analysis with multiple t-tests and the Holm-Sidak correction revealed that there were significant differences in every exposure group before SPS compared to after exposure in Sprague-Dawley rats (Control $t_{(12)}$ = 13.07 p < 0.0001; Control-SPS $t_{(8)}$ = 14.43 p < 0.0001; AIE $t_{(8)}$ = 12.69 p < 0.0001; AIE-SPS $t_{(8)}$ = 16.76 p < 0.0001) (**Figure 3-20**).

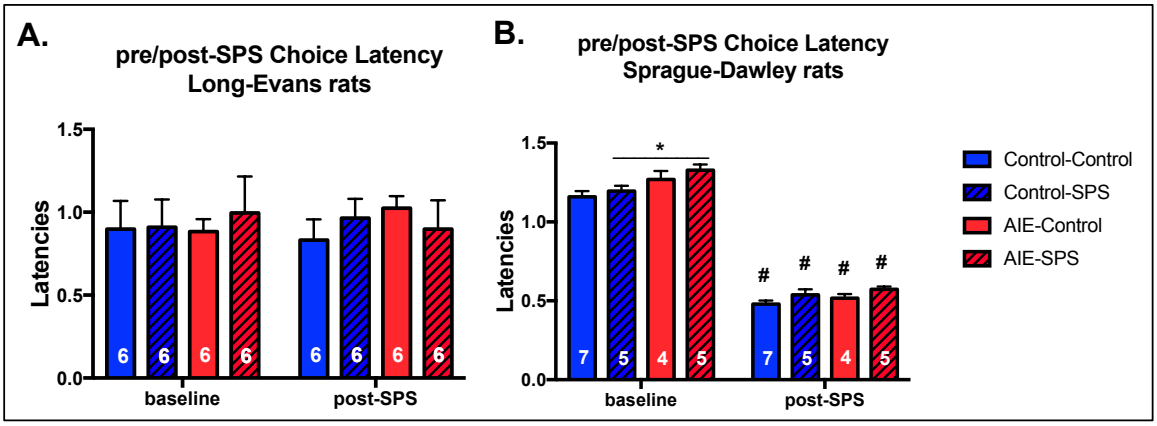

Figure 3-20. Sprague-Dawley rats displayed decreased choice latency in all exposure groups after SPS exposure. Average latencies to lever press during the PRL task over 4 days before and after SPS exposure for A) Long-Evans or B) Sprague-Dawley rats. * $p < 0.05$ # $p < 0.0001$ compared to baseline

Table 3-2. Significant results, separated by parameter, assessed during training for and testing of the PRL task in AIE and Control groups, as well as pre- and post-SPS exposure.

Parameter	Significant?	Exposure Group	>/<	Exposure Group	Strain	Phase
Reversals	Yes	AIE	<	Control	SD	16 days of PRL
	No* p = 0.069	Control-SPS & AIE-Control	<	Control-Control	LE & SD	post-SPS
Omissions	Yes	AIE	<	Control	LE	16 days of PRL
Win-stay ratio	Yes	AIE	<	Control	SD	16 days of PRL
	Yes	AIE-SPS	>	AIE-Control	LE	post-SPS
Lose-shift ratio	Yes	AIE	<	Control	SD	16 days of PRL
	Yes	AIE-SPS	>	AIE-Control	LE	post-SPS
Trials to criterion during discrimination	Yes	AIE	>	Control	LE	First day of PRL
Choice latency	Yes	AIE	>	Control	SD	16 days of PRL
	Yes	Control-SPS	<	AIE-SPS	SD	pre-SPS baseline
	Yes	all groups post-SPS	<	all gups pre-SPS	SD	post-SPS

DISCUSSION

Pre-SPS Observations

The results from these behavioral studies demonstrate that both Long-Evans and Sprague-Dawley rats performed the probabilistic reversal learning task at an equivalent level. On the first day of the task, there was a significant increase in the number of trials to criterion during the discrimination phase in AIE compared to Control groups in Long-Evans rats; however, there were no other

differences between AIE and Control groups in either Long-Evans or Sprague-Dawley rats on the first day of PRL testing. There were also no differences in either strain of rat between AIE and Control groups in terms of the number of days of training for the task; the number of reversals, omissions, or errors in either the discrimination or first reversal phases of the task; the win-stay or lose-shift ratios; or the choice latency.

Over 16 days of testing on the PRL task, there were several differences between AIE and Control groups, but they were strain-specific. In Long-Evans rats, the AIE group had fewer omissions per session compared to Controls, especially in the initial testing days. There were no differences between AIE and Control groups in Long-Evans rats in the number of reversals per session, the number of errors in the discrimination or first reversal phases, the win-stay or lose-shift ratios, the trials to criterion in either the discrimination or first reversal phases, or choice latency over 16 days of testing on the PRL task. In Sprague-Dawley rats, the AIE group had fewer reversals per session compared to the Control group. This difference was especially prominent in the first ten days of testing, which indicates that rats exposed to AIE may be slower to reach criterion on the PRL task. Together with the increased choice latency seen in AIE over 16 days of PRL testing, it could be hypothesized that AIE leads to decreased efficiency in strategy formation in Sprague-Dawley rats. There was no difference between the groups of Sprague-Dawley rats over 16 days of testing on the PRL task in terms of the number of omissions, errors in either the discrimination or first reversal phases, or trials needed to reach criterion in either the

discrimination or first reversal phases. However, AIE Sprague-Dawley rats did show reduced win-stay and lose-shift ratios over 16 days of PRL testing. Interestingly, Bari and colleagues (2010) demonstrated that acute administration of a moderate dose (10 mg/kg) of citalopram, a selective serotonin reuptake inhibitor (SSRI), decreased negative feedback sensitivity, whereas global cerebral serotonin depletion decreased the number of reversals completed as well as the win-stay ratio (Bari et al., 2010). These authors noted that acute modulation of serotonergic neurotransmission appeared to affect negative feedback sensitivity, whereas chronic perturbation altered reward sensitivity. A recent study demonstrated that AIE exposure decreased serotonin expression in adulthood in the raphe nucleus, as well as serotonergic projections to the hypothalamus and the amygdala (Vetreno, Yaxley, Paniagua, Johnson, & Crews, 2017). Taken together, these observations may suggest that AIE leads to reduced serotonergic neurotransmission in adulthood, which may in turn affect performance on the probabilistic reversal learning task investigated in the current study.

The lack of a frank effect of AIE exposure on reversal learning in the Long-Evans and Sprague-Dawley rat strains was surprising in light of several recent studies reporting impaired reversal learning after AIE exposure (Coleman, He, Lee, Styner, & Crews, 2011; Coleman et al., 2014; Fernandez, Lew, Vedder, & Savage, 2017; Fernandez & Savage, 2017; Fernandez, Stewart, & Savage, 2016). However, there are a number of significant differences in experimental design that may have contributed to different results, including the method of AIE

exposure, the length and timing of AIE exposure, the method of testing reversal learning, and the reinforcer used in the task. As discussed in more detail in chapter 6, these differences could account for why we did not observe impaired reversal learning.

Post-SPS Observations

Following SPS exposure, there was a trend ($p = 0.069$) for the Control-SPS and AIE-Control groups of rats, irrespective of strain, to complete fewer reversals per session compared to the Control group; this reduction in reversals per session was normalized in the AIE-SPS group. Although we do not believe these studies were underpowered, it is possible that the addition of more animals to these studies would pull out a small, but statistically significant, difference in this parameter. A decrease in reversals per session may indicate deficits in key brain regions for value updating, such as the OFC; however, this is unlikely to be the case as studies have shown that the OFC is only important for initial but not later phases of reversal learning (Boulougouris et al., 2007; Boulougouris & Robbins, 2009). This trend could also indicate a deficit in the nucleus accumbens shell (NAcS) or core (NAcC), as Dalton and colleagues (2014) demonstrated that inactivating the NAcC impaired reward approach, whereas inactivating the NAcS impaired probabilistic reinforcement learning. SPS has been shown to impair reversal learning as well (George et al., 2015), although the task involved reversals between sessions, unlike the procedure used in these studies. Interestingly, while the Control-SPS and AIE-Control groups appeared to be

somewhat impaired in the number of reversals completed per session compared to the Control-Control group, this impairment was not observed n in the AIE-SPS group. While this could be a trend of an effect, further studies of the effects of AIE and SPS on the number of reversals completed per session of the PRL task would be needed to determine if this trend would hold up with increased statistical power.

In Long-Evans rats, there was an increase in both win-stay and lose-shift ratios in the AIE-SPS group after SPS exposure. This effect was driven primarily by an increase in the lose-shift ratio, or increased negative feedback sensitivity. This increase in lose-shift behavior has been demonstrated following acute, global, cerebral serotonin reduction (Bari et al., 2010). Vetreno and colleagues (2017) have also shown that AIE leads to a reduced number of serotonergic neurons in the dorsal raphe nucleus, as well as decreased serotonergic projections to the amygdala. Although speculative, the combination of AIE and SPS exposure may have resulted in a reduction in serotonergic neurotransmission, and an increase in lose-shift behavior in Long-Evans rats. As discussed in a previous chapter, it has been reported that Long-Evans and Sprague-Dawley rats have differential behavioral and transcriptional responses to cocaethylene (the metabolic product of cocaine and ethanol) administration (Horowitz et al., 1997), which appears to be due to differences in their serotonergic systems (Baumann et al., 1998; Horowitz et al., 2002). Studies from this group demonstrated decreased behavioral sensitivity to cocaethylne in Long-Evans rats compared to Sprague-Dawley rats, apparently due to serotonin (5-

HT) availability, as fluoxetine (a 5-HT reuptake inhibitor) administration in Long-Evans rats eliminated the differences in behavior (Horowitz et al., 1997). Additional studies using immunohistochemical analysis of Fos, an immediate early gene product, revealed that Long-Evans rats had increased numbers of Fos immunoreactive-positive cells in the caudate nucleus and putamen compared to Sprague-Dawley rats (Horowitz et al., 2002). These differential responses of the serotonergic system in Long-Evans compared to Sprague-Dawley rats may have played a role in the differences seen in PRL performance after AIE and SPS exposure. Further studies may include examining the effect of administration of a 5-HT reuptake inhibitor on negative feedback sensitivity after AIE and SPS exposure in Long-Evans compared to Sprague-Dawley rats.

In Sprague-Dawley rats, there was a difference in baseline choice latency between Control-SPS and AIE-SPS groups; this was due to the difference in choice latency between AIE and Control groups prior to SPS exposure. All treatment groups (Control-Control, Control-SPS, AIE-Control, and AIE-SPS) exhibited significantly decreased choice latency after SPS exposure in Sprague-Dawley rats. While this effect was visually obvious and quite significant ($p < 0.0001$), it was unexpected for all four exposure groups to show similar reductions in choice latency despite differential exposure to both AIE and SPS. Therefore, it could be hypothesized that this effect was due to the time between testing sessions that all groups experienced (8 days) regardless of SPS exposure. This necessary gap in testing sessions may have led to increased anticipation of reward (20% sucrose) for all groups of Sprague-Dawley rats,

which in turn led to decreased choice latency after the re-initiation of the PRL task following the eight-day gap. However, Long-Evans rats experienced the same gap in testing, and displayed no decrease in choice latency following re-initiation of the PRL task following the eight-day gap. Although speculative, it may be that these rat strains respond differently to gaps in training with reward administration, and further testing to examine the choice latency on a simplified operant task after varying gaps in training could shed more light on this issue.

In conclusion, the results of the present set of studies demonstrated that Long-Evans and Sprague-Dawley rats respond differentially to AIE and SPS exposure as assessed via performance on the probabilistic reversal learning task. Long-Evans AIE compared to Control rats required increased trials to meet criterion during discrimination on the first day of PRL testing, and committed fewer omissions over 16 days of PRL testing. Additionally, Long-Evans AIE-SPS rats displayed increased win-stay and lose-shift ratios on the PRL task after SPS compared to AIE-Control rats. Sprague-Dawley AIE compared to Control rats completed fewer reversals per session, displayed decreased win-stay and lose-shift ratios, and displayed increased choice latency over 16 days of PRL testing. Additionally, all exposure groups of Sprague-Dawley rats displayed decreased choice latency after SPS exposure (regardless of SPS or Control exposure groups) compared to pre-SPS baseline choice latency. This pattern of differential effects of adolescent ethanol and adult SPS exposure in two different strains of rats demonstrates that AIE leads to distinct yet subtly different patterns of deficits on the PRL task without frank effects on the number of reversals completed in

Long-Evans and Sprague-Dawley rats. Additionally, the effects of SPS exposure in adulthood are modulated by AIE exposure and rat strain.

CHAPTER 4

ADOLESCENT BINGE-LIKE ETHANOL LEADS TO INCREASED CHOICE LATENCY ON THE PROBABILISTIC DECISION-MAKING TASK, & DECREASED RISKY CHOICE WITH SINGLE PROLONGED STRESS EXPOSURE

BACKGROUND & SIGNIFICANCE

One aspect of the suite of executive functions performed by the PFC is that of updating information. This can include updating the value and/or salience of external stimuli, updating information in the working memory, and updating behavioral strategies in order to maximize reward. These functions can be conceptualized as following after one another: using information from the working memory to modify the perceived value of a stimulus, and then adapting the current behavioral strategy accordingly. While these functions depend on brain regions and networks outside of the PFC to provide support for memory (the hippocampus), salience (the mesolimbic and mesocortical dopamine networks), and motor implementation (the basal ganglia and motor cortex), it is the PFC that orchestrates their functions to yield maximal return on investment. However, the PFC is one of the last areas of the brain to reach maturity in early adulthood, as the brain matures in a caudal to rostral direction. Adolescence is a time during which the PFC is still maturing, and not performing at its ultimate adult capacity. Therefore, adolescence is also a time of relative PFC hypo-functionality, in

addition to being a time of increased exploratory drive and risk-taking (L. P. Spear, 2000).

Due in part to this increase in exploratory drive and risk-taking, adolescence is a time of increased experimentation with drugs of abuse. One of the most commonly abused drugs in adolescence is ethanol, with two-thirds of adolescents reporting that they tried ethanol by 18 years of age (NIAAA, 2017b); additionally, 90% of the ethanol consumed by youth under 21 in the US is in the form of binge drinks, defined as 5 or more standard alcoholic drinks in one sitting (NIAAA, 2015b). Preclinical studies have shown that the prefrontal cortex is relatively more vulnerable to the effects of ethanol than other areas of the brain (Bava et al., 2009a; Fowler et al., 2014), and that binge-like ethanol exposure in humans in adolescence leads to impaired inhibitory control (Schweinsburg et al., 2004; Tapert et al., 2007) and an increased preference for large, risky rewards over small, certain rewards in an operant probabilistic decision-making task in rodents in adulthood (McMurray et al., 2016). The probabilistic decision-making task (PDT) used in the following experiments is the same procedure shown to be dependent upon the OFC and mPFC by St. Onge & Floresco (2010), and is based on an earlier operant procedure used by Cardinal & Howes (2005).

The dorsal anterior cingulate cortex (dACC) has been associated functionally with the appraisal of risk, and Claus and colleagues (2017) demonstrated that adolescent humans who frequently used ethanol and marijuana displayed decreased activation of the dACC and increased risk-taking. Other pre-clinical studies in rats have shown that reversible ACC inactivation did

not affect performance on an operant probabilistic decision-making task (Onge & Floresco, 2010). In the same study, orbito-frontal cortex (OFC) inactivation increased choice latency in the later blocks with lower probability of reward, without affecting risky choice. The authors also hypothesized that the medial prefrontal cortex (mPFC) served to update information in the PFC, such as the probability of reward associated with a stimulus, or a lever in the case of the probabilistic decision-making task (Onge & Floresco, 2010).

Several studies have shown that adolescent binge-like ethanol exposure leads to increased preference for large, risky rewards (McMurray et al., 2016; Nasrallah et al., 2011; Nasrallah et al., 2009), in Sprague-Dawley rats. However, other previous, unpublished studies in our lab have shown no differences in risky choice or choice latency following adolescent binge-like ethanol exposure in Long-Evans rats (**Figure 4-1**) (Centanni, 2015).

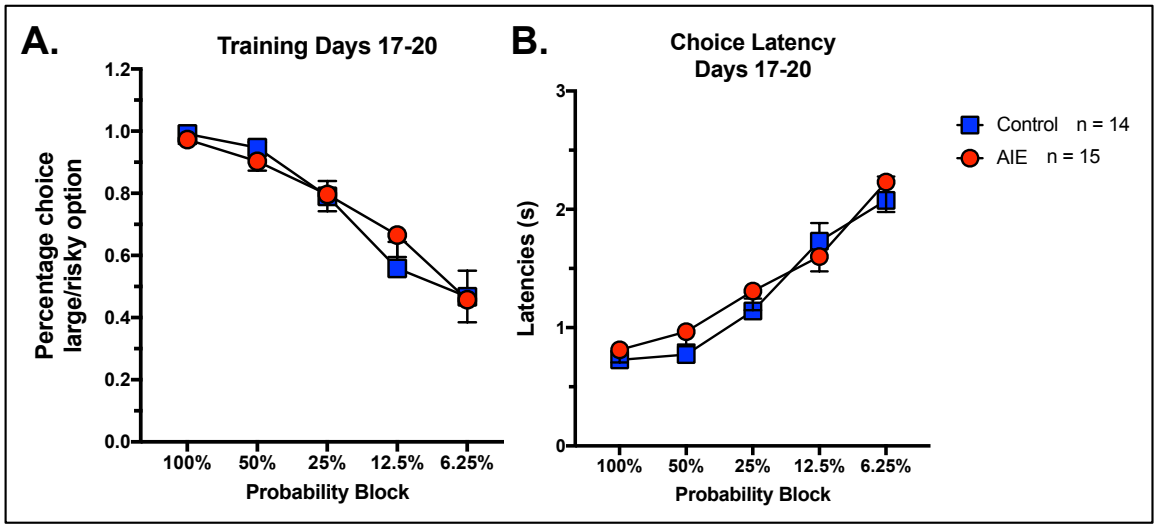

Figure 4-1. Long-Evans rats displayed no differences in A) risky choice or B) choice latency after AIE exposure.

As detailed in chapter 2, Sprague-Dawley and Long-Evans rats displayed differential anxiety-like behaviors following AIE exposure. Also recall the differences in performance between the two rat strains on the probabilistic reversal learning task from chapter 3. Therefore, taking these differences between the two rat strains in mind, we hypothesized that Sprague-Dawley rats may display deficits in risky choice after adolescent binge-like ethanol exposure.

Stress associated with repeated episodes of forced swimming has been shown to exacerbate cognitive dysfunction following binge-like ethanol exposure (Rodberg et al., 2017), and acute mild stress led to impaired set-shifting (Butts, Floresco, & Phillips, 2013). One model of acute stress is the single-prolonged stress (SPS) paradigm, which replicates several clinical symptoms of post-traumatic stress disorder including hyperarousal (Khan & Liberzon, 2004), impaired extinction retention (Knox et al., 2011), and impaired behavioral flexibility (George et al., 2015). Therefore, we hypothesized that Sprague-Dawley rats may display increased risky choice on the PDT following AIE exposure, and that SPS exposure would further exacerbate these deficits.

MATERIALS & METHODS

Animal care, AIE exposure, blood ethanol concentration (BEC) analysis, and SPS exposure are discussed in chapter 2 detailing general methods for chapters 3 and 4. The specific timeline of AIE exposure, PDT testing, SPS exposure, and PDT re-testing is detailed in **Figure 4-2**.

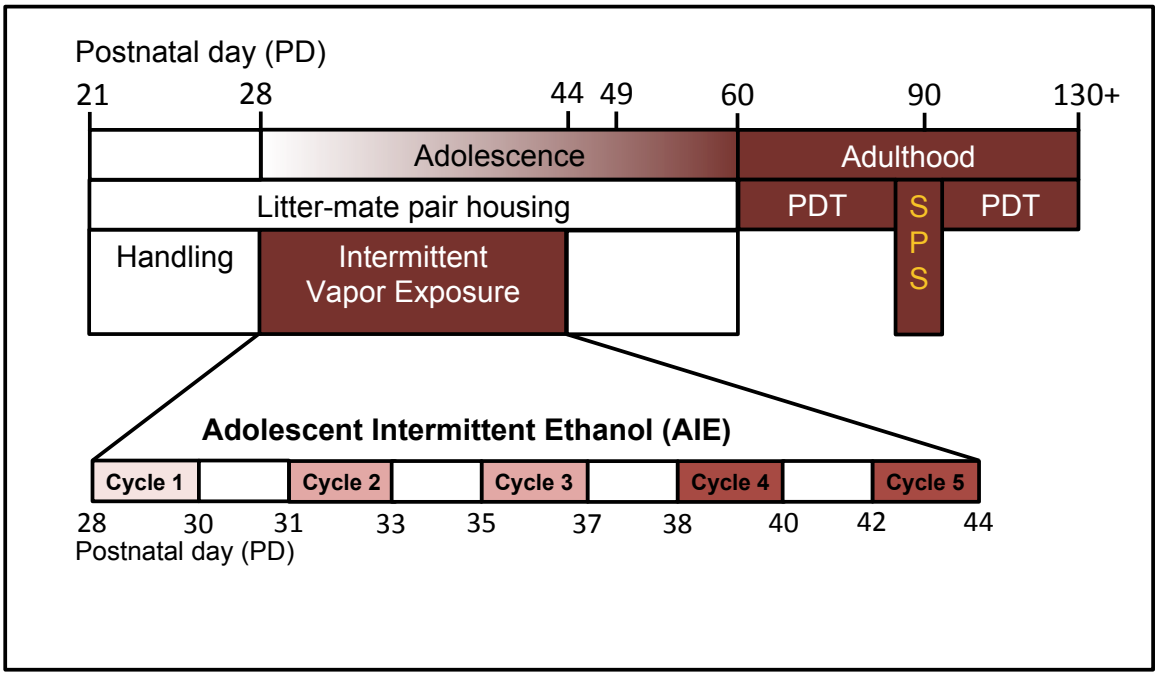

Figure 4-2. Experimental timeline of AIE exposure, pair housing, and operant testing

Operant Behavior

One week prior to the beginning of any operant training, rats were single housed and restricted to 85-90% of their *ad libitum* feeding weight. All operant training was started at PD70 or later. Additionally, rats were habituated to the process of moving to the behavior room for a minimum of two days before being placed in the operant box. Animals were also accustomed to the reward that would be used for operant training by receiving ~20 mL of 20% sucrose in their home cage Monday through Friday the week before operant training began. For the first day of operant training, a program was used which dispensed 10 µl of 20% sucrose at random intervals over the course of 30 minutes; 60 total reinforcements were dispensed. This was to habituate the rats to the sound of

the syringe pump turning on and off to deliver the liquid reward, and to associate the reward delivery well with a reward.

Operant boxes: Plexiglas operant boxes (Med Associates, St. Albans, VT) used in the present study measured 32 cm W x 25 cm D x 11 cm H and were located in melamine sound attenuating cubicles. Each cubicle was equipped with an exhaust fan to provide air circulation and mask external noise. Mounted on one wall of the self-administration chamber were two response levers that flanked a liquid receptacle connected to a single speed syringe infusion pump with polyethylene tubing. Located above the active lever was a 2.5 cm diameter stimulus light that was turned on during various phases of testing. Located at the top the chamber was a house light and a Sonalert speaker that emitted a tone (2900 Hz, ~65 dB) that were activated during various phases of testing. Chambers were interfaced to a PC computer that controlled experimental sessions and recorded data using commercially available software (MED Associates, MED-PC IV).

Probabilistic Decision-making Task

The probabilistic decision-making task (PDT), or "Risk" task, was originally described by Onge & Floresco (2009). The operant training consisted of 3 distinct phases: a Training phase in which rats were trained to lever press, a Probability Habituation phase in which rats learned to respond on a lever within a fixed amount of time (a trial); this phase also included the rats learning that each lever

had a 50% probability of delivering a reward. Finally, following side preference determination, there was a Probabilistic Discounting testing phase, where one lever was association with a small (1x volume), certain (100%) reward and the other lever was associated with a large (4x volume), risky reward. The probability for the large, risky reward decreased across blocks of trails from 100%, to 50%, to 25%, to 12.5%, to 6.25%.

Training phase: During this phase, rats were trained to respond to a lever with a lever press, on a fixed-ratio 1 (FR1) schedule of reinforcement. At the beginning of the first training session (30 min), one of the levers was extended and the house and receptacle lights were illuminated. Each lever press activated a syringe pump for 1.5 sec to deliver approximately 45 µl of 20% sucrose into the reward receptacle. Rats received daily 30 min sessions until stable responding was achieved, which was defined as receiving at least 50 reinforcements during a session for two consecutive training days. Training then proceeded to an FR1 schedule of reinforcement of whichever lever was not reinforced at first. Stable responses (receiving at least 50 reinforcements during a session for two consecutive training days) in this phase were required before progressing to the next part of the training.

Probability Habituation phase: This phase served to train the rats to respond with a lever press within a discrete amount of time (a trial), as well as to establish probabilistic reinforcement of lever presses. It consisted of 2 programs of

decreasing trial length time. Both programs had an inter-trial interval (ITI) of 40 seconds. Each program had the exact same order of all other components. At the start of each trial the house light would illuminate and one of the levers would extend. If the lever was selected then there was a 50% probability of activating a syringe pump for 1.5 seconds to deliver approximately 45 ul of 20% sucrose. If the lever was not selected then the lever would retract and all lights would be extinguished until the next trial. In the first program, there were 90 trials with 20 seconds during which a lever could be selected; in the second program, there were also 90 trials with 10 seconds during which a lever could be selected. Each rat had to omit fewer than 10 trials on 2 successive training days to progress from one program to another, and then to the next training phase.

Side preference determination: Following the last day of the probability habituation phase, the rats were assessed for preference for either lever. In the first trials, both levers were extended, and pressing either lever delivered a reward. Following a 20 second ITI, both levers were again extended. If the rat chose the same lever as the previous trial, then no reward was delivered. This continued until the rat chose the opposite lever of the one that initially delivered a reward; that would comprise one alternation. The program continued indefinitely until 7 alternations were completed. Assessing which lever was chosen first more often across the 7 alternations usually identified the side preference of the rat, although if the total lever presses for a lever were more than a 2:1 ratio

compared to the lever pressed first most often, then the lever that was pressed more frequently was considered the preferred lever.

Probabilistic Discounting Task testing phase: This phase consisted of a rat's preferred lever being associated with a small (1x volume), certain (100% probability) reward, and the non-preferred lever being associated with a large (4x volume), risky (decreasing from 100% to 6.25% probability) reward; this association remained the same for the duration of the testing phase. There were 5 blocks of trials, each consisting of 8 forced trials with only one lever extended, and 10 free choice trials where both levers were extended. This program had an ITI of 40 seconds, after which the houselights were illuminated and one or both levers were extended; each trial lasted for 10 seconds, and unless a selection was made, then both levers were retracted and all lights were extinguished. Selecting the small, certain lever would deliver a 30 ul reward of 20% sucrose, and selecting the large, risky lever may deliver a 120 ul reward. The probability for the small, certain lever remained 100% throughout the session, but the probability of the large, risky lever decreased over each successive block of trials from 100%, to 50%, to 25%, to 12.5%, to a 6.25% probability of reward in the 5^{th} and final block of trials in the session. Selecting the large, risky lever was most advantageous in the first two blocks of trials, and selecting the small, certain lever was most advantageous in the last two blocks of trials. This phase lasted for approximately 20 days, or until the cohort of test subjects reached a stable level of responding during each probability block over 3 days of testing. Stability

was determined by statistically analyzing the data for 5 blocks across 3 days with a 2-way ANOVA (day x block); if there was no statistical difference between days then the responding was determined to be stable. On the 8th day after SPS exposure, test subjects were re-started on the PDT program for 10 days, or until stable responding was achieved using the above methods of statistical analysis.

Data Analysis: For the 20 days of PDT testing and the 8 days of PDT testing after SPS exposure, the following parameters were assessed: percent risky choice by block, omissions, win-stay ratio, lose-shift ratio, and average latency to lever press. The percent risky choice by block was computed as the percent of trials in which the rat chose the risky lever within the 10 free-choice trials within each probability block. Omissions were the number of trials in which a selection was not made within the time limit of the trial. The win-stay ratio was the proportion of trials in which a rewarded, risky choice was followed by another choice of the risky lever. The lose-shift ratio was the proportion of trials in which an unrewarded, risky choice was followed by a choice of the certain lever. The average latency to lever press was the average amount of time between the initiation of a trial (lever extension) and lever selection.

Differences between AIE and Control groups were assessed using Students' t-tests, and the threshold for significance was set at $p < 0.05$. Differences between AIE-SPS, AIE-Control, Control-SPS, and Control-Control groups were assessed using ANOVA tests, RM-ANOVA tests, and/or Students' t-

tests, with the threshold for significance again being set at $p < 0.05$ or less. Data are presented as mean ± SEM.

RESULTS

The rats used in these studies (n = 22) were separated within litters into pair-matched groups of Control or AIE exposed animals, which were put through AIE exposure, the probabilistic decision-making task, SPS exposure, and then re-started on the PDT. Differences in probabilistic decision-making were assessed using the probabilistic decision-making task (PDT) (Cardinal & Howes, 2005; Onge & Floresco, 2009), wherein rats chose between a risky lever associated with a large reward (4x volume) and a certain lever associated with a small reward (1x volume); the probability of reward for the risky lever decreased within an operant session over 5 consecutive blocks: 100%, 50%, 25%, 12.5%, and 6.25%, but the identity of each lever did not change throughout the entirety of testing with this procedure. Each block began with 8 forced-choice trials with only one lever extended per trial to establish the new probability of reward associated with the risky lever as well as to re-establish the same probability of reward with the certain lever (always 100%). Next, 10 free-choice trials occurred where both levers were extended in each trial and the animal was allowed to choose between the large, risky lever and or the small, certain lever; it was from these free choice trials that the percent choice of the large/risky option was calculated for each probability block, on each PDT training day.

AIE did not impair operant learning, as there were no significant differences in the number of days needed to meet criteria in either the FR1 ($t_{(20)}$ = 0.197 p = 0.852) or probability ($t_{(20)}$ = 0.511 p = 0.852) phases of training (**Figure 4-3**).

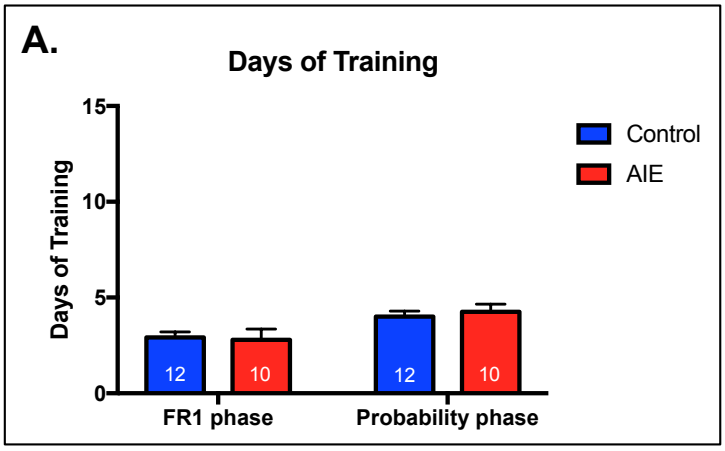

Figure 4-3. AIE is not associated with impaired operant learning A) Average days of operant training for Sprague-Dawley rats for the probabilistic decision-making task

AIE also had no effect on percent risky choice in the initial stages of PDT training. A 2-way ANOVA revealed that there were no significant effects of AIE exposure ($F_{(1,100)}$ = .307 p = 0.581) or probability block ($F_{(4,100)}$ = 0.416 p = 0.797) on percent risky choice on the first day of PDT training (**Figure 4-4**). Both AIE and Control groups progressed from expressing no preference for a certain lever on day one of training, to expressing a strong preference for the risky lever in the 100% and 50% probability blocks and a strong preference for the certain lever in the 12.5% and 6.25% probability blocks. The risky lever is the more efficacious choice in the 100% and 50% probability blocks. In the 25% probability block, both levers are equally valuable as the risky lever returns 4x the amount of reward as the certain lever. However, the certain lever is the more efficacious choice in the

12.5% and 6.25% probability blocks. Therefore, an appropriate strategy to maximize reward is to select the risky lever in the first two blocks, either lever in the third block, and the certain lever in the last two blocks. As expected, this pattern was not observed on the first day of PDT training (**Figure 4-4**), but did emerge as training progressed through day 20 (**Figure 4-5, 4-6, and 4-7**). Analyses via 2-way ANOVAs (AIE exposure by probability block) of percent risky choice averaged across four days of training revealed that there was an increasingly significant effect of probability block on percent risky choice on days 1-4 ($F_{(4,100)}$ = 4.66 p = 0.002) and 5-8 ($F_{(4,200)}$ = 120.8 p < 0.0001) (**Figure 4-5**), days 9-12 ($F_{(4,100)}$ = 166.7 p < 0.0001) 13-16 ($F_{(4,100)}$ = 217.7 p < 0.0001) (**Figure 4-6**), and days 17-20 ($F_{(4,100)}$ = 271.8 p < 0.0001), but no effect of AIE exposure, even on days 17-20 ($F_{(4,80)}$ = 1.791 p = 0.129) (**Figure 4-7**).

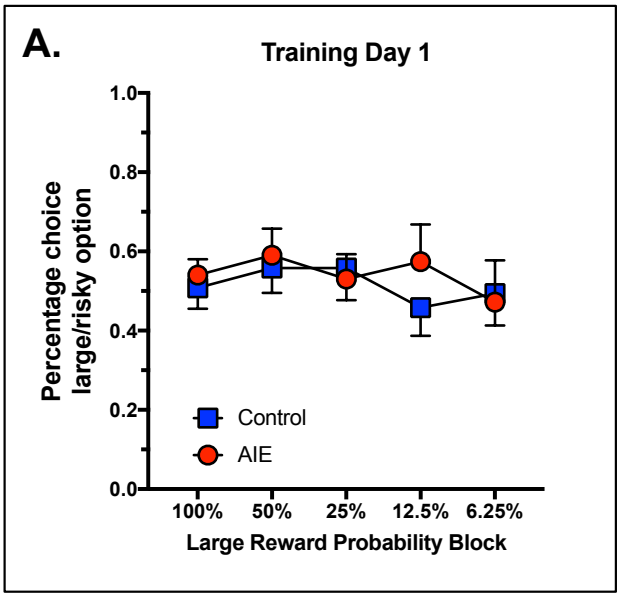

Figure 4-4. AIE is not associated with changes in risky choice on the first day of PDT training. Average percent choice of the large/risky lever during A) the first day of training for the probabilistic decision-making task. There was no effect of probability block on day 1 of training. AIE n = 12 Control n = 10

Interestingly, AIE appeared to have a biphasic effect on choice latency, in that the AIE group initially demonstrated increased choice latency (on days 1-4 and 5-8, **Figure 4-5**), but then displayed decreased choice latency (on days 13-16, **Figure 4-6**). However, by days 17-20 of PDT training, there was no effect of AIE on choice latency (**Figure 4-7**). A 2-way ANOVA (AIE exposure by probability block) revealed that there was a significant effect of AIE exposure, in that the AIE group displayed significantly different choice latency compared to the Control group on days 1-4 ($F_{(1,100)} = 52.92$ $p < 0.0001$), days 5-8 ($F_{(1,100)} = 9.93$ $p = 0.0021$) (**Figure 4-5**), and days 13-16 ($F_{(1,100)} = 13.11$ $p = 0.0005$) (**Figure 4-6**). A series of 2-way ANOVAs also revealed that there was a significant difference in choice latency due to probability block across all days of training.

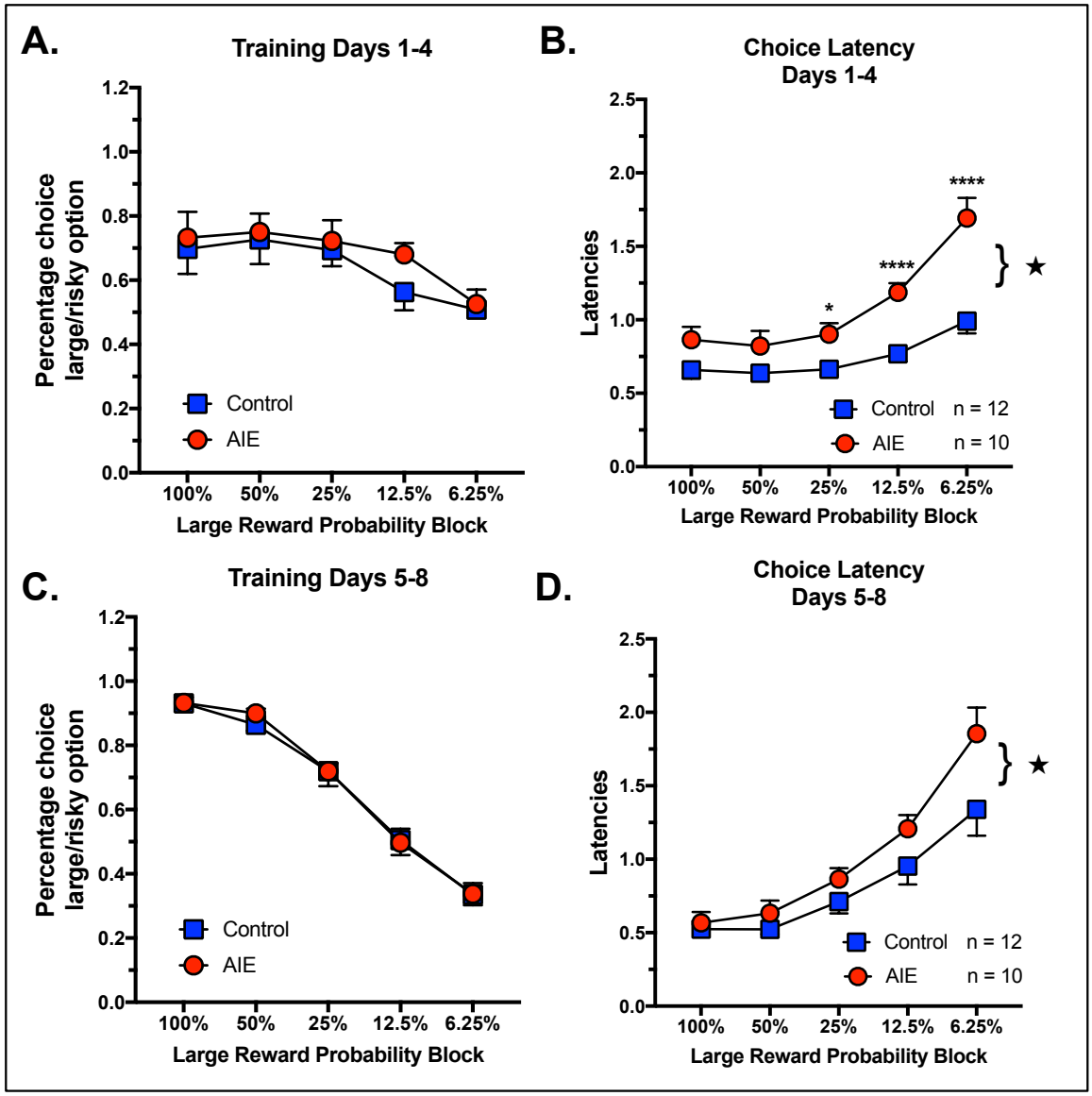

Figure 4-5. AIE resulted in increased choice latency on training days 1-4 and 5-8 of the probabilistic decision-making task. Average A) percent risky choice by block for training days 1-4, B) choice latency for days 1-4, C) percent risky choice by block for training days 5-8, and D) choice latency for days 5-8. * $p < 0.05$ **** $p < 0.001$ ★ main effect of AIE exposure

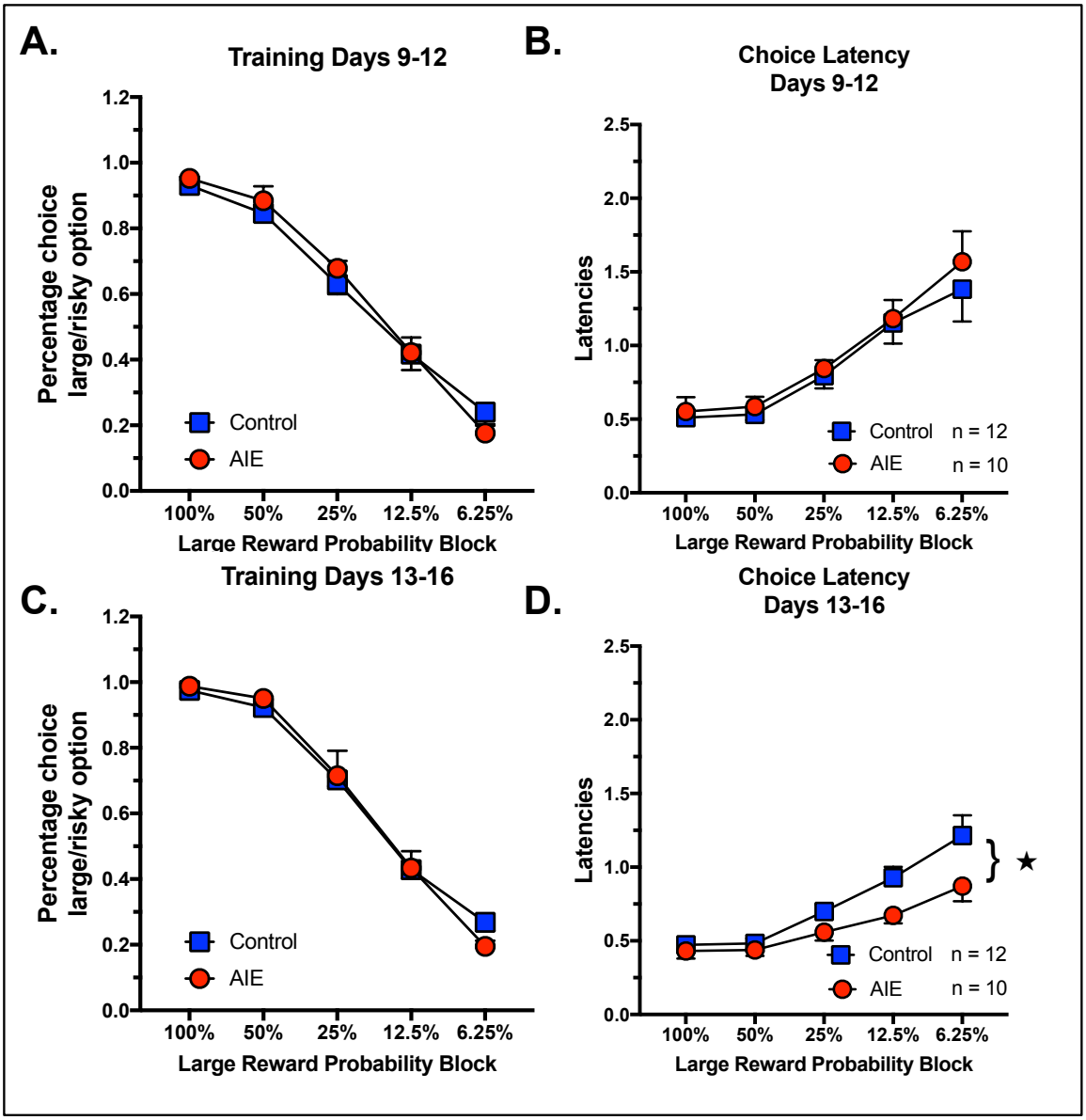

Figure 4-6. **AIE resulted in decreased choice latency on training days 13-16 of the probabilistic decision-making task.** Average A) percent risky choice by block for training days 9-12, B) choice latency for days 9-12, C) percent risky choice by block for training days 13-16, and D) choice latency for days 13-16. ★ main effect of AIE exposure

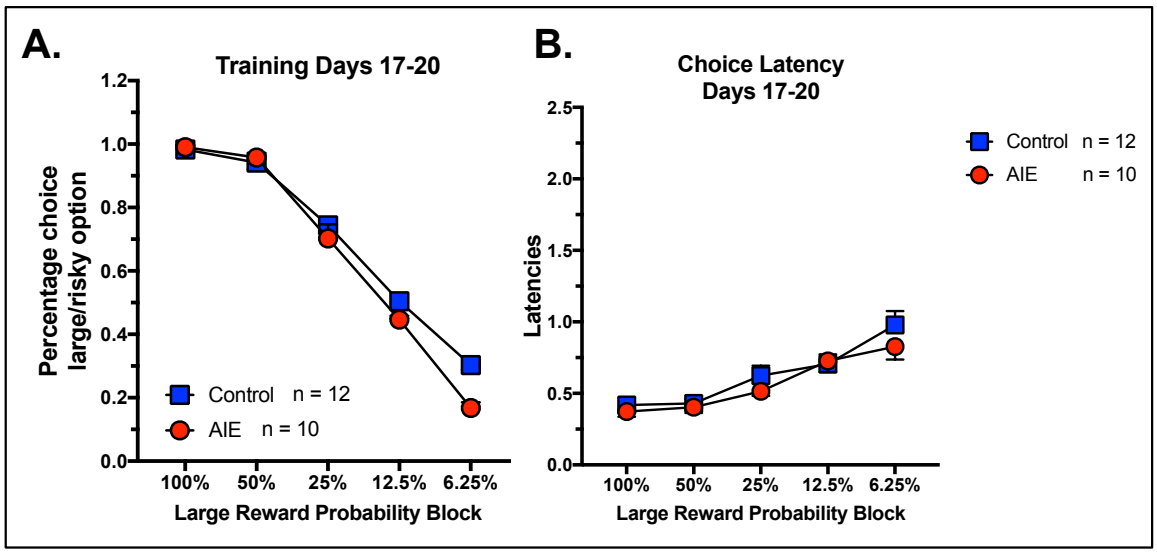

Figure 4-7. AIE is not associated with changes in risky choice or choice latency on training days 17-20 of the probabilistic decision-making task. Average A) percent risky choice by block, and B) choice latency on days 17-20 of the probabilistic decision-making task.

AIE had no effect on the number of omissions per session, either in the initial phases of training (days 1-4) ($t_{(9)}$ = 1.769 p = 0.111) or at the end of training (days 17-20) ($t_{(9)}$ = 0.474 p = 0.647) (**Figure 4-8**).

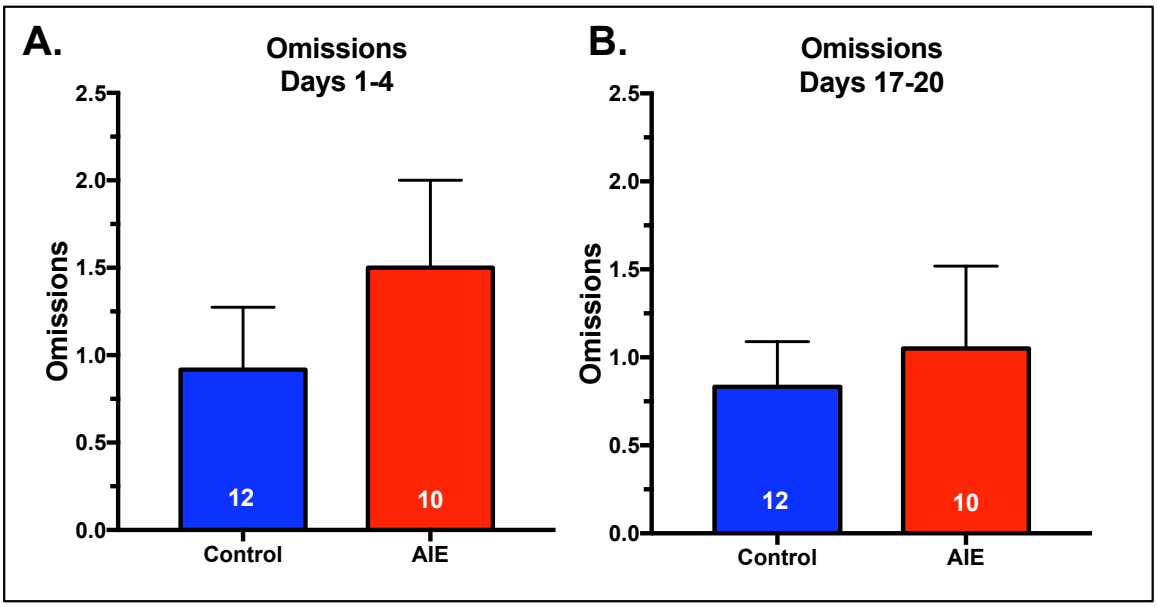

Figure 4-8. AIE is not associated with changes in omissions per session during training days 1-4 or 17-20 of the probabilistic decision-making task. Average omissions per session for A) days 1-4, and B) days 17-20 of the probabilistic decision-making task

Additionally, AIE had no effect on either the win-stay ratio ($t_{(9)}$ = 0.481 p = 0.642) or the lose-shift ratio ($t_{(9)}$ = 1.357 p = 0.208) during days 17-20 of the probabilistic decision-making task (**Figure 4-9**).

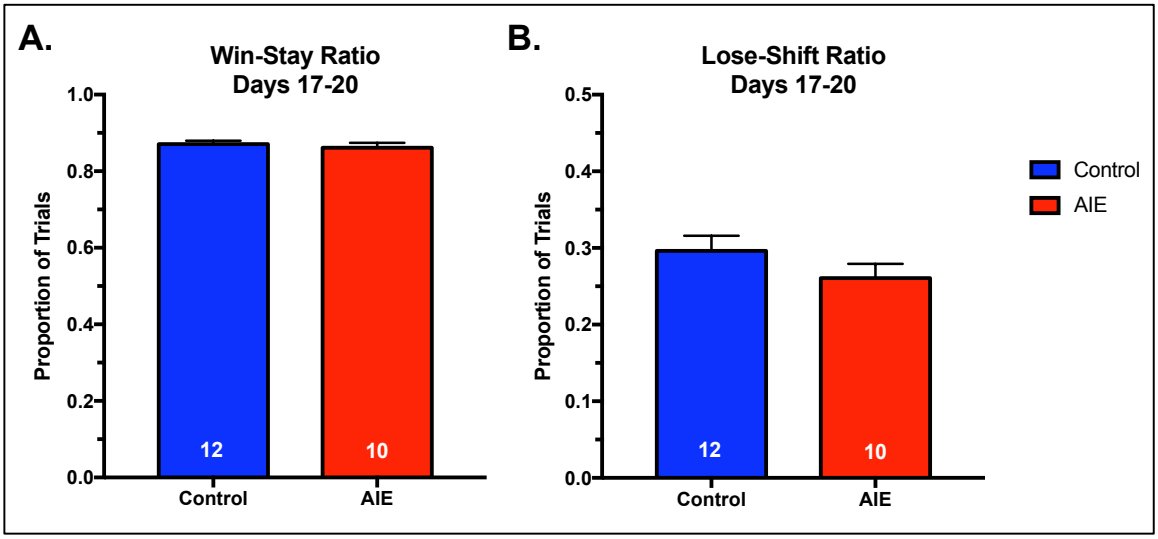

Figure 4-9. AIE is not associated with changes in the win-stay or lose-shift ratios on days 17-20 of the probabilistic decision-making task. Average A) win-stay ratio and B) lose-shift ratio for days 17-20 of the probabilistic decision-making task.

On the 21st day after initiation of PDT training, animals were exposed to either the single prolonged stress (SPS) procedure or a control exposure to light during their dark cycle (Control), as detailed in the methods section. They were then left undisturbed in their home cages for 7 days. Prior to any exposure, animals were separated into four groups (Control-Control, Control-SPS, AIE-Control, and AIE-SPS), and counterbalanced to minimize differences between SPS and Control groups within ethanol exposure groups. A one-way ANOVA of the four treatment groups' performance prior to SPS exposure revealed that there were no differences in win-stay ratios ($F_{(3,18)}$ = 0.237 p = 0.869), lose-shift

ratios ($F_{(3,18)}$ = 2.455 p = 0.096), or omissions ($F_{(3,18)}$ = 0.436 p = 0.730) between exposure groups prior to SPS exposure (**Figure 4-10**).

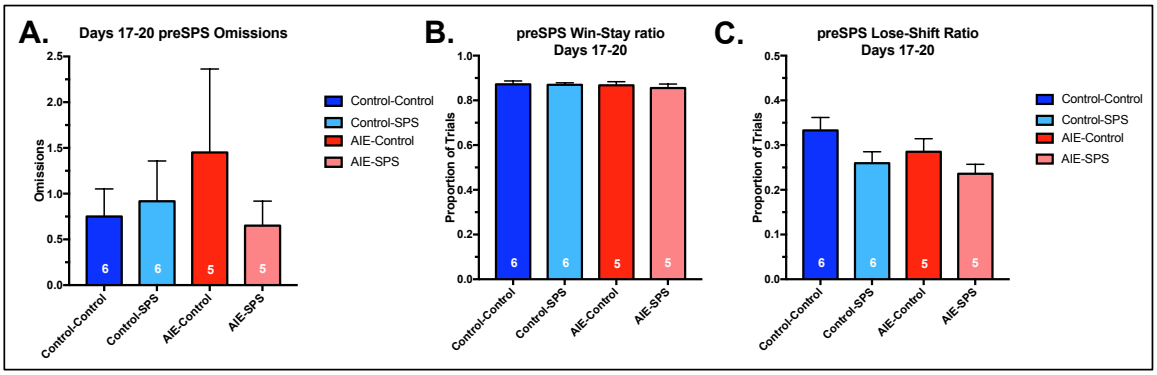

Figure 4-10. There are no differences in baseline performance between SPS exposure groups prior to SPS. Average A) omissions per session, B) win-stay ratio, and C) lose-shift ratio over days 17-20 of the probabilistic decision-making task expressed as the baseline performance before SPS exposure.

A 2-way ANOVA also revealed that there were no differences in percent risky choice ($F_{(3,90)}$ = 0.695 p = 0.558) or choice latency ($F_{(3,90)}$ = 1.747 p = 0.163) between treatment groups prior to SPS exposure (**Figure 4-11**).

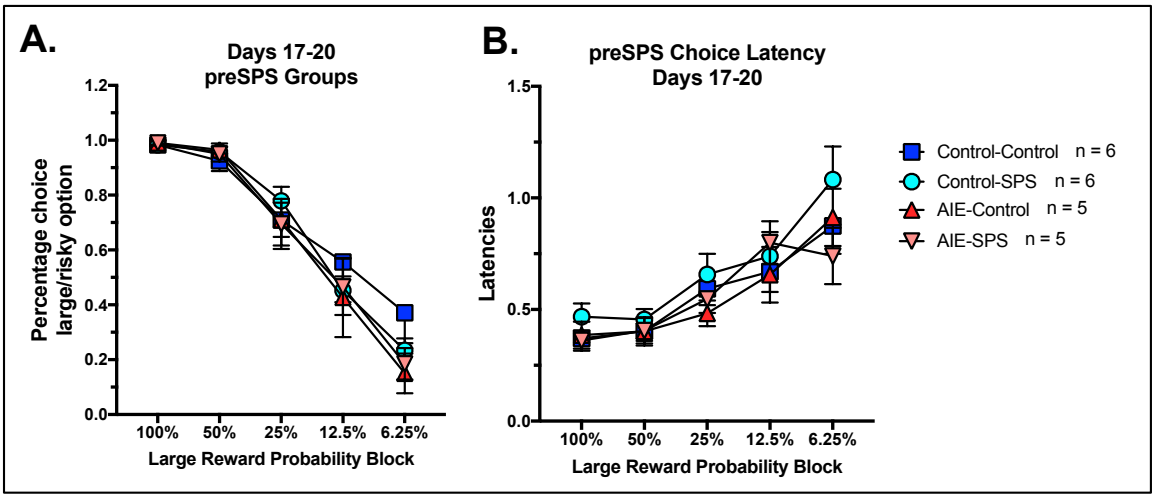

Figure 4-11. There are no baseline differences in percent risky choice or choice latency between SPS exposure groups prior to SPS. A) Average percent choice of the large/risky lever and B) choice latency during days 17-20 of the probabilistic decision-making task expressed as the baseline performance before SPS exposure.

A 4-way, multivariate, repeated measures analysis was conducted with phase (pre- and post-SPS) and probability block as within subject factors, and AIE and SPS exposure as between subject factors. This revealed that there was an interaction between phase, AIE exposure, and SPS exposure ($F_{(1,18)}$ = 4.446 p = 0.049). Follow-up analysis revealed that there were no significant effects prior to SPS exposure due to probability block, AIE exposure, or SPS exposure ($F_{(4,72)}$ = 1.117 p = 0.355). Further analysis of post-SPS percent risky choice via a 1-way ANOVA revealed that, in the Control-Control and AIE-Control groups (that is, groups not exposed to SPS), there was no difference between pre- and post-SPS performance ($F_{(1,9)}$ = 0.001 p = 0.974). Analysis of the SPS-exposed groups (Control-SPS and AIE-SPS) revealed that there was a significant difference between pre- and post-SPS percent risky choice ($F_{(1,9)}$ = 8.885 p = 0.015); rats in the AIE-SPS group were more risk averse (had lower percent risky choice) than rats in the Control-SPS group (**Figure 4-12**).

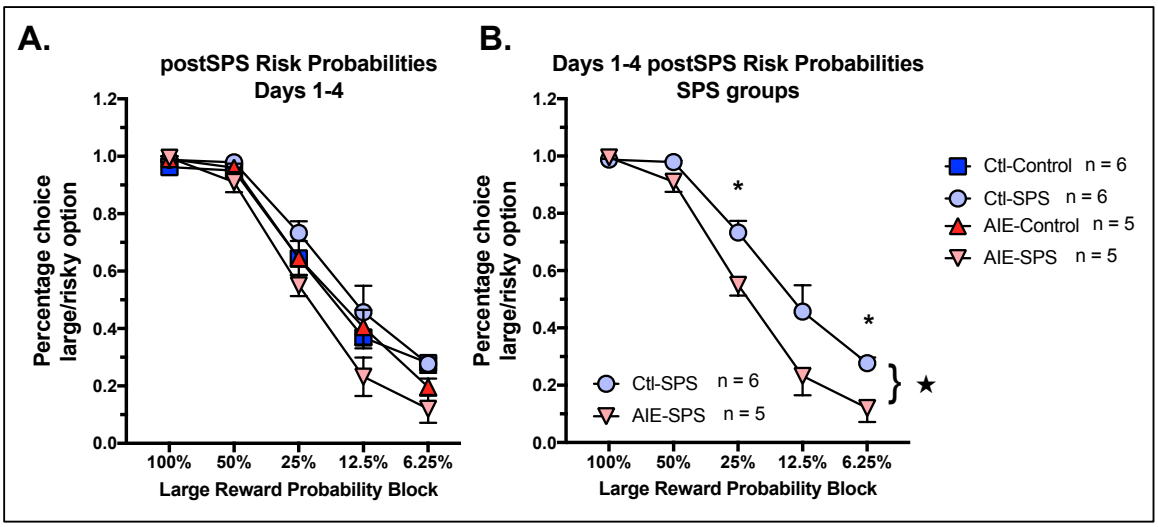

Figure 4-12. AIE resulted in increased risk aversion in SPS exposed animals on days 1-4 after SPS. Average percent choice of the large/risky lever on days 1-4 after SPS for A) all four exposure groups. B) For visual clarity, the same data is graphed as in A with only the Control-SPS and AIE-SPS groups displayed. * $p < 0.05$ ★ main effect of AIE exposure

However, this effect was only seen in the first four days of PDT testing after SPS exposure; there were no significant differences between groups on days 5-8 of PDT testing (**Figure 4-13**).

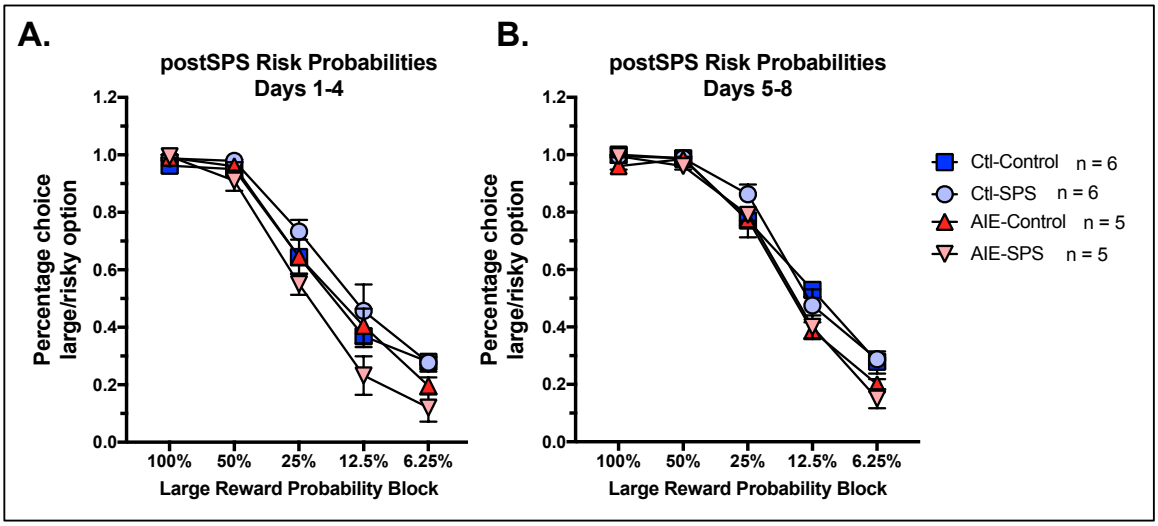

Figure 4-13. AIE and SPS are not associated with changes in percent risky choice more than four days after SPS exposure. Average percent choice of the large/risky lever on A) days 1-4 and B) days 5-8 after SPS exposure

Additionally, a 3-way ANOVA of the type of response (win-stay or lose-shift), AIE exposure, and SPS exposure, revealed that there were no significant differences between any of the groups in win-stay or lose-shift ratios pre- or post-SPS exposure ($F_{(1,18)}$ = 1.303 p = 0.289) (**Figure 4-14**).

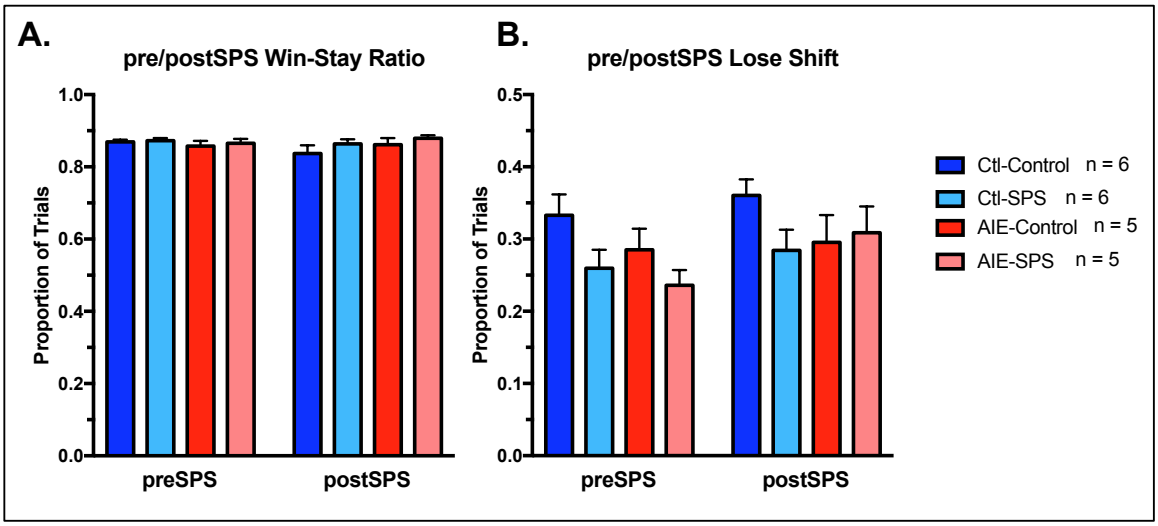

Figure 4-14. AIE and SPS are not associated with changes in win-stay or lose-shift ratios. The four-day average A) win-stay ratios and B) lose-shift ratios before and after SPS exposure

A 2-way ANOVA (AIE by SPS exposure) revealed that there were no differences between exposure groups in choice latency post-SPS exposure ($F_{(3,90)}$ = 0.783 p = 0.507), and another 2-way ANOVA revealed that there were no differences between exposure groups in omissions due to AIE ($F_{(3,36)}$ = 0.781 p = 0.512) or SPS ($F_{(1,36)}$ = 0.927 p = 0.342) exposure (**Figure 4-15**).

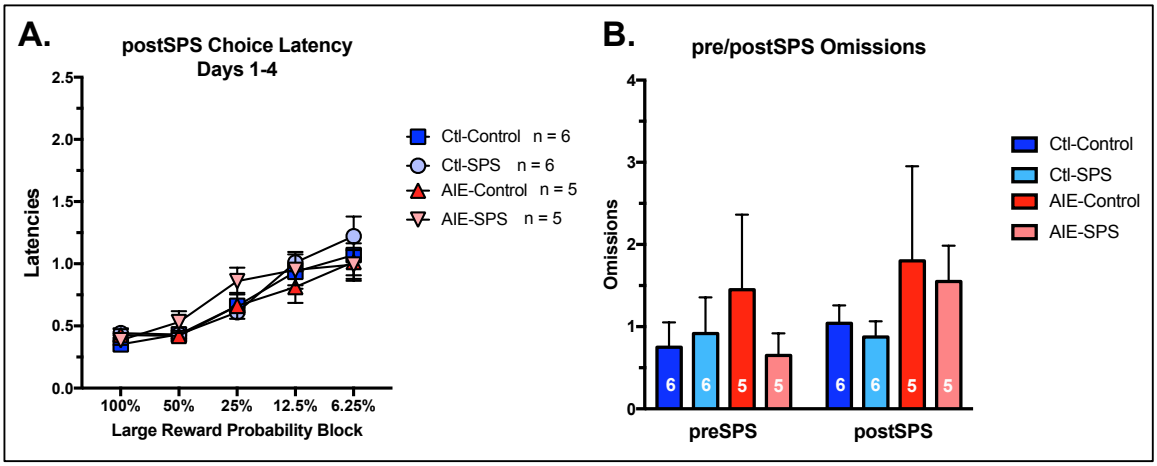

Figure 4-15. AIE and SPS are not associated with changes in choice latency or omissions. Average A) choice latency and B) omissions per session during days 1-4 after SPS exposure

Table 4-1. Significant results, separated by parameter, assessed during training for and testing of the PDT in AIE and Control groups, as well as pre- and post-SPS exposure.

Parameter	Significant?	In [group]	>/<	Compared to [group]	Phase
Percent risky choice	Yes	AIE-SPS	**<**	Control-SPS	post-SPS Days 1-4
Choice latency	Yes	AIE	**>**	Control	Days 1-8
	Yes	AIE	**<**	Control	Days 13-16

DISCUSSION

The results from these studies demonstrate that Sprague-Dawley rats were able to acquire adequate performance on the probabilistic decision-making task (Onge & Floresco, 2009). These results also reveal that AIE exposure led to increased choice latency on days 1-8 of PDT training without any significant difference in percent risky choice compared to the Control group. Additionally, there were no differences between Control and AIE groups in days of operant training leading up to the PDT, omissions per session, win-stay ratio, or lose-shift

ratio over 20 days of PDT training. In the 4 days following SPS exposure, the AIE-SPS group displayed decreased risky choice compared to the Control-SPS group; however, there were no additional differences between groups in choice latency, omissions, win-stay ratios, or lose-shift ratios.

The increase in choice latency over days 1-8 of PDT training may indicate impaired behavioral efficiency following AIE exposure, as was recently reported by Miller et al. (2017). Behavioral efficiency involves the maintaining the balance between speed and accuracy, and is best assessed in situations in which an organism must make a choice between competing stimuli. In their report, Miller and colleagues demonstrated that AIE led to increased choice latency (impaired behavioral efficiency) on the risky decision-making task (RDT). This task differed from the PDT in that it involved a risk of punishment instead of reward associated with the risky lever; that is, the risky lever always delivered a 4x reward compared to the small, certain lever, but with each change in probability block, the probability of punishment changed. The punishment used was relatively mild, a 0.35mA foot shock of durations ranging from 1.0-1.5s, as it is less than the stimulus traditionally used in fear conditioning (0.75mA for 2.0s), but enough to serve as an aversive stimulus nonetheless. This risk of punishment is evaluated with neural networks similar to those used in evaluating the risk of reward, but with opposite actions on neural activity.

During tasks with an appetitive stimulus, the basolateral amygdala (BLA) and its connections with the nucleus accumbens shell (NAcs) appear to facilitate selection of the large, risky choice over a small, certain choice, while the

prefrontal cortex (PFC) – specifically the medial PFC and the orbitofrontal cortex (OFC) – act as the brakes on this drive by tracking changes in reward probabilities and updating the value of an action, respectively. In contrast, during tasks with an aversive stimulus, the BLA appears to mediate the integration of information about reward magnitude and punishment probability, while the OFC seems to be critical for calculating punishment probabilities. Lesions of the BLA bias choice towards the risky option in aversive stimulus tasks, whereas they bias choice towards the certain option in appetitive stimulus tasks; contrastingly, lesions of the OFC bias choice towards the risky option in appetitive stimulus tasks and towards the certain option in aversive stimulus tasks (for review, see (Orsini, Moorman, Young, Setlow, & Floresco, 2015).

If the increase in choice latency seen during PDT training in AIE compared to Control rats demonstrates behavioral inefficiency, then a task with an aversive stimulus, such as the RDT, may shed more light on this effect of AIE than the current studies utilizing a task with an appetitive stimulus. However, it may also represent impaired ability to master the task, as was seen with the Sprague-Dawley animals in the last set of experiments (see Chapter 2). Recall that the Sprague-Dawley AIE group completed fewer reversals per session, and also displayed increased choice latency over 16 days of the PRL task compared to the Control group. In contrast, the Long-Evans AIE rats did not display impaired choice latency or reduced reversals. This could relate to genetic differences between the SD and LE rat strains. A more simple assessment of learning ability and recall may reveal more information about the learning impairment seen after

AIE exposure. In the next chapter, fear conditioning, extinction, and cue recall will be tested after AIE and/or SPS exposure; this more simple learning paradigm may shed light on the learning impairments demonstrated by the AIE compared to Control groups in this chapter, as well as chapter 3.

The AIE group did not display impaired risky choice on days 1-20 of the PDT compared to the Control group. This lack of effect of AIE on percent risky choice was surprising given recent studies showing that AIE exposure led to increased risky choice on a probabilistic decision-making task (Boutros, Der-Avakian, Semenova, Lee, & Markou, 2016; McMurray et al., 2016; Nasrallah et al., 2011; Nasrallah et al., 2009). However, there were several differences in experimental design that may have contributed to different results, including housing conditions during adolescence, AIE exposure method, and operant task design. These differences could account for why AIE rats in the current study did not display increased risky choice in the PDT, and are discussed in greater detail in chapter 6.

Compared to the Control-SPS group, the AIE-SPS group displayed decreased risky choice over the four days following SPS exposure. Choice under conditions of uncertain probabilities with an appetitive stimulus is thought to be mediated by a network of key brain regions, including, as mentioned previously, the BLA, the OFC, the mPFC, and the NAc. However, dopamine (DA) function within this network is critical to reward sensitivity, and balancing the drives between safety and exploration. D1-like receptors (D1Rs) are thought to, “aid in overcoming uncertainty costs,” to maintain an efficacious choice strategy despite

losses, and D2-like receptors underlie the exploration of choices in the face of changing reward probabilities (Orsini et al., 2015). Antagonizing D1Rs in the mPFC or the NAc has been shown to reduce risky choice in the PDT (Onge & Floresco, 2010; Stopper et al., 2014). Previous work from our lab has shown that AIE led to impaired D1R function in the prelimbic cortex of the mPFC (Trantham-Davidson et al., 2016). Additionally, SPS exposure has been reported to blunt responses to acute cocaine administration, which was hypothesized to result from decreased DA receptor function or expression following SPS (Eagle et al., 2015). Therefore, it may be that an AIE-induced reduction in D1R function in the mPFC was facilitated by SPS exposure, and contributed to decreased risky choice in the PDT on days 1-4 after SPS exposure.

In conclusion, the current set of studies demonstrated that AIE led to a significant increase in choice latency during days 1-8 of PDT training, without concurrent changes in percent risky choice, compared to the Control group. While this observation of a lack of increased risky choice in adult rats exposed to AIE was unexpected considering other studies in the literature that have observed increases in risky decision-making, differences in ethanol exposure, housing conditions in adolescence, and behavioral procedures may account for some or all of this. The increase in choice latency may reflect decreased behavioral efficiency, which could be clarified by additional studies utilizing a task with an aversive stimulus, such as the RDT. Additionally, following SPS exposure, the AIE-SPS group displayed decreased risky choice compared to the Control-SPS group in the four days following SPS exposure. This may be due to

a combination of AIE- and SPS-induced DA receptor dysfunction. Further studies examining the expression of DA receptor expression and function following SPS exposure could provide more information and clarity on this issue.

CHAPTER 5

ADOLESCENT BINGE-LIKE ETHANOL EXPOSURE LEADS TO CHANGES IN FEAR-RELATED BEHAVIOR

BACKGROUND & SIGNIFICANCE

Extinction learning can be conceptualized as a specific type of inhibitory learning (Hamilton & Brigman, 2015). It involves an initial phase of associative learning, in which a conditioned stimulus (such as a tone) is associated with an unconditioned stimulus (such as a mild shock). This is followed by an extinction phase, in which negative feedback is used to update the previously formed associative memory (i.e. a tone no longer predicts a shock). In the tone-shock pairing paradigm, lack of movement (freezing) is interpreted as the behavioral result of a successful association of the tone with the shock, as freezing indicates that the animal expects a shock. A lack of freezing during extinction indicates that the associative memory pairing the tone with the shock has been superseded by the extinction memory in which the tone is no longer paired with the shock. Pavlov originally theorized that the original associative memory would be inhibited by the extinction memory, as opposed to the erasure of the associative memory (Pavlov, 1927). In the years since Pavlov's original work, considerable progress has been made in detailing the specific circuitry of fear conditioning and extinction learning.

Fear conditioning is canonically thought to be dependent upon activity between amygdalar nuclei (basolateral, BLA, and central, CeA), the intercalated cells (ITC) of the amygdala, the hippocampus (HC), and prefrontal areas including the medial prefrontal cortex (mPFC) and orbitofrontal cortex (OFC). Information acquired during initial fear conditioning is transmitted from primary sensory cortices and the thalamus to the BLA, where reciprocal connections between the BLA and the ventral hippocampus (vHC), and the BLA and prelimbic mPFC, modulate fear-related neuronal plasticity. The BLA then projects to the CeA, and the CeA projects to the hypothalamus and brainstem nuclei, which mitigate fear-related behaviors (Tovote, Fadok, & Lüthi, 2015). Connections underlying extinction of fear behaviors include projections from the infralimbic (IL) mPFC to the ITC, and then to the CeA and brainstem nuclei to inhibit fear behaviors.

Activity within prefrontal areas is important for expression of fear behavior as well as consolidation of extinction learning, and disruption leads to deficits in these behaviors. Lesions of the lateral and ventral OFC have been shown to impair initial extinction learning in rats (West et al., 2013; Zelinski et al., 2010), and low frequency stimulation of the infralimbic PFC immediately after fear conditioning has also been shown to impair extinction learning in rats (Shehadi & Maroun, 2013). Inhibition of protein synthesis in the mPFC led to impaired fear extinction retention (Santini, Ge, Ren, de Ortiz, & Quirk, 2004), and temporary inactivation of the PL and IL prefrontal cortices prior to extinction impaired extinction retention as well as decreased freezing at all stages of fear

conditioning and extinction in rats (Sierra‐Mercado, Corcoran, Lebrón‐Milad, & Quirk, 2006). Taken together, these studies demonstrate that the PFC is critical for both fear behavior and extinction retention. Additionally, while studies utilizing circuit-specific disruptions have demonstrated deficits in fear-related behaviors following these disruptions, other systemic insults, such as those by drugs of abuse, can also lead to deficits in fear-related behaviors.

One such drug of abuse is ethanol, and its effects on the brain are widespread. However, its effects on fear conditioning and extinction can be quite specific. For example, rats exposed to intermittent ethanol during early adolescence displayed a deficit in context fear retention not seen in rats exposed to ethanol during later adolescence or adulthood, whereas rats exposed later in adolescence or adulthood displayed enhanced context extinction resistance not seen in rats exposed during early adolescence (Broadwater & Spear, 2013). Additionally, adolescent intermittent ethanol (AIE) exposure, an animal model of binge-like ethanol exposure encompassing early to middle adolescence, has been shown to impair extinction of ethanol-seeking behavior in rats (Gass et al., 2014). Interestingly, traumatic stress exposure has been shown to lead to similar deficits in fear-related behaviors.

As discussed in chapter 2, single prolonged stress (SPS) exposure is an animal model that exhibits a number of core symptoms of post-traumatic stress disorder (PTSD). These include hyper-arousal (Khan & Liberzon, 2004), increased negative feedback of the hypothalamic-pituitary-adrenal (HPA) axis (Liberzon et al., 1997; Liberzon et al., 1999), and impaired extinction retention

(Knox et al., 2011). Animals exposed to SPS displayed increased acquisition of fear conditioning with no differences in extinction learning; however, they displayed significantly impaired extinction retention, which parallels findings from PTSD patients (Liberzon & Abelson, 2016). In the studies presented in this chapter, we tested that hypothesis that AIE exposure would facilitate deficits in extinction retention following SPS exposure.

MATERIALS & METHODS

The animal care and AIE exposure model were identical to the methods detailed in chapter 2, with one exception. In contrast to 5 cycles of ethanol vapor inhalation used in all previous studies, the AIE exposure paradigm for experiments in this chapter encompassed post-natal days (PD) 28 through 53, and involved 8 cycles of 2 consecutive episodes of ethanol vapor inhalation. Rats were exposed to ethanol on PD28 & 29 (cycle 1), PD31 & 32 (cycle 2), PD35 & 36 (cycle 3), PD38 & 39 (cycle 4), PD42 & 43 (cycle 5, PD45 & 46 (cycle 6), PD49 & 50 (cycle 7), and PD52 & 53 (cycle 8) (**Figure 5-1**).

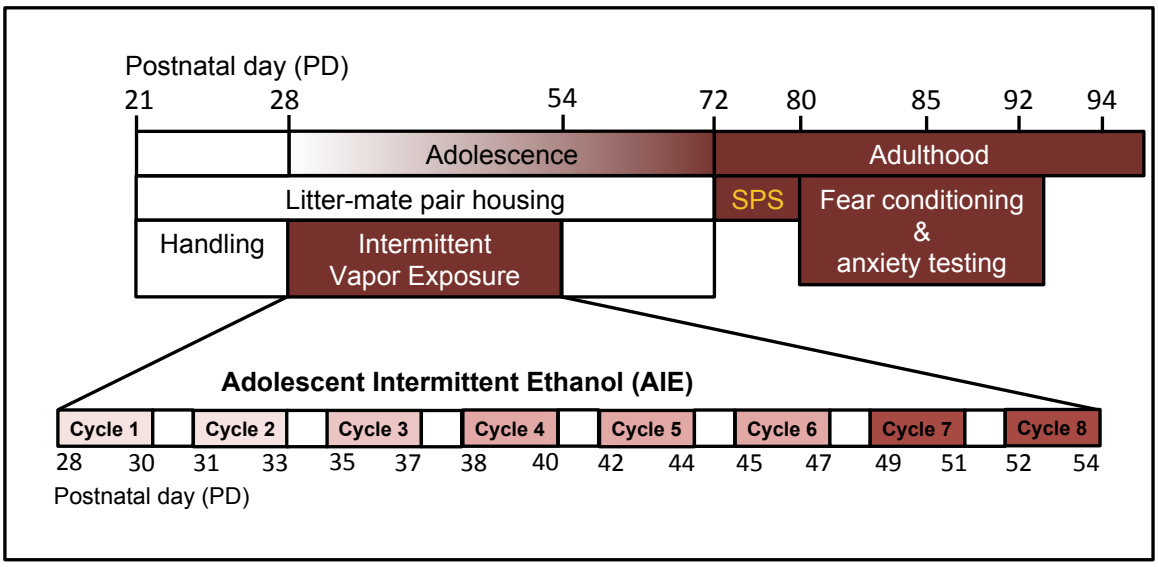

Figure 5-1. Experimental timeline of AIE exposure, pair housing, and operant testing

Single Prolonged Stress (SPS) exposure

In studies in this chapter that involve SPS, animals that were subjected to exposure were PD60 or older. The SPS procedure is based on previous work by Liberzon et al. (Liberzon et al., 1997; Liberzon et al., 1999). On the day of the SPS procedure, the control group was brought out of the animal facility to be kept in a lighted area outside of the exposure room in order to match the disturbance in light cycle that the SPS group would experience but without experiencing the smells and sounds from the stressed rats in the SPS group. Rats assigned to the SPS group were exposed to three successive stressors: 2 hours of restraint stress in a clear, acrylic cylinder; 20 minutes of forced swim in 23-25°C water that was deeper than the length of the rat; 15 minutes for recovery in a clean cage on a heating pad; followed by ether anesthesia to unconsciousness (approximately 5 minutes of exposure). Following this final stressor, the rats were allowed to recover in a clean cage on a heating pad until they were dry (approximately 2

hours), and then returned with the control group to the animal facility where they were left undisturbed for 7 days. Behavioral testing resumed on the 8th day after control or stressor exposures. Each of these exact stressors, in the order in which they are presented here, with the consolidation phase of 7 days after the stressors, have been shown to be vital to the development of behaviors which model some symptoms of PTSD in humans such as hyperarousal, extinction retention deficits, and enhanced negative feedback of the hypothalamic-pituitary-adrenal (HPA) axis (Knox et al., 2012; Liberzon et al., 1997; Liberzon et al., 1999).

Measures of Anxiety

Following AIE exposure and after PD60, two different behavioral measures were used to assess anxiety: the light/dark box (LDB), and the elevated plus maze (EPM) (**Figure 5-2**). Only one assessment was administered per day in order to reduce any confounds due to the sequence of behavioral tests.

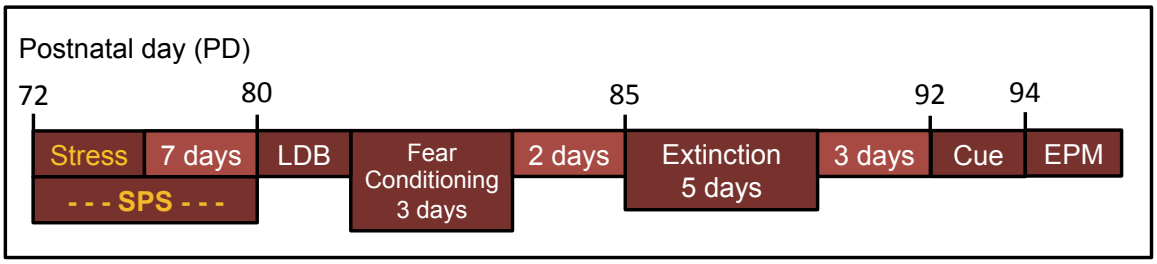

Figure 5-2. Detailed timeline of SPS stressor exposure, anxiety testing, and fear conditioning & extinction

The methods for assessment of anxiety-like behaviors were detailed in chapter 2. However, an additional measure was added for these studies. Since the same

animals can only be tested once with either of the tasks, rats were first tested on the light/dark box after AIE and SPS but prior to fear conditioning, and then tested after fear extinction using the elevated plus maze procedure as detailed below.

Elevated plus maze: The dimensions of the elevated plus maze were arm widths of 10 cm and length of 50 cm, closed arm wall heights of 40 cm, open arm wall heights of 1 cm, and a maze elevation height of 40 cm. The behavioral testing room was dimly lit with a red light. A white noise machine was used to obscure any distracting sounds. On test day, each rat was brought to the room individually in their home cage to habituate to the room for 5 minutes prior to the start of the test. To start the test, each rat was placed in the central square facing an open arm and allowed to explore the maze for 5 minutes. The entirety of the test was recorded via digital video at 60 frames per second for later analysis in Ethovision of the time spent in the open vs closed arms, and number of entries into the closed vs open arms. These values were averaged across treatment groups, and differences between groups were assessed via a 2-way ANOVA for exposure (AIE or Control) and stress (SPS or Control) with a significance threshold of $\alpha=0.05$. Between each test subject the entire apparatus was wiped with Cavicide and allowed to air dry.

Fear Conditioning with SPS & Measures of Anxiety

All animals that were treated with this exposure were PD60 or older. On the 8th day after SPS exposure, each subject was tested with the light/dark box test in order to assess anxiety-like behavior. On the following day, the first phase of fear conditioning began. It consisted of a 120 second acclimation period followed by three pairings of the Conditioned Stimulus (CS; 30s, 80dB, 3kHz tone) with the Unconditioned Stimulus (US; 2s, 0.75mA scrambled foot shock) during the last 2 seconds of the CS. This procedure was followed for three days. Two days later, extinction training began; this consisted of 120 seconds of acclimation followed by 10 presentations of the CS (30s tone). Each CS presentation was separated by 10-second intervals without any stimuli. This extinction training continued for 3-4 days, or until the subject froze less than 20% in response to the CS (30s tone) for 3 consecutive presentations. This model of extinction training in multiple blocks over 3-4 days (or until criteria is met) allows detailed analysis of within-session extinction (via trial blocks within one day) as well as extinction recall/retention the following day. Three days after extinction criteria was met, rats were tested for extinction recall and presented with the CS one time (**Figure 5-2**). Freezing behavior was determined from digitized videos using FreezeScan (Clever Systems, Inc.), and was determined as the complete absence of movement except for breathing, with parameters set by FreezeScan and verified by high inter-rater reliability prior to the initiation of the experiment. Percent freezing was calculated as the average amount of time the animal did not move within a trial divided by the total time of the trial. Finally, on the day

following the extinction recall test, each subject was assessed for anxiety-like behaviors using the elevated plus maze.

RESULTS

The Long-Evans rats used in these studies (n = 32) were separated within litters into pair-matched groups of Control or AIE exposed animals, and then sequentially subjected to AIE exposure, SPS exposure, and then fear conditioning. Intoxication scores measured at the end of each 14-hour ethanol vapor exposure period were averaged across all eight cycles. The average scores for day 1 of each of the eight cycles were 2.73 ± 0.34, 1.93 ± 0.15, 3.07 ± 0.21, 2.67 ± 0.16, 3.00 ± 0.09, 2.73 ± 0.12, 2.60 ± 0.27, and 2.67 ± 0.13, respectively, with a grand average across all eight cycles of 2.68 ± 0.07. The average scores for day 2, on which blood was obtained for BEC determination, of each of the eight cycles were 2.60 ± 0.21, 2.80 ± 0.11, 2.53 ± 0.17, 2.13 ± 0.24, 2.53 ± 0.13, 3.07 ± 0.07, 2.60 ± 0.16, and 2.47 ± 0.19, respectively, with a grand average across all eight cycles of 2.59 ± 0.07 (**Figure 5-3**).

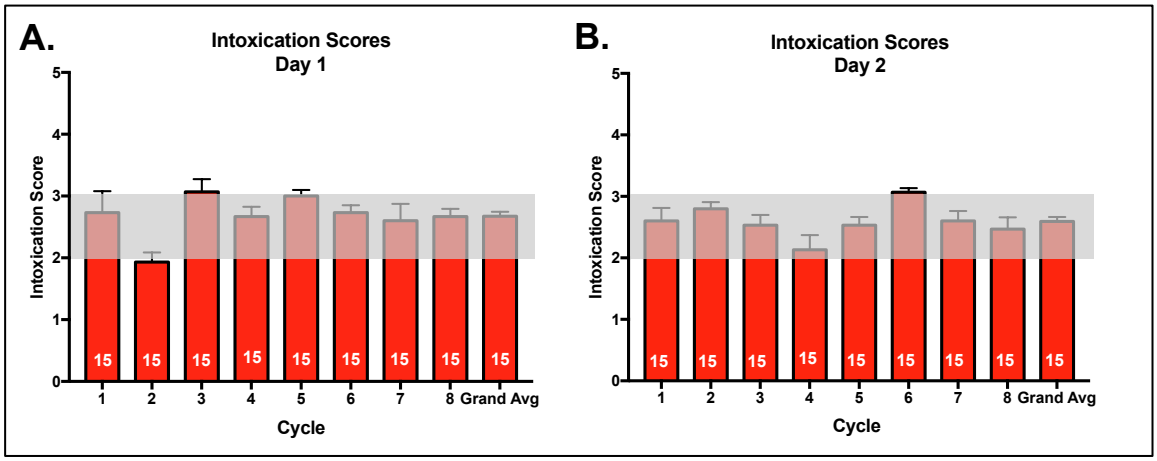

Figure 5-3. Average behavioral intoxication scores by cycle for A) day 1 or B) day 2 of each 2-day cycle, with the grand average across all first days of the 8 exposure cycles. The grey box denotes the target behavioral intoxication.

Intoxication scores for days 1 or 2 were collapsed across all eight cycles of ethanol vapor inhalation, revealing that the average scores for the cohort were 2.68 ± 0.07 for day 1, 2.59 ± 0.07 for day 2, with a grand average across both days of all eight cycles of 2.63 ± 0.07 (**Figure 5-4A**). Tail vein blood drawn at the end of each of the 2-day ethanol vapor exposure cycles revealed that the average BEC in each of the eight cycles was (in mg%) 443.2 ± 30.06, 421.84 ± 24.54, 394.37 ± 27.23, 311.40 ± 20.90, 399.77 ± 14.67, 426.81 ± 24.52, 431.46 ± 23.56, and 384.26 ± 24.15, respectively, with a grand average across all eight cycles of 401.64 ± 9.00 (**Figure 5-4B**).

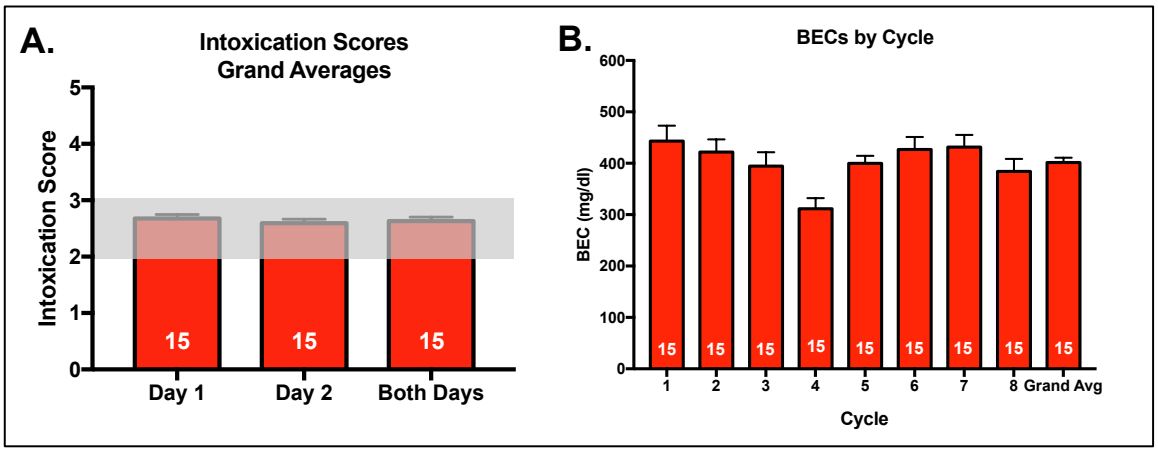

Figure 5-4. A) Average behavioral intoxication scores collapsed across all eight cycles for day 1, day 2, or for both days. B) Average BECs for each cycle, with a grand average across all eight cycles.

Following AIE and SPS exposures, anxiety-like behavior was assessed via the light/dark box task. A 1-way ANOVA of the four treatment groups (Control-Control, Control-SPS, AIE-Control, and AIE-SPS) revealed that there were no differences between groups in the amount of time spent in the light side of the box ($F_{(3,28)}$ = 1.184 p = 0.334) (**Figure 5-5**), the latency to first entry ($F_{(3,27)}$ = 0.566 p = 0.642), or in the number of entries into the light side of the box ($F_{(3,28)}$ = 0.891 p = 0.458) (**Figure 5-6**).

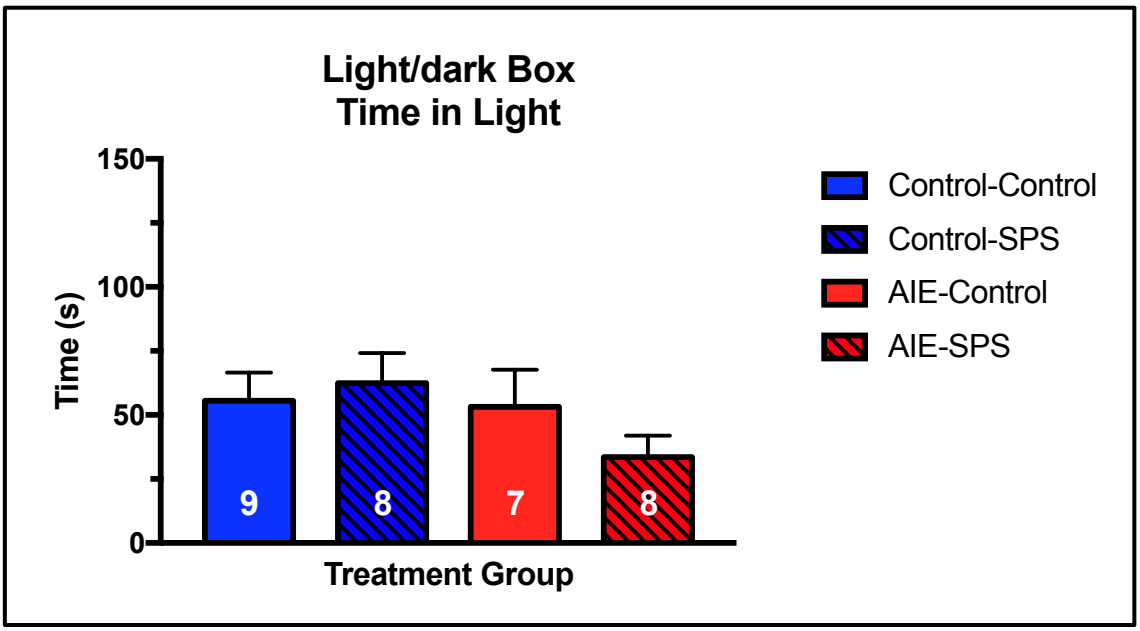

Figure 5-5. AIE and SPS are not associated with changes in time spent in the light side in the light/dark box task. Neither AIE nor SPS resulted in differences in the amount of time spent in the light side of the light/dark box.

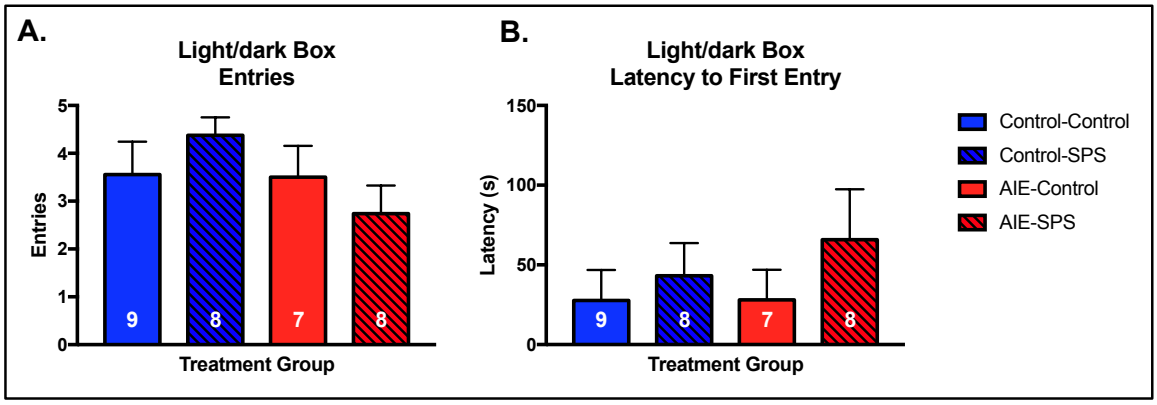

Figure 5-6. AIE and SPS are not associated with changes in latency to first entry or number of entries in the light/dark box task. Neither AIE nor SPS led to differences in A) latency to first entry or B) total entries in the light/dark box task.

This indicates that neither AIE exposure nor SPS exposure alone had an effect on anxiety-like behavior tested via the light/dark box task 8 days after SPS exposure.

After light/dark box testing, the effects of AIE and/or SPS exposures on cue-induced fear conditioning, extinction, and cue recall were tested. Fear

learning was assessed via three distinct phases: associative conditioning, in which a tone (CS) is associated with a mild foot shock (US); extinction, during which the tone is presented without the foot shock; and cue recall, in which the tone is presented without the foot shock. A 2-way ANOVA of treatment by day interactions revealed that the AIE-Control and AIE-SPS groups displayed increased freezing to the tone in the second and third ($F_{(6,84)}$ = 2.95 p = 0.0116) conditioning sessions compared to the Control-Control and Control-SPS groups (**Figure 5-7**).

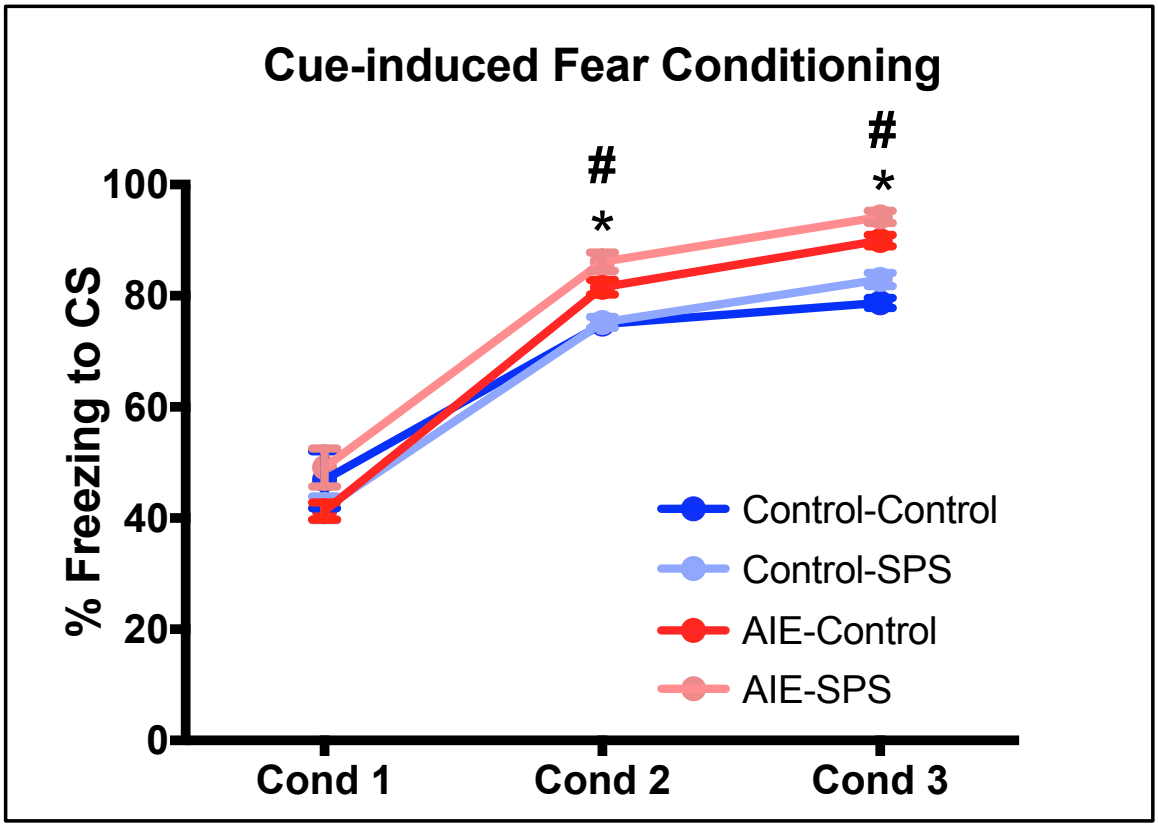

Figure 5-7. AIE and SPS facilitated acquisition of fear conditioning. AIE exposure led to increased freezing during fear conditioning sessions 2 and 3 compared to Control-control and Control-SPS groups, and SPS exposure exacerbated this effect. * $p < 0.05$ for AIE-Control compared to Control-Control # $p < 0.05$ for AIE-SPS compared to AIE-Control n = 7 - 9 per group

Following associative conditioning of the tone and the foot shock, extinction training was initiated. A 2-way ANOVA of treatment by day interactions with repeated measures revealed that, over five days of extinction, the AIE-control groups displayed increased freezing to the tone, and that SPS exposure exacerbated this effect ($F_{(12,144)}$ = 2.652 p = 0.013) (**Figure 5-8A-E**). Following extinction training, a cue recall test was administered. A one-way ANOVA revealed that the AIE-SPS and AIE-control groups were significantly different from the Control-Control and Control-SPS groups ($F_{(3,12)}$ = 19.53 p = 0.0001), and visual inspection revealed that the AIE-SPS and AIE-Control groups displayed increased freezing compared to the other groups (**Figure 5-8F**). When taken together with the increased extinction resistance observed earlier in the AIE-SPS and AIE-Control groups, these observations are consistent with an inability to extinguish freezing behavior following AIE exposure. Of particular interest is that there appeared to be a synergistic interaction of AIE and SPS.

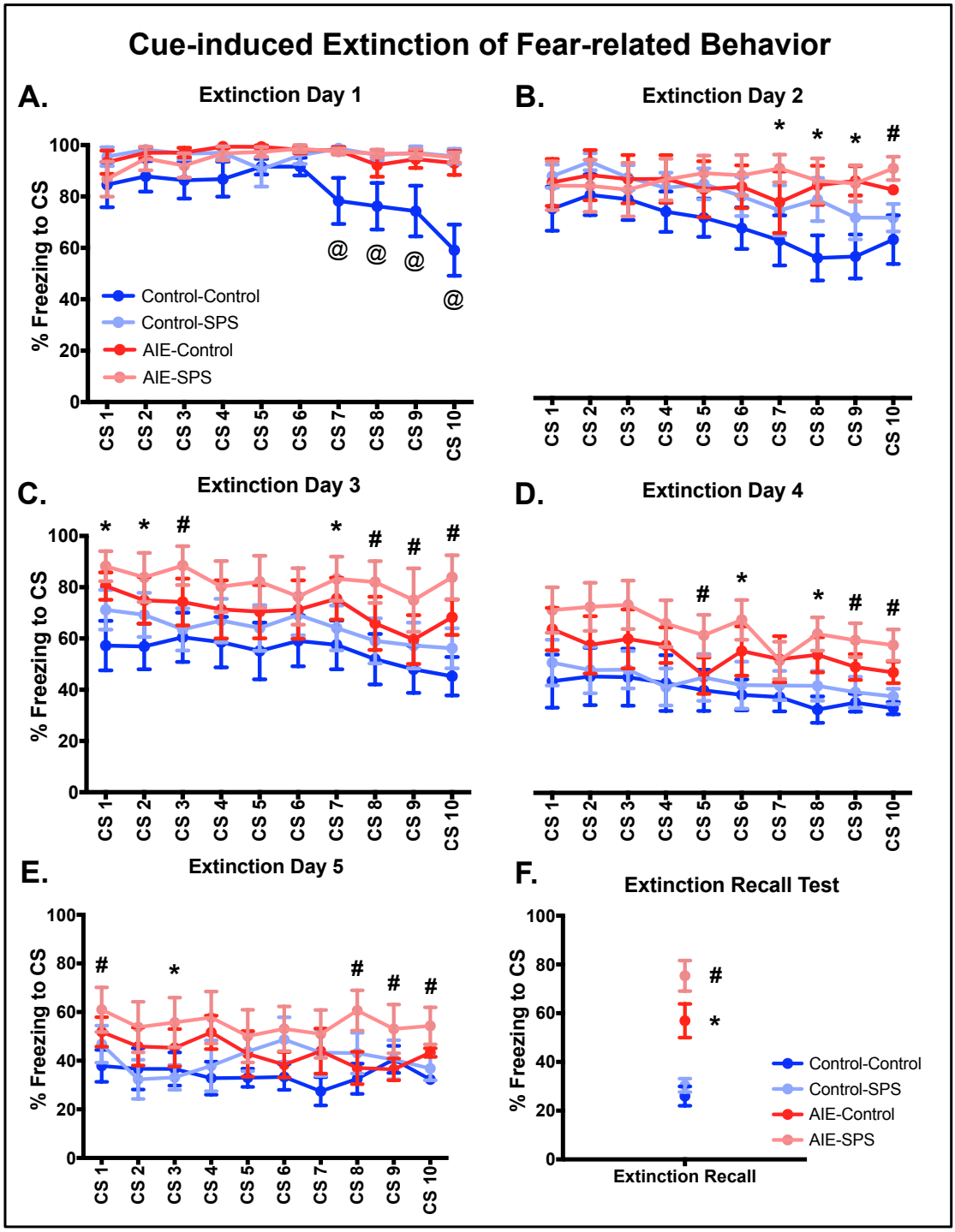

Figure 5-8. AIE and SPS resulted in increased resistance to fear extinction. AIE exposure led to increased freezing behavior compared to Control-Control or Control-SPS groups during multiple days of extinction training, and SPS exposure exacerbated this effect. Extinction sessions following fear conditioning for A) Day 1, B) Day 2, C) Day 3, D) Day 4, E) Day 5, and F) the extinction recall test. @ $p < 0.05$ for Control-Control compared to all other exposure groups * $p < 0.05$ for AIE-Control compared to Control-Control # $p < 0.05$ for AIE-SPS compared to AIE-Control n = 7 - 9 per group

To examine the effect of SPS on anxiety behaviors in these rats, one day after the cue recall session, anxiety-like behavior was tested via the elevated plus maze task. A one-way ANOVA revealed that there were no differences between groups in the amount of time spent in the open arms ($F_{(3.28)}$ = 0.139 p = 0.936) (**Figure 5-9**), or in the total number of entries into the open arms ($F_{(3,28)}$ = 0.436 p = 0.729) for any of the treatment groups. However, there was a significant difference between groups in the latency to first open arm entry ($F_{(3,27)}$ = 3.826 p = 0.021). Multiple comparisons with Holm-Sidak correction revealed that the Control-Control group was significantly different from the AIE-Control group ($t_{(27)}$ = 3.072 p < 0.05), and visual inspection revealed that the AIE-Control group displayed significantly decreased latency to first open arm entry compared to the Control-Control group (**Figure 5-10**).

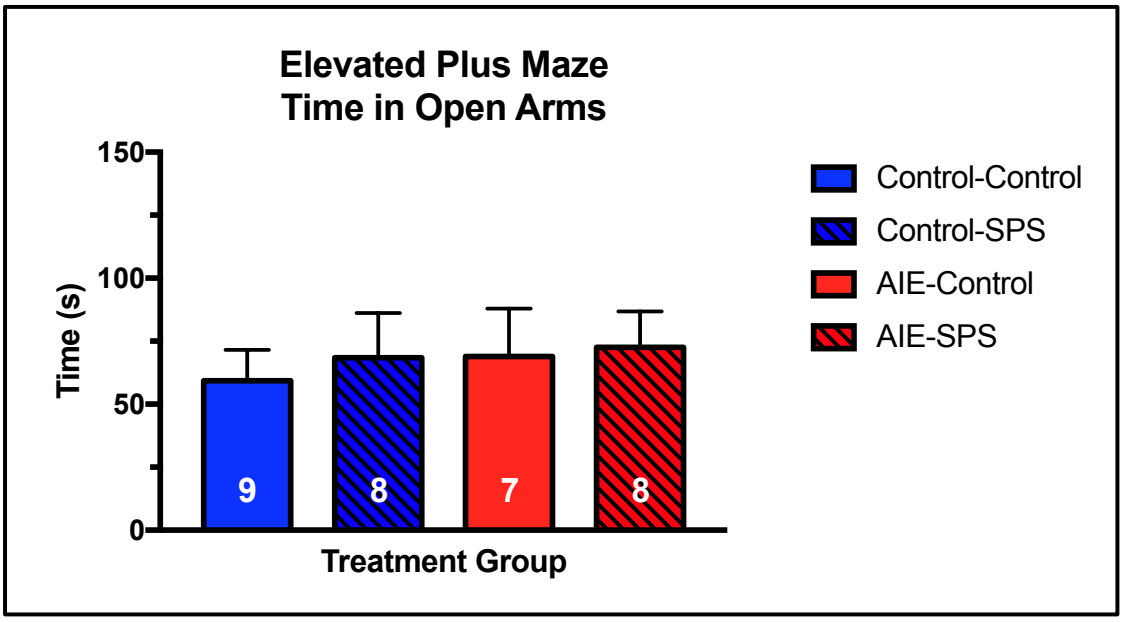

Figure 5-9. AIE and SPS are not associated with changes in time spent in the open arms of the elevated plus maze. Neither AIE nor SPS exposures led to differences in the amount of time spent in the open arms of the elevated plus maze.

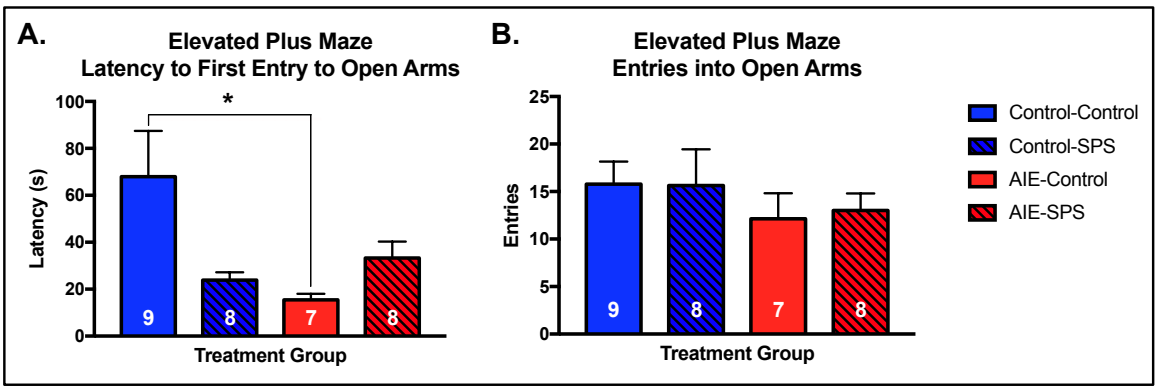

Figure 5-10. AIE resulted in decreased latency to first open arm entry on the elevated plus maze. AIE led to a significant decrease in the latency to first open arm entry compared to the Control-Control group. There were no differences between other groups in the latency to first entry, and no differences between groups in total number of entries into the open arms. * $p < 0.05$

DISCUSSION

The results from these studies demonstrate that both AIE and SPS exposures led to significant alterations in fear-related behaviors. Prior to fear conditioning, neither AIE nor SPS were associated with changes in anxiety-like behavior as assessed via the light/dark box task. However, AIE exposure led to increased acquisition of fear conditioning, and SPS exposure exacerbated this effect. During extinction learning, AIE was associated with extinction resistance, an effect that was exacerbated by SPS. In the extinction retention test, AIE exposure was associated with impaired extinction retention, which was exacerbated by SPS. Finally, after the extinction retention test, neither AIE nor SPS exposures were associated with alterations in anxiety-like behavior as assessed via the elevated plus maze.

The lack of effect of SPS on anxiety-like behavior on the light/dark box task prior to fear conditioning was unexpected, as a previous study reported that

SPS exposure led to increases in anxiety-like behavior on both the light/dark box and elevated plus maze tasks (Shafia et al., 2017). However, a potentially important difference between that study and the current one is that it used a 14-day period of consolidation in the home cage, and tested the rats for anxiety-like behavior on the elevated plus maze and light/dark box tasks six weeks after SPS exposure.

Three weeks after SPS exposure and one day after the extinction retention test, there were no differences between the groups in the amount of time spent in the open arms of the elevated plus maze. However, this observation may have been confounded by a floor effect, as each group spent ~20% of the total time (~60 out of 300 seconds) in the open arms, and a more extensive habituation to the testing conditions prior to testing might increase time spent in the open arms of the maze, as this would negate a potential floor effect. This could reveal differences in anxiety between the groups that were masked by the insufficient habituation in the present set of studies. For example, a prior study utilized a habituation procedure involving one hour of habituation to the testing conditions the day before testing, and five minutes of habituation on test day (Gass et al., 2014). It is important to note that this testing was conducted after almost three weeks of handling for fear conditioning testing; it could be that extended handling confounded the results of this test for anxiety-like behavior following SPS exposure. Interestingly, there was a significant difference in the latency to first open arm entry; the AIE-Control group had a significantly decreased latency to first open arm entry compared to the Control-Control group.

However, since this difference between groups was not associated with changes in time spent in the open arms or number of entries into the open arms of the maze, it is unclear what, if anything, this change in latency means.

Another potential explanation for the lack of differences observed in anxiety-like behavior following SPS exposure could be that these types of differences following SPS (and AIE) are not as evident in the strain of rat used in this study (Long-Evans). Data was presented in chapter 2 demonstrating that Long-Evans rats did not display increased anxiety-like behavior following AIE, whereas Sprague-Dawley rats did. Perhaps the use of Sprague-Dawley instead of Long-Evans rats may reveal differences in anxiety-like behavior following SPS exposure using the light/dark box and elevated plus maze tasks.

A particularly interesting observation was that AIE exposure led to increased acquisition of fear-related behavior over three sessions of fear conditioning, and SPS exposure exacerbated this effect. A similar effect was reported by Azzinnari et al. (2014), in which mice exposed to 15 days of chronic social defeat (CSD) were assessed for fear-related behavior in a contextual fear conditioning paradigm. The mice exposed to CSD froze for a significantly increased time during that intertrial interval compared to controls. While this study used contextual fear conditioning (in contrast with cued fear conditioning used in the present studies), they also observed increased acquisition of fear conditioning consistent with our observations following either AIE alone or in combination with SPS exposure. However, other studies using AIE exposure (Bergstrom et al., 2006; Broadwater & Spear, 2013; M. Broadwater & L. P.

Spear, 2014) have not reported increased acquisition of fear-related behavior. The increased acquisition of fear conditioning in the present study following AIE exposure may be the result of ethanol exposure encompassing early to late adolescence (PD28-53), as other studies that did not report a change in acquisition of fear conditioning following AIE used exposures that only encompassed either early to mid- or mid- to late adolescence. Perhaps binge-like ethanol exposure during the entire period of adolescence is necessary for increased acquisition of fear conditioning as reported in the present study. Additionally, while other studies using SPS exposure (Knox et al., 2011; Knox et al., 2012) have not reported increased acquisition of fear conditioning, it was only the AIE-SPS group that exhibited this increase, and not the Control-SPS group. It is possible that the combination of binge-like ethanol exposure throughout most of adolescence with SPS exposure in adulthood is necessary to promote increased acquisition of fear conditioning.

AIE also led to increased resistance to extinction over five days of extinction testing, and SPS exposure exacerbated this effect. Consensus in the literature is lacking on the effects of AIE on extinction and extinction retention of fear-related behavior. Broadwater & Spear (2014) reported decreased retention of context-induced fear, but a separate study by Broadwater & Spear (2014) reported that AIE exposure during mid- to late-adolescence led to increased resistance to extinction of context-induced fear. Additionally, Bergstrom et al. (2006) reported that adolescent ethanol was associated with impaired extinction of cue-induced fear in male rats.

It is important at this juncture to note the difference in context-induced and cue-induced fear conditioning. Context-induced fear conditioning associates foot shocks with a specific environment, and is predominantly dependent on hippocampal connections with the amygdala and prefrontal cortex for conditioned fear expression and extinction (Morgan & LeDoux, 1999; Orsini, Kim, Knapska, & Maren, 2011). In contrast, cue-induced fear conditioning associates foot shocks with a cue (i.e., a tone), and this is relatively more dependent on connections from the basolateral amygdala (BLA) to the prefrontal cortex for conditioned fear expression and extinction (Blair, Schafe, Bauer, Rodrigues, & LeDoux, 2001). It has been reported that BLA projections to the mPFC are not fully developed until adulthood (Cunningham, Bhattacharyya, & Benes, 2002), and that chronic ethanol administration alters the expression of NMDA and GABA-A receptors in the BLA (Floyd et al., 2004; McCool, Frye, Pulido, & Botting, 2003). Therefore, adolescent ethanol exposure may impair development of these BLA connections to the mPFC, and that may in turn impair cue-induced fear-related behavior.

While AIE has been shown to lead to impaired extinction of fear-related behavior, a similar resistance to extinction of fear-conditioned freezing has been reported following SPS exposure (Knox et al., 2012), although only one day of extinction training was conducted before testing for extinction retention. Additionally, the authors note that while this phenomenon was observed in one cohort in their study, it was not replicated in unpublished pilot experiments in their lab, leading these authors to conclude that the enhanced conditioned fear memory performance during extinction is likely not a stable phenomenon (Knox

et al., 2012). Regarding the possible mechanisms underlying this effect, SPS has been shown to result in decreased activity in the infralimbic (IL) cortex as well as disinhibition of the BLA during extinction training (Knox et al., 2016). Inhibition of the BLA by the IL cortex is important for extinction retention (Herry et al., 2008; Knapska & Maren, 2009; Sierra-Mercado, Padilla-Coreano, & Quirk, 2010; Sierra‐Mercado et al., 2006). Thus, decreased IL activity during extinction training in the SPS-exposed groups may have led to disinhibition of the BLA, and thus impaired extinction training and retention (Knox et al., 2016). Taken together, decreased activity in the IL cortex following SPS exposure may manifest as disinhibition of the BLA during extinction training and retention, and disruption of extinction of fear-conditioned freezing.

Another interesting observation was that AIE also led to decreased recall of the extinction memory, and this effect was exacerbated by SPS. It is important to note the difference in impaired extinction and impaired extinction recall. Extinction is the process by which a memory (i.e. a tone is not associated with foot shock) is formed that serves to inhibit a previously formed excitatory memory (a tone is associated with foot shock). When extinction is examined over several days with multiple sessions per day, the first session of each day (excluding the first day) serves as a recall test for the inhibitory memory learned the day before. Therefore, impaired extinction would manifest as significantly increased freezing during sessions within one day, whereas impaired extinction retention would manifest as significantly increased freezing in the first session of a day compared to the last session of the day before. Impaired extinction is indicative of impaired

behavioral flexibility, whereas impaired extinction retention is indicative of impaired consolidation of the new inhibitory memory. Accordingly, the deficit in extinction retention in the AIE-Control and AIE-SPS groups indicates impaired consolidation between the last extinction session and the extinction recall test. As discussed earlier, AIE during early to mid-adolescence has been shown to impair retention of context-induced fear conditioning (Broadwater & Spear, 2013), but not AIE during mid- to late adolescence. Another report by Broadwater & Spear (2014) showed that AIE exposure in early to mid-adolescence reduced retention of fear to context, but enhanced retention of cued fear. However, both of these studies reported changes in retention of fear conditioning, and not changes in extinction retention, following AIE exposure. It could be argued that the impairment in extinction retention observed in the AIE-Control group was simply due to a failure to extinguish cue-induced freezing. However, the AIE-Control group did display reduced levels of cue-induced freezing at levels similar to those observed in the Control-Control and Control-SPS groups, and it was only the AIE-Control and AIE-SPS groups that exhibited a significant increase in cue-induced freezing during the extinction retention test.

Decreased extinction retention following SPS exposure has been reported in a number of previous studies (Knox et al., 2011; Knox et al., 2012; Knox et al., 2016), and they have suggested it is mediated by enhanced conditioned fear memory performance (Knox et al., 2011). Extinction retention relies on reciprocal connections between the ventral hippocampus (vHPC), the IL, and the BLA (Burgos-Robles, Vidal-Gonzalez, Santini, & Quirk, 2007; Corcoran & Quirk, 2007;

Knox et al., 2016; Milad & Quirk, 2002; Santini et al., 2004; Sierra-Mercado et al., 2010; Sierra‐Mercado et al., 2006). Inhibition of the BLA by the IL cortex is important for extinction retention (Do-Monte, Manzano-Nieves, Quiñones-Laracuente, Ramos-Medina, & Quirk, 2015; Herry et al., 2008; Knapska & Maren, 2009; Sierra-Mercado et al., 2010; Sierra‐Mercado et al., 2006), and reciprocal connections between the BLA and vHPC are critical for increasing activity of "fear" neurons (Herry et al., 2008). However, extinction suppresses activity in the BLA and vHPC (Knox et al., 2016). SPS exposure abolishes this suppression during extinction, which may be one of the mechanisms through which SPS leads to deficits in extinction retention (Knox et al., 2016). SPS has also been reported to increase apoptosis in the BLA (Ding, Han, & Shi, 2010; Xiao, Yu, Wang, Han, & Shi, 2011). If apoptosis in the BLA following SPS is selective for inhibitory neurons, then this could explain the loss of suppressed neural activity during extinction in the BLA, and the impaired extinction retention following SPS exposure (Knox et al., 2016). Furthermore, as SPS increases apoptosis in the IL cortex (Knox, Perrine, George, Galloway, & Liberzon, 2010; Zhao, Zhou, Xu, & Zhang, 2014), this could lead to decreased output from the IL to the BLA, which could also impair extinction retention.

In conclusion, while neither AIE nor SPS led to alterations in anxiety-like behavior as assessed via the light/dark box (tested before SPS) or elevated plus maze (tested after SPS) tasks, AIE exposure alone led to increased acquisition of fear-related behavior, increased resistance to extinction, and decreased

extinction retention, and SPS exposure exacerbated each of these effects. Although speculative, the absence of changes in anxiety-like behavior observed in the present study may relate to the strain of rat used (Long-Evans). This strain did not show increases in anxiety-like behavior following AIE, whereas Sprague-Dawley rats did (see chapter 2). Perhaps use of the Sprague-Dawley strain would reveal anxiety-like behavioral changes not evident with Long-Evans rats following SPS exposure. AIE exposure in Long-Evans rats was associated with increased acquisition of fear-related behavior. However, the AIE exposure paradigm used in the studies presented in this chapter encompassed almost the entirety of early to late adolescence (PD 28-53). Therefore, it may be that the increased extent of AIE exposure led to the observed changes in acquisition of fear-related behavior, and this was exacerbated by SPS exposure in adulthood. The resistance to extinction following AIE exposure observed in the present study has been observed previously, in particular after ethanol exposure in mid- to late adolescence (Bergstrom et al., 2006; Broadwater & Spear, 2013; M. A. Broadwater & L. P. Spear, 2014); this effect has also been reported following SPS exposure (Knox et al., 2011; Knox et al., 2012). This increased resistance to extinction following AIE and SPS exposures may be due to impaired connections between the BLA and the mPFC, as well as impaired inhibition of the BLA by the IL cortex. Finally, results from the present study demonstrated that AIE exposure led to impaired retention of extinction of cue-induced fear-related behaviors, and SPS exposure exacerbated this effect. It has been reported previously that SPS leads to impaired extinction retention, which may be due to disrupted

connections between the BLA and vHPC, decreased inhibition of the BLA by the IL cortex, or selective loss of inhibitory interneurons in the BLA. These mechanisms of impaired extinction retention may also underlie the deficits demonstrated in the present study. Taken together, these effects on fear-related behavior suggest that AIE may lead to impaired connections between the BLA and the mPFC, and impaired inhibition of the BLA by the IL cortex; SPS exposure might further disrupt these connections, which would result in the effects on fear-related behavior demonstrated in the present study.

CHAPTER 6

DISCUSSION & FUTURE DIRECTIONS

The studies presented in this dissertation provide support for the idea that adolescent binge-like ethanol exposure leads to persistent cognitive deficits in adulthood, specifically in behavioral flexibility, and that a traumatic stress exacerbates these deficits. Additionally, adolescent binge-like ethanol exposure has been associated with changes in anxiety-like behavior in adulthood, and data presented following an animal model of binge-like ethanol exposure (adolescent intermittent ethanol exposure, AIE), show differential responses of anxiety-like behavior in two different, outbred strains of rats. This was the basis for the initial hypothesis that AIE may also lead to differential effects on behavioral flexibility in adulthood in Long-Evans (LE) versus Sprague-Dawley (SD) rats and that a traumatic stress (single prolonged stress, SPS) would exacerbate these effects. Therefore, several tasks dependent on behavioral flexibility and PFC function were used to assess both strains of rats in adulthood following AIE and SPS exposures. The results are summarized and discussed below.

AIE differentially affects anxiety-like behavior in LE versus SD rats

Following AIE exposure, the light-dark box task was used to assess anxiety-like behavior. This revealed that LE AIE-exposed rats spent more time in

the light side of the box compared to control rats. In contrast, SD AIE-exposed rats spent more time in the dark side of the box compared to controls. This indicates an increase in anxiety-like behavior in SD rats, but not in LE rats, which is consistent with previous observations. It could be argued that the AIE LE rats demonstrated a decrease in anxiety-like behavior, but it could also be interpreted as an increase in disinhibitory behavior. Increased disinhibition would also manifest as more time spent in the light side of the box, and several studies have reported increased disinhibition following AIE exposure (Ehlers et al., 2011; Gass et al., 2014; Gilpin et al., 2012). It is also important to consider the possibility that both increased disinhibition and decreased anxiety could contribute simultaneously to this behavior. A recent review examined the lack of consensus in the literature regarding whether AIE led to increased anxiety-like behavior or increased disinhibitory behavior (Crews et al., 2016). The authors concluded that the use of certain ethanol exposure paradigms (such as IP or IG) and strains of rats (such as SD) are more likely to be associated with increased anxiety-like behavior, whereas the use of other ethanol exposure paradigms (such as ethanol vapor) and rat strains (such as LE and Wistar) are more likely to yield increased disinhibitory behavior. Further testing with a separate task that more specifically examines increased disinhibition versus decreased anxiety, such as the open field conflict avoidance task (Ehlers et al., 2011), may yield a more clear answer. However, the authors of the review ultimately concluded that, “the nature of rodent assessments prevents a clear determination of how AIE impacts these two traits,” (Crews et al., 2016). Nevertheless, the results from the light/dark box

in the current set of studies indicated differential effects of AIE on LE versus SD rats, which provided support for the hypothesis that the performance of LE and SD rats on tasks of behavioral flexibility would also be differentially affected following AIE exposure.

AIE differentially affects probabilistic reversal learning in LE versus SD rats

The deficits seen in the probabilistic reversal learning (PRL) task following AIE exposure were subtly different between LE and SD rats. For example, the LE AIE group only showed a deficit in the number of trials to reach criterion during the discrimination phase of the first day of PRL testing, and the number of omissions over 16 days of PRL testing compared to the LE control group. The former result indicates impaired discrimination learning with probabilistic reinforcement, but not reversal learning. This is contrary to some reports in the literature that suggested that AIE impairs reversal learning in adulthood (Coleman et al., 2011; Coleman et al., 2014; Fernandez et al., 2017; Fernandez & Savage, 2017; Fernandez et al., 2016). The latter result may indicate increased compulsivity or impulsivity, but not without a concomitant change in choice latency (which was not reported in LE rats in these studies). In contrast, the SD rats showed no deficits following AIE on the first day of PRL testing, but several deficits following 16 days of PRL testing. SD AIE rats displayed a decreased number of reversals completed per session, increased choice latency, decreased win-stay ratio, and decreased lose-shift ratio compared to the SD control group. The combination of fewer reversals per session and increased

choice latency suggests a deficit in behavioral efficiency. However, tasks with an appetitive stimulus, such as the PRL task, are not ideal for testing this parameter. Behavioral efficiency is best tested with a task with an aversive stimulus (Miller et al., 2017), such as the risky decision-making task (RDT).

The lack of an effect on reversal learning in either LE or SD rats on day one of the PRL task, and the lack of an effect after 16 days of the PRL task in LE rats, was unexpected given the reports in the literature (Coleman et al., 2011; Coleman et al., 2014; Fernandez et al., 2017; Fernandez & Savage, 2017; Fernandez et al., 2016). As discussed below, there are a number of differences in experimental design that may have contributed to different results.

Method of ethanol administration

The present study exposed rats to binge-like levels of ethanol via chambers of vaporized ethanol, whereas the studies by Coleman et al. (Coleman et al., 2011; Coleman et al., 2014) used an intragastric mode of exposure. Forced ethanol administration through oral gavage can be associated with experimenter handling stress, whereas passive exposure via vaporized ethanol chambers allows for similar levels of closely monitored intoxication without the possible handling stress that may be associated with oral gavage. Adolescence is an ontogenetic period in which the stress system (hypothalamic-pituitary-adrenal, or HPA, axis) is undergoing significant development. Foilb et al. (2011) demonstrated that the typical adult response to stress (30 minutes of restraint stress in this study) in male rats developed as indexed by plasma

adrenocorticotropic hormone (ACTH) levels between 40 and 50 days of age. The typical adult response to stress as indexed by plasma corticosterone (CORT) levels appeared to develop between 30 and 40 days of age in male rats. Additionally, many studies have shown that an acute stressor exerts a response from the HPA axis as indexed by ACTH and CORT levels that is twice as extensive in prepubertal compared to adult rats (Doremus-Fitzwater, Varlinskaya, & Spear, 2009; Goldman, Winget, Hollingshead, & Levine, 1973; Romeo, 2016; Romeo, Patel, Pham, & So, 2016). Therefore, stress associated with experimenter handling during oral gavage administration of ethanol may exert effects in addition to those from adolescent binge-like ethanol administration. This could be a contributing factor to the deficit in reversal learning seen in the studies from Coleman et al. (Coleman et al., 2011; Coleman et al., 2014), Fernandez & Savage (2017), and Fernandez et al. (2017).

Length & timing of ethanol administration

The binge-like ethanol exposure in the present studies encompassed early to middle adolescence (PD28-44). However, the exposures from Coleman et al. (2011 & 2014) also encompassed early adolescence (PD28-37), while the exposures from Fernandez & Savage (2017) and Fernandez et al. (2017) covered early to late adolescence or adulthood: PD25-55 in the former, and PD28-53 & PD35-88 in the latter study. Development of the PFC spans the entirety of adolescence, and therefore ethanol exposures that encompass more of adolescence may lead to more severe cognitive deficits in adulthood.

However, both studies from Coleman et al. (Coleman et al., 2011; Coleman et al., 2014) covered a relatively shorter range of days than either the current studies or the other studies referenced previously, yet they still demonstrated impairment in reversal learning in adulthood. A recent review (L. Spear, 2015) hypothesizes that early to mid-adolescent insults (such as binge-like ethanol exposure) may disproportionately affect self-administration and social/affective behaviors, whereas insults during later adolescence and early adulthood may disproportionately affect cognitive functions supported by neural substrates that are still undergoing maturation. While length and timing of ethanol exposure may be another contributing factor to the lack of effect of AIE on reversal learning seen in the present studies, it is by no means the prime candidate given the deficits seen in reversal learning by Coleman et al. (Coleman et al., 2011; Coleman et al., 2014) after early to middle adolescent ethanol exposure. As discussed below, those studies differ from the present studies primarily in the method of reversal learning testing.

Method of testing reversal learning

The present studies use an operant reversal learning paradigm with probabilistic reinforcement, which differs significantly from the paradigms used in other previously published studies. Several of these used spatial-based reversal learning tasks: Coleman et al. (2011) used the Morris water maze, while Coleman et al. (2014) and Fernandez & Savage (2017) used the Barnes maze. Reversal learning paradigms such as these are more dependent on the

hippocampus, as they test not only reversal learning, but also spatial orientation and spatial memory. Therefore, perhaps tasks requiring spatial reasoning abilities are more sensitive to the effects of AIE.

After completing 16 days of PRL testing, one half of the LE and SD rats underwent SPS exposure, resulting in four treatment groups for each rat strain: Control-Control, Control-SPS, AIE-Control, and AIE-SPS. After four days of PRL testing following SPS exposure, LE AIE-SPS rats displayed increased win-stay and lose-shift ratios compared to the LE AIE-control groups. The increase in win-stay ratio was statistically significant, but was small, and thus this deficit may not be functionally meaningful. However, the increase in lose-shift ratio for the LE AIE-SPS group was more robust, and was a 26.7% increase in the AIE-SPS group compared to the AIE-Control group. This increase in lose-shift ratio is thought to reflect an increase in negative feedback sensitivity, which is also seen in depressed patients, and following global, acute, cerebral serotonin reduction (Bari et al., 2010). Therefore, it seems reasonable to conclude that this effect may be due to a change in serotonergic neurotransmission following both AIE and SPS exposures. Future studies to address the underlying mechanism could include characterization of markers of serotonergic neurotransmission and innervation in the PFC, OFC, and nucleus accumbens in AIE-SPS compared to AIE-control LE rats. Additionally, it may be valuable to test the effects of administration of a selective serotonin reuptake inhibitor on LE AIE-SPS rats during the PRL task.

In contrast to LE rats, exposure of SD rats to SPS was associated with a decrease in choice latency across all treatment groups compared to the baseline values prior to SPS exposure. Sometimes animal behavior can be affected by the activity in the building in which they are housed or perform their behavior. Anecdotally, there may be changes in performance on a Monday after a day off on Sunday. However, following SPS exposure, half of the SD rats were re-started on the PRL task on a Thursday, and the other half were re-started the following day, on a Friday. Therefore, it is unlikely that the decrease in choice latency across all treatment groups was due to the day of the week. As this effect was significant across treatment groups following SPS exposure, it is also likely not due to either AIE and/or SPS exposure.

AIE leads to increased choice latency on the probabilistic decision-making task

Previous data from the lab demonstrated that there is no change in risky choice or choice latency as assessed via the probabilistic decision-making task (PDT) in AIE compared to control LE rats. However, it was hypothesized that SD rats may be differentially affected on the PDT following AIE exposure. Following AIE exposure, SD AIE rats displayed increased choice latency across all probability blocks on days 1-4 and days 5-8 of training compared to the SD control group. However, this was unaccompanied by changes in risky choice either during training, or after reaching a stable level of performance (17-20 days of training). Additionally, there were no differences between SD AIE and control

groups in the win-stay or lose-shift ratios, or the number of omissions per session. The AIE group did not display impaired risky choice on days 1-20 of the PDT compared to the Control group. This lack of effect of AIE on percent risky choice was surprising given recent studies showing that AIE exposure led to increased risky choice on a probabilistic decision-making task (Boutros, Semenova, Liu, Crews, & Markou, 2015; McMurray et al., 2016; Nasrallah et al., 2011; Nasrallah et al., 2009). However, these studies differed from the current set of experiments in several important ways.

Method of ethanol administration

All of the referenced studies used ethanol exposure periods that were similar, encompassing early, mid, and some of late adolescence (~PD29-49). However, not all of the exposure paradigms used similar ethanol administration methods. The Boutros group (2015) used intra-gastric gavage, while the Nasrallah (Nasrallah et al., 2011; Nasrallah et al., 2009) and McMurray (2016) groups used ethanol-laced gelatin self-administration. While models of self-administration of ethanol in rodents are more representative of how humans typically consume ethanol, they have a number of drawbacks for studying the effects of ethanol. Rats may consume ethanol to reach BECs of 80mg/dl (the legal limit of intoxication for driving) or higher, but will usually not self-administer to the levels associated with binge-intoxication seen in humans. Additionally, not all of the rats within an experimental cohort will consume similar amounts of ethanol. As seen in the paper from McMurray et al. (2016), it is common to have

part of the group consume significantly more ethanol (the high consumption group), and the rest of the group consume less ethanol (the low consumption group), sometimes with several animals consuming little to no ethanol at all. This is one example of why many studies utilize experimenter-administered ethanol, such as intra-gastric gavage (Boutros et al., 2015), or chambers of vaporized ethanol as in the current set of studies.

There are several common critiques of experimenter-administered ethanol in studies of the effects of binge-like ethanol exposure. First, these studies should be designed to shed light on the processes involved in human abuse of ethanol, which is largely self-administered. However, given the relatively low intoxication rates associated with rodent ethanol self-administration (compared to humans), it is sometimes more relevant to employ experimenter-administered ethanol to achieve BECs similar to those seen in humans who engage in binge drinking, as is modeled in the current studies. Second, the route of experimenter-administered ethanol is not always the same as human ethanol use. While all of the studies referenced previously involve ethanol intake via the gastrointestinal tract, the present set of studies used chambers of vaporized ethanol. Ethanol vapor administration is not very popular among humans, but it is used sparingly with nebulizers or heated vaporizers of ethanol (Glatter, 2013; Press, 2006). More importantly, it is a method with minimal handling for rodents of achieving BECs associated with binge-like ethanol consumption in humans without the handling-associated stress and/or pain of injections (IP ethanol), gavage (IG ethanol), or the uncertainty of ethanol self-administration levels.

Isolation during adolescence

Another important factor that can impact differences in results, especially in relation to PFC function and anxiety-like behavior, is social isolation. The previous studies from Nasrallah et al. that were cited above (Nasrallah et al., 2011; Nasrallah et al., 2009) employed individual housing during adolescence (starting at PD27 or 30, respectively), while the current study employed pair housing with a littermate until adulthood (up to PD60). Rodents are naturally social creatures, and social isolation during adolescence has been used as a model of anxiety as well as vulnerability to alcohol use disorders (Butler, Karkhanis, Jones, & Weiner, 2016). Social isolation in adolescence has been shown to increase anxiety-like behaviors, as well as lead to increased ethanol consumption in adulthood. Therefore, adolescent social isolation should not be considered a standard housing practice, and may have contributed significantly to both the amount of ethanol gelatin consumed, as well as the increase in risky decision-making in both studies from Nasrallah et al. (Nasrallah et al., 2011; Nasrallah et al., 2009).

Between- or within-session changes in reward probability

There are two broad categories of changes in behavioral procedures: between-session and within-session changes. A change in the behavioral paradigm, such as the probability of reward on a risky lever, which happens between discrete sessions of behavioral testing is denoted as a “between-session” change. In contrast, changes that occur within a discrete behavioral

session are “within-session” changes. The studies detailed in this chapter utilize within-session changes in reward probability, which familiarize the animal with all levels of reward probability in one session, and allow for extended training with all of these probabilities. However, both studies from Nasrallah et al. (Nasrallah et al., 2011; Nasrallah et al., 2009), as well as the study from McMurray et al. (2016), utilized between-session changes in reward probability for the risky lever. The result of this experimental design is that the animal only experienced one reward probability per day, and required significantly more days of training to both familiarize the animal with each distinct probability of reward, as well as to examine changes in task performance over the course of multiple days of testing. Another implication is that the animal would have to remember information from one day with one reward probability to the next (and likely several more) to form the most efficacious strategy for reward. This may have placed an increased cognitive load on short- as well as long-term memory, memory consolidation, reward evaluation, and strategy formation, which may have introduced a confound to assessment of PFC function.

AIE leads to changes in fear-related behavior

Reports in the literature on changes in cue-induced fear-related behavior following AIE exposure is sparse, and there have been no studies to date that we are aware of examining the combined effects of AIE and SPS exposures on cue-induced fear-related behaviors. Following AIE and SPS exposures in the present study, LE rats were assessed for anxiety-like behavior in the light/dark box task.

Surprisingly, there were no differences between any treatment groups. However, other studies have found increases in anxiety-like behavior in the light/dark box task following SPS exposure and a longer consolidation period (Shafia et al., 2017). Perhaps a longer consolidation period after administration of the stressors and before light/dark box testing would reveal differences in anxiety-like behavior not seen in the present study. After assessment of cue-induced fear-related behavior, a separate assessment of anxiety-like behavior was carried out using the elevated plus maze. However, there were no consistent, significant differences between groups. Again, a longer consolidation period may have revealed deficits in anxiety-like behavior not seen here with the canonical seven-day consolidation period. Additionally, as reported in chapter 2, LE rats did not display an increase in anxiety-like behavior following AIE exposure, whereas SD rats did display this. Accordingly, changes in anxiety-like behavior following AIE and SPS exposure may be revealed through the use of SD instead of LE rats.

Despite not observing changes in anxiety-like behavior, there were significant differences in cue-induced fear-related behavior following AIE and SPS exposure. Both the AIE-Control and AIE-SPS groups showed increased acquisition of cue-induced freezing compared to the Control-Control and Control-SPS groups, and thus it is likely that AIE exposure (as opposed to SPS exposure) is necessary to elicit this difference in fear-related behavior.

There have been relatively few studies of the acquisition of fear-related behavior following AIE. There has not been a report of increased acquisition of fear-related behavior, but the present set of studies utilized a model of AIE that

encompassed early (PD28) to late (PD54) adolescence. This is a more extensive ethanol exposure compared to other studies that have not reported an increase in acquisition of fear-related behavior, and it may be that this more extensive exposure over most of adolescence is the reason for the increase in acquisition of cue-induced freezing. Follow-up studies could examine the effects of exposures varying both in length of time and age of initiation to ascertain if the extended AIE exposure is the cause of increased acquisition of cue-induced freezing.

One of the most interesting observations in the present set of studies was that AIE led to an increased resistance to extinction training, and that SPS exacerbated this effect. On the first day of extinction, only the Control-Control group displayed a decrease in cue-induced freezing. However, after five days of extinction training with multiple cue presentations per day, all groups demonstrated extinguished cue-induced freezing except for the AIE-SPS group. Several studies have reported increased resistance to extinction following AIE exposure (Bergstrom et al., 2006; Broadwater & Spear, 2013). Cue-induced fear-related behavior is dependent on connections between the basolateral amygdala (BLA) and the PFC (Blair et al., 2001), and these connections continue to develop throughout adolescence (Cunningham et al., 2002). Perhaps AIE exposure disrupts communication between these regions, and this in turn leads to impaired extinction retention (and possibly also increased acquisition of cue-induced freezing). Relevant follow-up studies might include examining cellular activation within the BLA during cue-induced fear conditioning and extinction

training with protein markers of recent cellular activity, such as cFos. Additionally, studies detailing the development of connections between the BLA and PFC, and whether AIE affects this, would be informative.

SPS exacerbated the resistance to extinction seen in the AIE-Control group. This effect of SPS exposure has been noted in the literature (Knox et al., 2012). It has also been reported that SPS exposure leads to decreased activity in the infralimbic (IL) cortex, which has reciprocal projections with the BLA. IL inhibition of the BLA is critical for extinction retention, so decreased IL activity may lead to disinhibition of the BLA, and impaired extinction training as demonstrated in the present set of studies. Follow-up studies to confirm this hypothesis could involve analysis of cellular activation of the cellular population that projects to the BLA during extinction training and retention in the IL, and examining the effects of an artificial increase in the activity of these neurons to alleviate the resistance to extinction observed following AIE and AIE-SPS exposures.

Finally, the present studies also revealed that AIE exposure led to impaired extinction retention, and that SPS exposure following AIE exacerbated this effect. Impaired extinction retention following SPS exposure has been well documented (Knox et al., 2011; Knox et al., 2012; Knox et al., 2016), but very few studies have investigated the effects of AIE exposure on extinction retention. The literature regarding SPS exposure suggests that impairments in IL and BLA neural activity (and projections between the two regions) similar to those proposed to underlie the resistance to extinction also mediate the deficit in

extinction retention (for review, see D. Knox et al., 2016). However, it should be noted that neither the AIE-Control nor the AIE-SPS groups failed to extinguish; all treatment groups reduced cue-induced freezing by at least 50% over the course of extinction training, but only the AIE-Control and AIE-SPS groups displayed significantly increased freezing during the extinction retention test three days later. Follow-up studies to further detail the effects of AIE and SPS exposures on IL and BLA activity could include optogenetic or chemogenetic inhibition of IL to BLA projections during extinction training and extinction recall to recapitulate the deficits reported here, or excitation of this circuit to alleviate the resistance to extinction training and extinction recall reported here.

In conclusion, the studies included in this dissertation demonstrate a subtle yet significant impact of adolescent binge-like ethanol exposure on three separate measures of behavioral flexibility in adulthood. While SPS exposure exacerbated some of these effects, the effects of both AIE and SPS exposures varied by rat strain. Differing susceptibilities to anxiety-like behavior for each rat strain may play a role in the observed differences in performance. These studies demonstrate the synergistic effects of binge-like ethanol and traumatic stress exposure, and contribute to the growing literature examining the effects of comorbid ethanol abuse and traumatic stress. They also contribute to the mounting evidence that adolescent ethanol abuse can have lasting, if not permanent, effects on cognitive function in adulthood.

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